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Research Article

Anticancer activity of *Mandragora autumnalis*: an in vitro and in vivo study

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Abstract

Ethanol crude extract and four solvent fractions were extracted from *M. autumnalis* leave to evaluate their anticancer effect in both models: in vitro and in vivo. The in vitro assessments were conducted using the MTT method in five cancer cell lines and one normal cell line. Ethanol extract and n-hexane fraction showed antitumor activity against MCF-7 breast cancer cells with IC_{50} values of 0.1 and 0.4 mg/ml, respectively, and low cytotoxicity against normal VERO cell line (IC_{50} value > 4 mg/ml). Furthermore, expression levels of VEGF were tested in MCF-7 cells treated with *M. autumnalis* using Human VEGF Simple Step ELISA Kit. The results indicated downregulation of VEGF expression in the treated cells compared to the control group. Additionally, the LD_{50} of *M. autumnalis* ethanol extract was calculated by applying an acute toxicity assay. Balb/C mice were inoculated with EMT6/p breast cancer cells and then treated with (137 mg/kg/day intraperitoneal injection) of ethanol extract for ten days. Interestingly, a significant ($p < 0.001$) reduction occurred in the tumor size of the treated group comparing to the control. Treatment in vivo toxicity was evaluated by measuring liver and kidney parameters. The tumor-bearing mice treated with the *M. autumnalis* extract showed normal serum level of AST, ALT, and creatinine. The results indicate that *M. autumnalis* has antitumor properties in both models in vitro and in vivo. However, further molecular investigation is required to understand the molecular bearings of *M. autumnalis*' activity.

Keywords

anticancer activity, *M. autumnalis*, VEGF, MTT assay, LD_{50}

Introduction

Cancer is considered to be one of the leading causes of mortality and morbidity over the world (McDaniel et al. 2017). According to the International Agency for Research on Cancer (IARC), the worldwide cancer burden is estimated to have increased to 19.3 million new cases and 10.0 million deaths in 2020 (Sung et al. 2021). It is recognized as a serious, public health concern of social significance and ranked as the second leading cause of death in the United States (Siegel et al. 2019).

The bioactivity of phytochemicals affects diseases either directly or indirectly by targeting their underlying causes or by boosting the immune system. In particular, they may inhibit carcinogens from reaching targeted sites and enhance the detoxification of highly reactive molecules. Specific phytochemicals also improve innate immune surveillance and increase body's ability to eliminate malignantly transformed cells. Furthermore, plant-derived molecules have various impacts on intrinsic DNA repair mechanisms and may influence tumor suppressors and prevent cellular proliferation pathways (Kotecha et al. 2016). One of the effective methods to develop new drugs and improve health care is the potential isolation and use of novel plant-derived bioactive products. The selection of promising anticancer and antimicrobial phytochemicals

needs to be verified in multiple in vitro and in vivo tests to assure their activity and safety (Talib 2011).

The current available anticancer medications have many side effects that may be overcome by applying plant-derived molecules as a single treatment or in combination (Dutt et al. 2019). The plant kingdom is the primary source of the natural secondary metabolites, which are being investigated for their anticancer properties leading to the discovery of new clinical drugs (Greenwell and Rahman 2015). Since ancient times, *Mandragora autumnalis* plant was considered one of the most important medicinal plants and had significant cultural value as a herb. Its use throughout history was remarkable. Mandragora is known for its various properties like healing, hallucinogenic, increasing fertility, and poisonous effects (Zohary 1982). Moreover, another property of *Mandragora autumnalis* is its narcotic effect which is probably due to the presence of a different form of alkaloids (Bierbaumer et al. 2009). Mandrake is the common name for the plant genus *Mandragora* and belongs to the Solanaceae family (Hanuš et al. 2006). Down the ages, different parts of *Mandragora* species like root, fruit, and leaves have been used as a treatment for diseases like insomnia, eye health problems, inflammation, and ulcers (Zohary 1982). Despite the importance of the mandrake plant over the centuries as a traditional and valuable herbal medicine, little work has previously been published on its biological activity and potential phytochemicals.

Materials and methods

Plant materials

Fresh leaves of *Mandragora autumnalis* were collected from Ajloun Forest Reserve in Jordan. The taxonomic identity of the plant was authenticated by Royal Society for the Conservation of Nature institute in Jordan.

Preparation of the extract and fractions

Steps of extract preparation were according to Talib et al. (Talib and Mahasneh 2010) with some modifications. The fresh plant was cleaned; leaves were separated, dried at room temperature and ground mechanically. Solvents with different polarities were used to prepare different fractions. Ethanol extract was prepared by macerating the dried powder in 95% ethanol (1L per 100 g) for two weeks with daily stirring. After that, the residue was removed; the supernatant was filtered, concentrated using rotary evaporator, fully dried using a lyophilizer. The dried ethanol extract was further subjected to solvent-solvent partitioning between water and chloroform. Both fractions were dried under reduced pressure using a rotary evaporator. The resulted water fraction dried completely using lab oven with fan at 40 °C. Solvent fractions of *n*-hexane and 10% aqueous/methanol were prepared by dissolving dried chloroform extract in *n*-hexane, fractionate it using aqueous/methanol and both fractions (*n*-hexane and aqueous/methanol) concentrated using a rotary evaporator. Ethanol crude extract and fractions which are water, *n*-hexane and aqueous/methanol were kept at -20 °C until used.

Phytochemical screening

The phytochemical examination was performed for *M. autumnalis* extract and fractions. Various types of active chemical constituents such as tannins, terpenoids, alkaloids, phenols, flavonoids, and steroids were detected using qualitative chemical tests. Identification of the extracted components was achieved according to the standard methods described by Saqallah et al. (Saqallah et al. 2018).

GC-MS analysis of *M. autumnalis* ethanol extract

Analysis was performed by using GC-2010 plus gas chromatography (Shimadzu, Japan) coupled to a GCMS-QP2010 SE mass spectrometry detector (Shimadzu, Japan) and equipped with an AOC20i auto-injector (Shimadzu, Japan). A capillary Rtx-5MS column (30 m × 0.25 mm i.d × 0.25 μm film thickness, Restek, USA) was utilized for separation. Helium (at a flow rate of 1.0 ml/min) was used as carrier gas. The temperature was fixed at 60 °C for 5 min and programmed to reach 240 °C at the rate of 3 °C/ min. The samples were injected at a temperature of 250 °C. The injection amount was 1.0 μl in a 1:30 split ratio. The mass spectra were applied with electron impact ionization (70 Ev) at full scan mode (40 to 500 *m/z*), using an ion source at 200 °C. The identification of compounds was detected using the NIST (National Institute of Standards and Technology, USA) mass spectral library.

Cell lines and cell culture conditions

Human breast cancer (MCF-7 and MDA-MB-231), human colon cancer (HCT-116), human lung adenocarcinoma (A549) cell lines, and monkey normal cells (VERO) cell lines were provided by the University of Jordan. Mouse mammary sarcoma cell line (EMT6/P) was purchased from the European Collection of Cell Cultures (Salisbury, UK). The cells were cultured in a complete medium and incubated at 37 °C in 5% CO_2, 95% humidity incubator. MCF-7 was cultured in a complete RPMI 1640 medium. On the other hand, HCT-116, MDA-MB-231, A549 cell lines were cultured in a complete DMEM medium. EMT6/P was cultured in complete MEM medium. All culture media were supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% gentamycin solution.

Animals

Forty eight Balb/C female mice (4–6 weeks old, weight 21–25 g) were used in this study. Animals were kept under

specific conditions: 25 °C with a 12 h light/dark cycle, and continuous air ventilation in the animal house. All animal experiments were approved by the Research and Ethical Committee of Applied Science University.

Antiproliferative assay

The cytotoxicity of *M. autumnalis* was measured using MTT (the tetrazolium salt, 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay kit (Bioworld, UK). Cancer cells were harvested by trypsinization and seeded into 96-well flat-bottom plates at a density of 1.5×10^4 cells/well for overnight incubation. After incubation, the media was completely removed from each well and the adherent cells were treated in triplicates with decreasing concentrations of *M. autumnalis* extract and fractions (4–0.06 mg/ml). Cells were incubated for 48 h. After that, 10 µl of MTT solution was added to each well, and incubated for 3 h. Followed by the addition of 100 µl of DMSO and cultivated for an hour. A microplate reader (Biotek, Winooski, VT, USA) was used to measure the resulting color at 550 nm. Percentage cell survival was determined for all treatments and compared to the negative control (untreated cells). Vincristine sulfate was used as a positive control.

Determination of VEGF expression in MCF-7 cells

Actively growing MCF-7 cells were harvested using trypsin-EDTA treatment. RPMI medium was used to prepare cell suspension, and then cell viability was assessed utilizing trypan blue dye exclusion method. Cells were dispensed into four separated tissue culture flasks at a concentration of 1.5×10^5 cells/10 ml of complete tissue culture media and incubated for 24 h. Then, culture media was completely removed and replaced with fresh media containing one of the following treatments: ethanol extract (0.5 mg/ml), *n*-hexane fraction (0.6 mg/ml), vincristine as a positive control (0.02 mg/ml), and media as a negative control (Talib and Al Kury 2018). Cells were incubated for 48 h, and then harvested and tested using Ab222510 Human VEGF Simple Step ELISA Kit version 1 catalog (Abcam, UK). A standard curve was acquired using different concentrations of human VEGF, and then the resulted data are expressed as equivalent of VEGF (pg/ml) for each milliliter of the extract.

Acute toxicity of *M. autumnalis* ethanol extract

A limit test was carried out on a small group of mice to select the dose ranges for actual LD_{50} (median lethal dose) determination. Briefly, ethanol extract was dissolved in PBS containing (5–10%) of tween 20. Two female mice (6 weeks old, 20–23 g weight) were injected intraperitoneally with a dose of the extract estimated from a previous study (Everett and Gabra 2014). Then, animals were observed for 24 hours for any mortality. The next doses were modified according to "up and down" method described previously by Akhila et al. (Akhila et al. 2007). For the main test, LD_{50} determination, four groups (n = 4) of mice were injected intraperitoneally with different concentrations (1000, 1,500, 2000, and 2,500 mg/kg) of ethanol extract within the higher and lower range that has been determined in the limit test. The untreated fifth group (n = 4) was used as a negative control and injected IP with PBS and 5% tween 20. Mice were observed for 24 hours for mortality and general behavior. The LD_{50} was indicated by the concentration that showed 50% mortality applying the arithmetical method of Karber (Akhila et al. 2007).

Antitumor activity on experimental animals

The mouse mammary tumor cells EMT6/P were collected by trypsinization and tested for their viability using the trypan blue exclusion method. Balb/C female mice were injected subcutaneously in the abdominal area with a tumorigenic dose of 1×10^6 cell in 0.1 ml. After 14 days, tumors size was measured using a digital caliper. Then, tumors volume was calculated according to the following formula: $(A \times B^2 \times 0.5)$. Where A was the length of the longest aspect of the tumor and B was the length of the tumor aspect perpendicular to A (Agrawal et al. 2004). Tumor-bearing mice were randomly divided into two groups, each one with 10 mice. The control group was injected intraperitoneally with a vehicle of 5% tween 20 in PB. Then, the treatment group was injected intraperitoneally with 137.4 mg/kg/day (10% of the calculated LD_{50}) of *M. autumnalis* ethanol extract. All treatments continued for 10 days. At the end of the study, blood samples were withdrawn for each group, and tumors were measured. Then mice were sacrificed, and their tumors dissected and stored in 10% formalin.

Assessment of liver and kidney toxicity

Liver and kidney toxicity were assessed after the treatment with *M. autumnalis*. Aspartate transaminase (AST), alanine transaminase (ALT), and creatinine in serum samples were measured to evaluate liver and kidney functions by following the instructions in the relevant kits (BioSystems, Barcelona, Spain).

Statistical analysis

Data were presented using the mean ± SEM (Standard Error of Mean). The statistical significance among the groups was determined using SPSS one-way analysis of variance (ANOVA) and student's t-test. Differences between groups were considered significant when the p-value was less than 0.05 ($p < 0.05$). IC_{50} values were calculated using non-linear regression in SPSS (Statistical Package for the Social Science, Chicago, Illinois version 24).

Results

Phytochemical screening and GC-MS analysis of *M. autumnalis*

The qualitative phytochemical screening results showed that all the tested phytochemicals were detected in the ethanol crude extract, while steroids and alkaloids were found only in ethanol extract and n-hexane fraction. Tannins and phenols were found in both extract and fractions except the *n*-hexane fraction (Table 1).

Table 1. Phytochemical screening results obtained from *M. autumnalis* ethanol crude extract and its solvent fractions.

Phytochemical screening tests	Ethanol extract	*n*-hexane fraction	Aqueous/ methanol fraction	Aqueous fraction
Tannins (Braemer's test)	+	-	+	+
Terpenoids (Salkowski test)	+	+	+	+
Alkaloids (Mayer's test)	+	+	-	-
Phenols (Ferric chloride test)	+	-	+	+
Flavonoids (Sodium hydroxide test)	+	+	-	+
Steroids (Liebermann-Burchardt test)	+	+	-	-

*results were rated as: + (positive), - (negative).

Further analysis of the ethanol extract using GC-MS revealed the presence of high concentrations of eicosane, linoleic acid, ethyl butanoate, and caprylic acid with the following percentages: 12.1%, 11.5%, 10.6%, and 10.3%, respectively (Table 2). Some other compounds were detected in lower concentrations like palmitic acid 9.1%, borneol 8.11%, and squalene 7.12% (Table 2).

Table 2. Major compounds identified in *M. autumnalis* ethanol crude extract using GC-MS method. M.W: molecular weight, R.T: retention time.

No	Compound	Formula	M.W	R.T	%
1	Vianol	C15H24O	220	6.14	3.8
2	Capric acid	C10H20O	172	7.52	2.6
3	Stearic acid	C18H36O2	284.5	8.62	2.11
4	Margaric acid	C17H34O2	270.5	9.8	1.96
5	Palmitic acid	C16H32O2	256	13.80	9.1
6	Lauric acid	C12H24O2	200	17.99	0.91
7	Caprylic acid	C8H16O2	144	18.1	10.3
8	Methyl caprate	C11H22O2	186	19.82	6.8
9	Eicosane	C20H42	282.5	20.93	12.1
10	Squalene	C30H50	410.7	23.4	7.12
11	Linoleic acid	C18H32O2	280	34.97	11.5
12	Cinnamic acid	C9H8O2	148	38.76	1.02
13	Methyl stearate	C19H38O2	298.5	39.89	1.22
14	Cyclododecane	C12H24	168	41.77	1.36
15	Ethyl palmitate	C18H36O2	284.5	42.9	1.02
16	Methyl laurate	C13H26O2	214	43.5	1.01
17	B-belladonnine	C34H42N2O4	542.7	44.0	0.41
18	Apoatropine	C17H21NO2	271.35	45.73	0.31
19	Borneol	C10H18O	154	46.2	8.11
20	3a-Tigloyloxytropan	C13H21NO3	239.31	46.90	0.88
21	Scopolamine	C17H21NO4	303.35	47.30	0.46
22	Ethyl butanoate	C6H12O2	116	49.5	10.6

Antiproliferative activity of *M. autumnalis* extract and fractions

Dose-dependent inhibition of cell proliferation was observed after treatment of various cell lines with increasing concentration (0.06–4 mg/ml) of *M. autumnalis* extract and fractions (Fig. 1A–D). Both ethanol extract and n-hexane were more cytotoxic against MCF-7 cells with IC_{50} values of 0.10 and 0.48 mg/ml, respectively (Table 3). On the other hand, aqueous and aqueous/methanol fractions exhibited lower activity with IC_{50} values range between 3 to 4 mg/ml. For the VERO cell line, the cytotoxicity of the crude extract and fractions was limited with IC_{50} values of more than 4 mg/ml (Table 3).

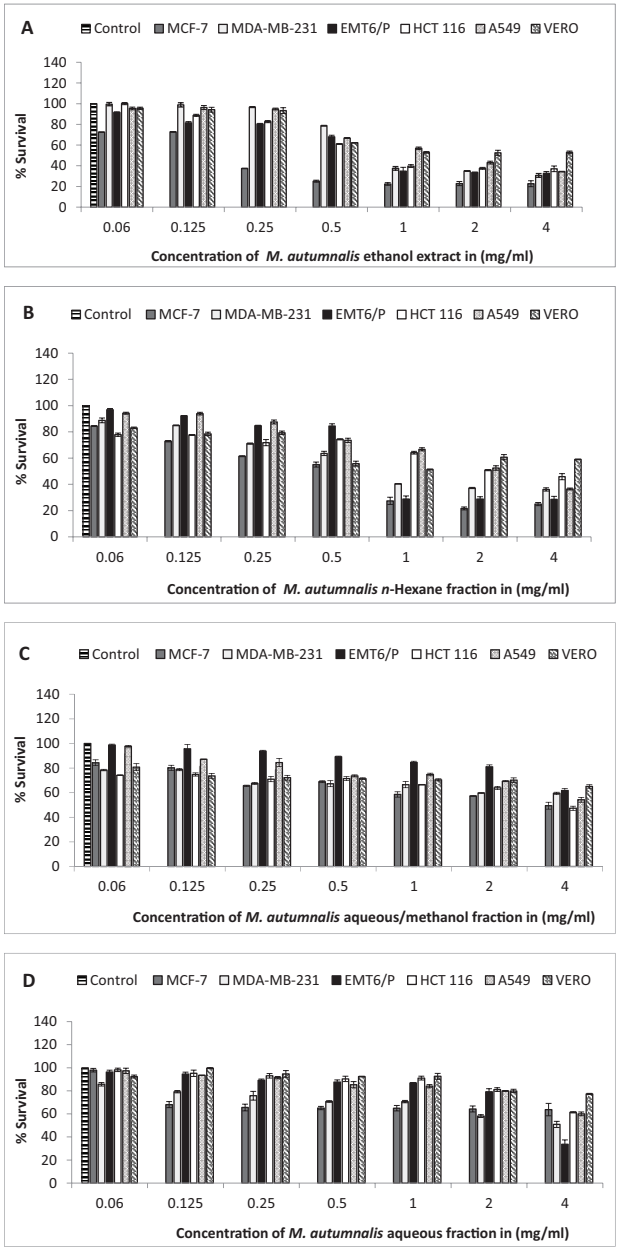

Figure 1. (**A**) Antiproliferative activity of ethanol crude extract of *M. autumnalis* on MCF-7, MDA-MB-231, EMT6, HCT 116, A549 and VERO cell lines. (**B**) Antiproliferative activity of n-hexane fraction of *M. autumnalis* on MCF-7, MDA-MB-231, EMT6, HCT 116, A549 and VERO cell lines. (**C**) Antiproliferative activity of aqueous/methanol fraction of *M. autumnalis* on MCF-7, MDA-MB-231, EMT6, HCT 116, A549 and VERO cell lines. (**D**) Antiproliferative activity of aqueous fraction of *M. autumnalis* on MCF-7, MDA-MB-231, EMT6/P, HCT 116, A549 and VERO cell lines. Results expressed as means (bars)± SEM (lines).

Table 3. The IC_{50} (mg/ml) of *M. autumnalis* ethanol extract and its solvent fractions, tested on six different cell lines (MCF-7, MDA-MB-231, EMT6/P, HCT 116, A549 and VERO).

Plant extract and fractions	MCF-7 IC_{50} (mg/ml)	MDA-MB-231 IC_{50} (mg/ml)	EMT6/P IC_{50} (mg/ml)	HCT 116 IC_{50} (mg/ml)	A549 IC_{50} (mg/ml)	VERO IC_{50} (mg/ml)
Ethanol	0.10 ± 0.01	0.86 ± 0.01	0.74 ± 0.09	0.81 ± 0.1	1.88 ± 0.3	> 4
n-Hexane	0.48 ± 0.02	0.73 ± 0.1	0.82 ± 0.07	1.99 ± 0.8	3.11 ± 1.7	> 4
Aqueous/ Methanol	3.95 ± 1.8	> 4	> 4	3.81 ± 0.2	> 4	> 4
Aqueous	> 4	3.06 ± 1.6	3.56 ± 0.1	> 4	> 4	> 4
Vincristine (positive control)	0.01 ± 0.08	0.02 ± 0.02	0.04 ± 0.04	0.02 ± 0.2	0.008 ± 0.1	> 4

IC, inhibitory concentration.

Effects of *M. autumnalis* on VEGF Expression in MCF-7 Cells

The expression of VEGF was measured to evaluate whether the suppression of angiogenesis may contribute to the observed antiproliferative effect of the ethanol and n-hexane fraction. The results have shown that the negative control group has the highest expression of VEGF (364 pg/ml). Compared to that, all the applied treatments appeared to lower the level of VEGF., The most prominent effect was observed for the ethanol extract lowering the cellular levels of VEGF by about 30%(224 pg/ml), followed by the n-hexane fraction (241 pg/ml) (Fig. 2). Vincristine, used as a positive control, exhibited the lowest VEGF concentration of 161 pg/ml.

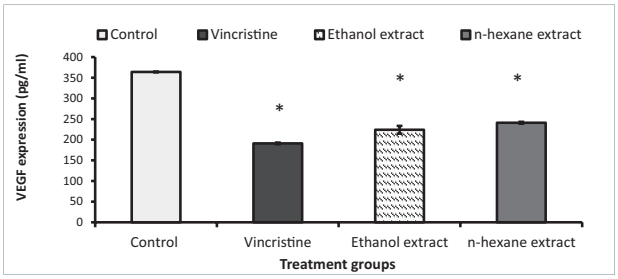

Figure 2. The effect of IC_{50} concentration of *M. autumnalis* ethanol crude extract (0.5 mg/ml), n-hexane fraction (0.6 mg/ml) and vincristine (0.02 mg/ml) on VEGF expression in MCF-7 cell line. (* $P < 0.01$) compared to the control.

Acute toxicity evaluation of *M. autumnalis* ethanol extract

The limit test of *M. autumnalis* ethanol extract revealed that the highest nonlethal concentration was 460 mg/kg and the lowest lethal concentration was 2,025 mg/kg (Table 4). According to the arithmetical method of Karber, the LD_{50} value of ethanol extract was 1,375 mg/kg (Table 5).

Table 4. The outcomes of the limit study of different doses of *M. autumnalis* ethanol crude extract in mice.

Groups (N = 2)	Dose (mg/kg)	No. of death	% of Mortality
1	300	0	0
2	460	0	0
3	1,350	1	50
4	2,025	2	100

The highest non-lethal dose is 460 mg/kg and the lowest lethal dose is 2,025 mg/kg. Percentage of mortality = Total number of deaths / Total number of mice in the group * 100.

Table 5. Acute toxicity assay of *M. autumnalis* ethanol extract after intraperitoneal injection in mice (N = 4) and observation for 24 hours.

Groups (N = 4)	Dose (mg/kg)	No. of mortality	Dose difference (a)	Mean mortality (b)	Probit (a × b)
1 (Control)	PBS + 5%tween 20	0		0	0
2	1000	0	0	0	0
3	1,500	0	500	0	0
4	2,000	3	500	1.5	750
5	2,500	4	500	3.5	1,750

Dose difference (a) = higher dose – lower dose. Mean mortality (b) = (mortality in the second concentration + mortality in the first concentration) / 2. LD_{50}= least lethal dose- $\Sigma(a \times b) / N$, where N = total number of mice in each group.

In vivo antitumor effects of *M. autumnalis* ethanol extract on EMT6/P cells implanted in mice

Tumor size in mice treated with *M. autumnalis* ethanol extract was significantly ($P < 0.001$) decreased compared to the negative control (Figs 3, 4). Ethanol extract showed significant inhibition of tumors where a reduction in tumor size was recorded (-35.99%) compared with the untreated control group (+107.02%) (Table 6). For both groups, there was no death recorded. The percentage of mice with no detectable tumor in the ethanol extract-treated group was 30%, and mice showed normal activity with no side effects.

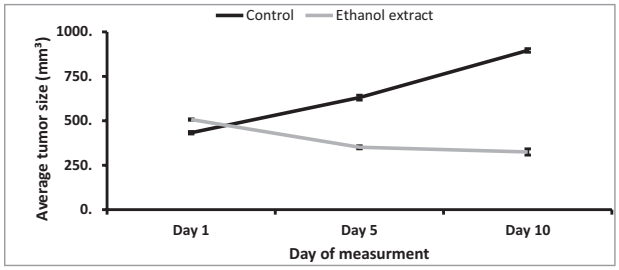

Figure 3. A plot of change in average tumor size (mm^3) vs. time in (days) of treatment in EMT6/P cell line. ($P < 0.001$) compared to the control group.

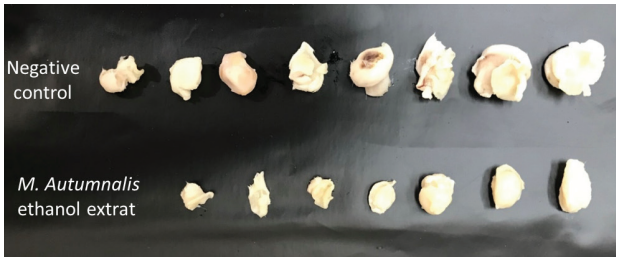

Figure 4. Effect of *M. autumnalis* ethanol extract on tumor size and cure percentage. Treatment with *M. autumnalis* ethanol extract resulted in reducing tumors size and increasing cure percentage compared to the negative control. (N = 10 mice) in each group.

Table 6. The effect of *M. autumnalis* ethanol extract on tumor size and weight in mice (N = 10).

Treatment groups (n = 10)	Av. Initial tumor size (mm^3)±SEM	Av. Final tumor size (mm^3)±SEM	% Change in tumor size	% of mice with no detectable tumor	Average tumor weight (g)
Control	432.62 ± 1.19	895.61 ± 1.08	107.02	20%	0.72
M. autumnalis (ethanolic extract)	506.83 ± 2.35	324.40 ± 1.45	-35.99	30%	0.44

(mm^3: cubic millimeter). % change in tumor size = (Final size – Initial size)/ Initial size *100.

Effects on serum level of AST, ALT, creatinine

The plasma levels of AST, ALT, and creatinine for tumor bearing mice groups were elevated compared to normal mice. However, the mildest elevation of the three parameters was observed in the experimental group treated with the *M. autumnalis* ethanol extract (Fig. 5A). The highest level of creatinine was detected in the ethanol groupcompared to the normal mice (Fig. 5B).

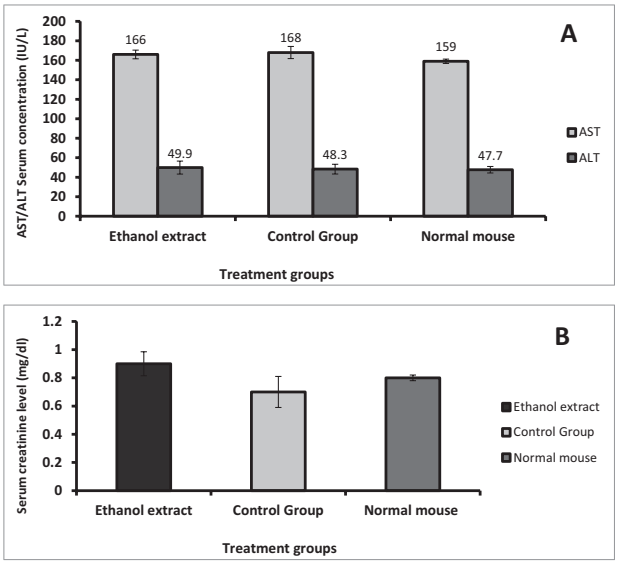

Figure 5. Effect of *M. autumnalis* treatment on serum levels of: (**A**) AST, ALT and (**B**) Creatinine. Concentrations of AST and ALT are expressed by IU/L and serum creatinine level by mg/dl. Mice were treated with (137.4 mg/kg) of ethanol extract. Creatinine, ALT, and AST levels in normal healthy mouse were 0.8 mg/dl, 47.7 IU/L, 159 IU/L, respectively. Results are expressed as means (bars) ± SEM (lines). ALT, alanine transaminase; AST, aspartate transaminase.

Discussion

Cancer is a serious health problem that continues to be the main reason for death worldwide. Recently, a considerable number of anticancer drugs have been discovered and developed due to the escalating progress in understanding the molecular mechanism of cancer growth. However, the use of synthetic chemotherapeutic agents has not significantly improved the overall survival rate over the past few decades. Thus, new strategies and novel chemopreventi-on agents are needed to enhance the efficiency of current cancer therapies (Choudhari et al. 2020).

In the present study, crude extract and solvent fractions from *Mandragora autumnalis* leaves were prepared and their anticancer activity was evaluated in vitro and in vivo. Despite the traditional importance of *M. autumnalis*, there is virtually no information on its antitumor potency. In this study, *M. autumnalis* exhibited the ability to inhibit cancer cell growth in a dose-dependent manner. The results of the phytochemical screening agree with a previously conducted study that showed the leaves acetone extract of *M. autumnalis* contained the highest amount of phenols, while methanol extract had the highest content of flavonoids (Uysal et al. 2016). Another study demonstrated that *M. autumnalis* leaves ethanol extract had high contents of phenols and flavonoids with the presence of other phytochemicals like terpenoids, anthraquinones, coumarins, phlobatannins, and tannins (Jodallah 2013). Interestingly, most phytochemicals affect cancer by regulating molecular pathways that are involved in the growth and progression of the tumor. The specific mechanisms include antioxidant enhancement, carcinogen inactivation, inhibiting proliferation, induction of cell cycle arrest and apoptosis, and modulation of the immune system (Choudhari et al. 2020). Plant phenolic compounds and herbal extracts rich in phenolic compounds regulate cell proliferation, survival, and apoptosis through modulating the levels of reactive oxygen species (ROS) in cells. Also, many in vitro observations, preclinical and epidemiological studies have demonstrated the association of plant phenolic acids in reducing cancer growth (Anantharaju et al. 2016). Reactive oxygen species (ROS) promote carcinogenesis by inducing genetic mutations, activating oncogenes, increasing oxidative stress, which all affect cell proliferation, survival, and apoptosis (NavaneethaKrishnan et al. 2019). According to previous studies, *M. autumnalis* leaves extracts exhibited antioxidant activity (Jodallah 2013; Uysal et al. 2016), which might be behind the ability of our crude extract and fractions to reduce cancer cells viability and promote apoptosis. Moreover, different types of terpenoids have shown cytotoxicity against various tumor cell lines and chemopreventive as well as anticancer efficacy in a preclinical animal model (Thoppil and Bishayee 2011). The activity of flavonoids as cancer preventive compounds represented by interaction between different types of genes and enzymes (Chahar et al. 2011). Besides, their anticancer activity is related to their modulation of signal transduction pathways within cancer cells. As a result, flavonoids can inhibit cell proliferation, angiogenesis, and metastasis, as well as promoting apoptosis (Abotaleb et al. 2019). Alkaloids, on the other hand, are one of the important phytochemicals in plants. Many alkaloids isolated from natural herbs exhibited antiproliferation and anti-metastasis effects on different types of cancers, both in vitro and in vivo (Lu et al. 2012). Seven types of alkaloids were identified by Al-Khalil and Alkofahi (1996)

from roots, leaves, unripe fruits, and seeds of *M. autumnalis*, which grows naturally in Jordan. Regarding ethanol and n-hexane fraction, this previous study agrees with our phytochemical screening results, which positively indicate the presence of alkaloids in both ethanol extract and n-hexane fraction. However, according to Bekkouche et al. (1993) investigation results, the highest level of alkaloids was found in the roots of samples during the flowering period of the plant. Since alkaloids are more soluble in a non-polar solvent (Georgiev and Pavlov 2017), this may explain the antiproliferative activity of n-hexane fraction against all tested cancer cell lines.

According to the reported results, *M. autumnalis* ethanol extract revealed high activity almost in all experimental assays in the current study. As a result, further analysis was applied using Gas Chromatography-Mass Spectrometry (GC-MS) to determine the most important phytochemicals in the extract (Table 2). The identified phytocompounds comprised of hydrocarbons, fatty acids, alcohols, esters, and alkaloids. These findings were relevant to previous literature where *M. autumnalis* leaves, and fruits have been analyzed using GC-MS (Baser et al. 1998; Hanuš et al. 2006; Jodallah 2013).

Based on previous studies, eicosane exhibited antitumor activity and potent cytotoxicity in the human cancer screening program (Yu et al. 2005) as well as antiproliferative activity against different cancer cell lines (Walia et al. 2012) and cytotoxic effect both in vitro and in vivo against human ovarian carcinoma cells (Xie et al. 2007). Linoleic acid (LA) is a polyunsaturated omega-6 fatty acid found mostly in plant oils. It has shown antiproliferative activity against hormone-independent PC-3 prostate cancer cell line (Bratton et al. 2019). Another study has demonstrated that LA was able to suppress colorectal cancer cell growth by inducing cell apoptosis, enhancing cellular oxidant status, and promoting mitochondrial dysfunction (Lu et al. 2010). On the other hand, caprylic acid was significantly able to reduce cancer cell viability by 70% to 90% compared to controls. Its anticancer effect was due to the down-regulating cell cycle regulatory genes and up-regulating genes involved in apoptosis (Narayanan et al. 2015). Borneol is a terpene derivative and a bicyclic organic compound. The cytotoxicity of borneol and its DNA-damaging effects has been studied in malignant HepG2 hepatoma cells, malignant Caco-2 colon cells, and non-malignant human VH10 fibroblasts. Borneol showed cytotoxicity in all cell lines (Slameňová et al. 2009). Based on another study, borneol potentiates selenocysteine-induced apoptosis in human hepatocellular carcinoma cells by the enhancement of cellular uptake and activation of ROS-mediated DNA damage (Su et al. 2013). On the other hand, squalene, a triterpene and a precursor of different hormones in animals and sterols in plants, was reported to have an anticancer effect against ovarian, breast, lung, and colon cancer (Lozano-Grande et al. 2018). According to (Palaniyandi et al. 2018), squalene was able to significantly reduce the cell viability of gastric adenocarcinoma cell line and induce apoptosis. Crude extract and solvent fractions of *M. autumnalis* showed low activity against Vero normal cell line, which is presented by high IC_{50} values; this may indicate the safety of the crude extract and fractions against normal cells. In order to understand the mechanism of action of *M. autumnalis* as an antiproliferative agent, further analysis was conducted to determine the effect of ethanol extract and *n*-hexane fraction on cancer cells' angiogenesis process. Angiogenesis, the growth of new blood vessels, plays a crucial role in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) and its receptors are one of the major angiogenic activators and highly overexpressed in a variety of tumors (Prager et al. 2012).

The current study has found that *M. autumnalis* ethanol extract and *n*-hexane fraction were significantly ($P < 0.01$) able to reduce VEGF secretion in the MCF-7 cell line compared to the control group (Fig. 2). This consistent with previous studies that confirm the potential activity of polyphenols in decreasing the development and progression of many types of cancer via their ability to reduce tumor cell viability and VEGF expression (He et al. 2015). Moreover, it was found that flavonoids have the ability to inhibit VEGF secretion and suppress in vitro angiogenesis (Mirossay et al. 2017). Lupeol and stigmasterol, major phytosterols in various herbal plants, have shown anti-angiogenic activity through decreasing the downstream effector levels of VEGFR-2 signaling (Kangsamaksin et al. 2017).

According to the results of the acute toxicity assay, the LD_{50} value was 1,375 mg/kg (Table 5). In the literature, no previous study has reported the LD_{50} of *Mandragora autumnalis*. It was reported that the lethal dose (LD_{50}) of the 'Great Rest', a popular medieval opiate drug containing *M. autumnalis*, was calculated. It has composed of three alkaloid-rich plants; raw opium, henbane, and mandrake (Everett and Gabra 2014). In reviewing the literature, no previous studies have shown the activity of *M. autumnalis* against cancer cells in an in vivo model. Based on the GC/MS analysis and phytochemical screening, ethanol extract contains various bioactive compounds which may explain its activity against cancer cells. Gallic acid (phenolic compound) was able to decrease xenograft tumor growth in a dose-dependent manner (Liang et al. 2012). On the other hand, a study reported that methanol extract of the *Erucaria pinnata* plant was rich in flavonoids and has the ability to inhibit the concentration of VEGF-A in vivo (Zakhary et al. 2020). Another study has demonstrated the activity of natural borneol (NB) as a chemosensitizer that enhanced temozolomide-induced anticancer efficacy against human glioma cells. Their combination treatment significantly inhibited tumor volume and tumor weight compared to that in treatment with NB or temozolomide alone (Liu et al. 2018).

Conclusion

The present study was designed to evaluate the anticancer activity and the chemical compositions of

Mandragora autumnalis. The findings have demonstrated that *M. autumnalis* have the potential to reduce cancer cell viability of various cancer cell lines. Besides, ethanol extract and n-hexane fraction have reduced the expression of VEGF in breast cancer cell line (MCF-7). Moreover, *M. autumnalis* ethanol crude extract exhibited anticancer activity in both models: in vitro and in vivo and this might be explained by the presence of various phytochemicals in *M. autumnalis* leaves. On the whole, the results suggested that *Mandragora autumnalis* has valuable activity, and it could be a source of natural remedies in pharmaceutical applications and adjuvant therapies. However, further molecular evaluation is needed to understand the particular mechanism of action of *M. autumnalis*.

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