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Evaluation of the cytotoxic activity of extracts from six species of *Phlomis* genus

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ABSTRACT

The present study was designed to investigate the cytotoxic activity of the total extract (methanol 80%) and fractions from six *Phlomis* species, including *P. anisodontea*, *P. bruguieri*, *P. caucasica*, *P. olivieri*, *P. persica* and *P. kurdica* against four human cancer cell lines (HepG2, MCF7, HT29 and A549) and one normal cell line (MDBK). The *in vitro* cytotoxicity of different concentrations (31.25 - 1000µg/mL) of the extracts and fractions evaluated by the MTT assay. Data revealed that all six extracts exhibited cytotoxic activity against at least one cancer cell line. The interesting cytotoxic activity on MCF7 cell line, respectively, was observed for dichloromethane fractions of *P. anisodontea* and *P. Kurdica*, and ethylacetate fractions of *P. kurdica, P. anisodontea and P. bruguieri*. In addition, the highest cytotoxic activity against A549 cells was found for ethylacetate fraction from *P. caucasica* and *P. kurdica*. Overall, the results of this study suggest that among selected plants, *P. kurdica*, *P. anisodontea* and *P. caucasica* are the best candidates for further investigations on the mechanisms of cytotoxic action and isolated the active compounds.

INTRODUCTION

Medicinal plants have been used as traditional treatments for most of the diseases through centuries. It is estimated that out of 250,000 species of plants, one thousand possess remarkable anticancer activity (Mukherjee *et al.*, 2001). Some of these plants are regarded as important sources of potent molecules used in cancer therapy. Now a days, approximately 60% of drugs used to treat cancer are derived from natural products, such as vincristine and vinblastine isolated from *Catharantus roseus* and Taxol from *Taxus brevifolia* (Newman and Cragg, 2012). Despite the large number of anticancer drugs, there is still a big requirement of new medicines with less adverse effects and more efficacies.

The genus *Phlomis* includes up to 100 species that are distributed in Euroasia (Fedorov, 2000). From these species, 17 are endemic to Iran (Morteza-Semnani and Saeedi, 2005), which have been widely distributed in north, west and center of Iran (Naghibi *et al.*, 2005). Phytochemical studies on the plants belonging to the genus *Phlomis* showed that these species contain different classes of phytoconstituents, including flavonoids, iridoids, phenyl ethanoid/propanoid glycosides, essential oils, lignans, diterpenoids and alkaloids (Amor *et al.*, 2009, Bader *et al.*, 2015). In an earlier study, cytotoxic activity of different fractions of *P. lanceolata* showed that the petroleum ether fraction had high cytotoxic activity on three cancer cell lines, including HT29, T47D and Caco2 and normal cell NIH3T3 (Soltani-Nasab *et al.*, 2014). Recently, Salimi et al. (2016) confirmed that the methanol extract of *P. kurdica* was cytotoxic on human melanoma cells (SKMEL-3). Furthermore, some isolated phenolic and polyphenolic compounds from *Phlomis* plants have been found to be strong antioxidant and/or cytotoxic agents such as chlorogenic acid,

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verbascoside (acetoside), kaempferol 3-O-glucoside, luteolin 7-O-β-dglucopyranoside**,** naringenin and forsythoside B (Badria *et al.,* 2007, Ahmed et al**,.**2009 **,** Lee *et al.,* 2016, Harmon and Patel 2004).

Considering previous studies, in this survey, cytotoxic activity of the total extracts and different fractions of *P. anisodontea, P. bruguieri, P. caucasica, P. oliveri***,** *P. persica* and *P. kurdica* was investigated against four cancer cell lines (HepG2, MCF7, HT29, A549) and MDBK as a normal cell line for the first time.

MATERIALS AND METHODS

Plant material and extract preparation

The aerial parts of *Phlomis* species (Lamiacea) were collected from Azerbaijan (*P. olivieri, P. caucasica)* and Kurdistan *(P. anisodontea, P. bruguieri, P. Kurdica, P. persica*), provinces in Iran and identified by Mr. Yousef Ajani. The voucher specimens have been deposited at two Herbariums located at Faculty of Pharmacy, Tehran University of Medical Sciences, and Institute of Medicinal Plants (IMP), Iranian Academic Centre for Education, Culture and Research (ACECR) in Iran.

The dried powder of *Phlomis* species (100 grams) were extracted twice with MeOH (80%, 1500 mL) in percolator for one week. The combined methanol extracts were evaporated by Rotary Evaporator. Dried crude extract was successively fractionated using dichloromethane, ethylacetate and *n*-butanol, respectively.

Cell culture

MCF7 (breast adenocarcinoma), HepG2 (hepatocellular carcinoma), HT29 (colon carcinoma), A549 (lung carcinoma) and MDBK (bovine kidney) cells, obtained from National Cell Bank of Iran, were used to evaluate cytotoxic activity of plant extracts and their fractions. Cancer cell lines were cultured in DMEM (Gibco) supplemented with 5% FBS (Gibco) and MDBK cells were grown in RPMI 1640 (Gibco) supplemented with 5% FBS. Penicillin (100 U/mL) and streptomycin (10 µg /mL) were added to all media. All the cells were maintained at 37ºC in a 5% CO_2 incubator.

MTT assay

The cells were seeded in 96-well plate at $1x10^4$ cells/well and incubated for 24 hours. After that cells were washed and exposed to the different concentration of the total extracts and fractions and incubated for 72 hours, under 5% CO_2 at 37ºC. The initial concentration of samples was 1000 µg/mL and serial dilution was made in culture medium to yield six different concentrations of samples.

The final concentration of DMSO was less than 1% in all treatments. Cytotoxicity of samples was measured by MTT assay (Carmichael *et al.,* 1987). At the end of 72 hours incubation of treated cells, the medium in each well was replaced with MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazodium bromide) and plates were incubated for 4 hours. After this period the medium was discharged and DMSO was added to dissolve formazan crystals produced by viable cells. Plates were gently shaken for 20 min and the absorbance was measured by a microplate reader at 570 nm. IC_{50} was calculated as the concentration of samples which inhibited 50% of cell viability.

Statistical analyses

All samples were presented as mean ± SD for three measurements. Unpaired Student's t test was used to calculate $P < 0.05$ for each compound against the control. IC_{50} values were calculated using dose-response curve for each sample.

RESULTS

The cytotoxic activity of the total extract (MeOH 80%) from *P. olivieri, P. caucasica, P. anisodontea, P. bruguieri, P. Kurdica* and *P. persica* has been evaluated against MCF-7, HepG2, HT29, A549 and MDBK cell lines at different concentrations by MTT assay. The IC_{50} values are reported in Table 1.

Data revealed that all total extracts exhibited the highest cytotoxic activity against A549, MCF7 and MDBK cells. *P. caucasica*, *P. anisodontea* and *P. bruguieri* showed cytotoxic activity (IC_{50} < 1000 µg/mL) on A549, MCF7 and MDBK cells. The total extract of *P. caucasica* indicated the highest cytotoxicity on A549 (309.15 µg/mL) and MDBK (397.5 µg/mL) cells. *P. Kurdica* and *P. persica* extracts were cytotoxic against MCF7 and MDBK cells while, *P. olivieri* total extract showed cytotoxic activity only against MDBK normal cell line. However, the proliferative activity of HT29 and HepG2 cell lines was not affected by the maximum dose used (1000 µg/mL) of the total extracts. Active total extracts were fractionated based on increasing polarity solvents (dichloromethane, ethylacetate and *n*-butanol, respectively)

The cytotoxic activity of the fractions at different concentrations (31.25-500 µg/mL) were obtained by MTT assay against A549, MCF7 and MDBK. Cytotoxicity of the fractions reported with IC_{50} values and showed in Table 2.

The results indicated that most of the fractions exhibited cytotoxic effect against MDBK cells. MCF7 cell line was sensitive to ethylacetate and dichloromethane fractions of *P. anisodontea* (IC_{50} = 129.3, 80.75 µg/mL, respectively), ethylacetate fraction of *P. bruguieri* (IC_{50} = 168.9 µg/mL) and n-butanol, ethylacetate and dichloromethane fractions of *P. kurdica* (IC_{50} = 328.6, 92.21 and 91.57 µg/mL, respectively). The best cytotoxic activity on A549 tumor cells was observed by ethylacetate fraction of *P. caucasica* with IC_{50} value 64.58 µg/mL. In addition, ethylacetate and *n*-butanol fractions of *P. kurdica* (IC_{50} 127 and 418.8 µg/mL, respectively) and ethylacetate fraction of *P. olivieri* and *P. persica* also showed cytotoxic effect (IC_{50} =315.47 and 489.5 µg/mL, respectively) against A549 cell line.

Table 1: Cytotoxicity (IC_{50} values [µg/mL]) of the total extracts from selected *Phlomis* species against tumor and normal cell lines. Data are expressed as mean ± SD, n=3.

	Cell lines				
samples	**HT29**	**A549**	**HepG2**	**MCF7**	**MDBK**
Phlomis anisodontea	>1000	798.8±17.3	>1000	586.7±6.3	529.5±28.2
Phlomis bruguieri	>1000	663.5±12	>1000	726.3±12.7	928.8±2.1
phlomis caucasica	>1000	309.15±2.1	>1000	670.8±2.8	397.5±17.6
Phlomis oliveri	>1000	>1000	>1000	>1000	524.7±55.1
Phlomis persica	>1000	>1000	>1000	890±20.5	468.1±7.7
Phlomis kurdica	>1000	>1000	>1000	686.2±19.7	503.3±14.1

*Concentrations used in this experiment were 1000, 500, 250, 125, 62.5 µg /mL.
*IC_{50} values were calculated using dose-response curve for each sample.

Table 2: Cytotoxic activity (IC_{50} values (µg/mL); mean±SD, n=3) of the fractions from selected *Phlomis* species against tumor and normal cell lines.

		Cell lines		
samples	**Fractions**	**A549**	**MCF7**	**MDBK**
Phlomis anisodontea	n-butanol	-	-	229±14.1
	Ethyl acetate	-	129.3±1.4	82.52±7.7
	Dichloromethane	n.d	80.75±4.9	103.8±1.4
Phlomis bruguieri	n-butanol	-	-	173.2±9.8
	Ethyl acetate	-	168.9±4.9	115.7±31.8
	Dichloromethane	-	n.d	n.d
phlomis caucasica	n-butanol	-	-	-
	Ethyl acetate	64.58±1.4	-	83.66±7
	Dichloromethane	nd	-	n.d
Phlomis oliveri	n-butanol	-	-	226.7±7.7
	Ethyl acetate	315.47±4.9	n.d	-
	Dichloromethane	n.d	n.d	76.71±1.4
Phlomis persica	n-butanol	-	-	375.6±17.6
	Ethyl acetate	489.5±25.4	-	n.d
	Dichloromethane	n.d	n.d	n.d
Phlomis kurdica	n-butanol	418.8±7	328.6±4.9	-
	Ethyl acetate	127±4.9	92.21±4.9	135.7±14.8
	Dichloromethane	n.d	91.57±2.8	n.d

*Concentrations used in this experiment were 500, 250, 125, 62.5 and 31.25 µg /mL.
*IC_{50} values were calculated using dose-response curve for each sample.
*n.d: IC_{50} not determined due to sample precipitation
*Inactive fractions are shown by (-) sign.

DISCUSSIONS

Cancer is a leading cause of death globally and as reported by WHO it caused more deaths than AIDS, tuberculosis, and malaria in 2012. Among the 14 million incidence of cancer in 2012, common diagnosed cancers were lung, breast and colorectal and the most common causes of cancer death were lung, liver and stomach cancer (WHO, 2013)

Herbal medicine has provided a valuable guide of drug discovery through the years (Sahranavard *et al.*, 2009). Almost one third of prescribed drugs in the world are derived from plants and anticancer properties of over 3000 plant species have been indicated (Ghafari *et al.*, 2015).

The *Phlomis* genus has been a good source of herbal medicinal products (Kim, 2006) and has long history to treat different pathological conditions such as diabetes, (Hasani-Ranjbar *et al.*, 2008) gastritis (Digrak *et al.*, 1999), burns, lesions and dermal infections (Liolios *et al.*, 2007, Amor *et al.*, 2009).Previous studies on some *Phlomis* species such as *P. lanceolata* and *P. armeniaca* have shown cytotoxic activity against tumor cell lines (Soltani-Nasab *et al.*, 2014, Saracoglu *et al.*, 1995).

Petroleum ether fraction of *P. lanceolata* showed the potential cytotoxic activity against HT29, Caco2, T47D and NIH3T3 cell lines, while the cell lines did not respond to the polar fractions. Methanolic extract of *P. kurdica* has been proven to be effective against human melanoma skin cancer and melanogenesis process (Salimi *et al.*, 2016).

Based on these previous findings, in this study we investigated the cytotoxic activity of six *Phlomis* species which their antiproliferative effect have not been evaluated. Cytotoxic activity of total extract of *P. olivieri*, *P. caucasica*, *P. anisodontea*, *P. bruguieri*, *P. Kurdica* and *P. persica* on the HepG2, MCF7, HT29, A549 and MDBK cells was evaluated using MTT test. Data revealed that *Phlomis* total extracts did not show cytotoxic activity against HepG2 and HT29 cell lines, therefore the cytotoxic effect of the fractions of selected species were not tested on these cell lines.

Among the fractions of *Phlomis* species, the ethylacetate fraction of *P. kurdica* showed cytotoxic activity against all the tested cell lines. The highest cytotoxicity against A549 and MCF7 cancer cell lines, respectively, were found for ethylacetate fractions of *P. caucasica* and *P. kurdica*. These results are

probably related to the presence of polar compounds in this fraction such as flavonoid and phenolic glycosides (Delazar *et al.*, 2008). On the other hand, dichloromethane fractions of *P. anisodontea* and *P. kurdica* showed good cytotoxicity against MCF7 cells. This effect might be due to the toxic terpenoides and lipophilic compounds which exist in large amounts in *phlomis* genus.

These data is also in accordance with the cytotoxic action of non polar fraction from *P. lanceolata* against cancer cell lines which has been assigned to the presence of lipophilic and triterpene compounds. (Soltani-Nasab *et al.*, 2014). In addition, data showed that most of the *n*-butanol fractions didn't exhibited significant cytotoxic activity on cancer cell lines.

In the present study, most of the samples showed cytotoxic activity against MDBK cell lines. Previous reported findings confirmed that many current chemotherapy cancer treatments affect both normal and cancer cells. For that reason, concomitant toxicity of chemotherapeutic drugs in normal cells has been one of the major fields of research in recent decades. For instance, recent studies have shown that long- term use of tamoxifen can cause endometrial and hepatic cancer in breast cancer patients. Moreover, in another cytotoxic study, the hamster lung normal fibroblast cells (V79) as a normal cell line, was the most sensitive line to tamoxifen in comparison with nine tumoral cell lines, (Petinari *et al.*, 2004)

However, cancer cells generally undergo rapid cell division and are often more sensitive to drugs than normal cells, therefore, tumor cells are preferentially damaged in comparison with normal cells.

In our study, data revealed that while some total extracts showed cytotoxic activity against some cancer cell lines (effects of *P.caucasica* on MCF7 and *P. berguieri* on A549 cell lines), their fractions were not active against the same cell lines. This effect might be due to the synergistic effect of phytochemical constituents present in total extract. On the other hand, fractions of some inactive total extracts showed antiproliferative effect against certain cell lines (effect of *P. kurdica* fractions on A549 cell line). These data confirmed that the selection of potential plants based on the screening of total extract can lead to the loss of some active constituents.

Overall, the results of this study showed that *P. kurdica*, *P. anisodontea* and *P. caucasica* are considerable source of natural bioactive substances with antiproliferative activity on MCF7 and/or A549 cancer cell lines.

CONCLUSION

Among the *Phlomis* genus studied for the antiproliferative activity against cancer cell lines, interesting cytotoxic activity was observed for dichloromethane fractions of *P. Kurdica* and *P. anisodontea*, and ethylacetate fractions of *P. kurdica*, *P. anisodontea* and *P. caucasica.* Following this study, further investigation using different cancer cell lines and evaluation of the mechanism of cytotoxic induction and isolation and structure elucidation of active compounds from these fractions are suggested.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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