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The effects of the Methanolic Extracts of Prangos Uloptera and Crossoptera on the Growth, Mutagenicity and Proliferation of Human Lymphocytes, Based on Ames Test

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ABSTRACT

BACKGROUND AND OBJECTIVE: The genus Prangos is among plants with numerous species. The positive medicinal effects of this herb have been demonstrated in multiple studies. The aim of this study was to evaluate the effects of methanolic extracts from Prangos uloptera and crossoptera on the growth and proliferation of human lymphocytes and determine their mutagenic potentials.

METHODS: In this experimental study, the plants were dried and milled after determining their species. The methanolic extracts of Prangos uloptera and crossoptera were prepared by immersion and were diluted to final concentrations of 10, 100, 500, 1000, 1500, 2000 and 2500 mg/ml, using sterile phosphate-buffered saline. The effects of the extracts on the growth and proliferation of human-extracted lymphocytes were evaluated by lymphodex. The samples cultured in RPMI medium were evaluated by MTT assay, and the mutagenic potential was measured by Ames test.

FINDINGS: The results showed that the extracts from both Prangos species increased the growth and proliferation of lymphocytes (by 5-300 %) and exhibited no mutagenic potentials. The seed and leaf extracts from both species had the least and most significant impacts on the growth and proliferation of lymphocytes (5-7% and 2.1-3.1 % times, respectively), respectively.

CONCLUSION: The obtained results showed that the two evaluated species of Prangos are safe and lack any mutagenic potentials. They also enhanced the growth and proliferation of human lymphocytes.

KEY WORDS: Ames Test, Species, Methanolic Extract, Lymphocyte.

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Introduction

The use of new pharmaceutical compounds, which can modulate the immune system, is a new approach against infectious and immunosuppressive diseases. Among these compounds, natural compounds, particularly plants, are of great importance by affecting the growth and proliferation of immune system cells (1). In general, modulator compounds in the immune system are divided into two groups (causing an increase or a decrease in immune responses). The first group is used in a variety of infectious diseases and the second group is applied for organ transplants and autoimmune diseases (2).

In recent years, a great number of pharmaceutical studies have concentrated on identifying compounds which can stimulate or suppress the immune system for purposes such as finding anti-cancer compounds and effective drugs for autoimmune diseases and strengthening immune responses (3). Medicinal herbs have a wide range of secondary metabolites with different medicinal effects. The effectiveness of various plants has been demonstrated in numerous studies. However, these plants may contain toxic and hazardous compounds. Therefore, the assessment of toxicity and mutagenic potential is of great importance (4).

The prevalence of new diseases, drug resistance, high cost of chemical medicines and their adverse side-effects have led to extensive research on the favorable effects of medicinal herbs (5, 6). In recent years, several laboratory studies have confirmed the therapeutic effects of plants including anti-microbial (7), anti-cancer (8) and anti-diabetic properties (9), as well as valuable pharmaceutical compounds in these herbs (10).

The Apiaceae family is distributed in various regions and has many medicinal applications in Iran. This family has more than 300 genera and 2,500 species, worldwide. Overall, about 113 genera and 320 species of this plant have been found in Iran (11). Prangos is one of the most important genera of Apiaceae family with about 30 species, among which 15 are grown in Iran and 5 are endemic Iranian species (12).The medicinal properties of Prangos species can be categorized as anti-inflammatory, anti-parasitic, anti-virus, anti-bacterial and carminative features. These species can strengthen the nervous system and help with the extraction of urinary tract stones. These properties have been confirmed in many laboratory studies (13-15).

Prangos uloptera (P. uloptera) is one of the most important Prangos species with a wide distribution in Mediterranean regions and Central and Western Asia including Iran. This species, which normally grows on cliffs, is used in traditional medicine for the treatment of digestive disorders, wounds and leukoplakia (16 , 17). The presence of effective pharmacological compounds including various types of coumarins, furanocoumarins, pinenes and other secondary metabolites has triggered a lot of scientific interest in the medicinal effects of these species (18). Laboratory studies on the medicinal properties of this plant have shown several properties including anti-cancer, anti-bacterial and antioxidant features (19).

Moreover, Prangos crossoptera (P. crossoptera), which is a Prangos species from Zagros mountains, is another endemic Iranian species, with a wider distribution in western regions (20). Despite numerous studies on the therapeutic effects and active constituents of Prangos species, no study has been conducted on the therapeutic and medicinal properties of P. crossoptera; in fact, research on this species is limited to botanical characteristics (21-23).

Considering the variety of secondary metabolites with medicinal properties in Prangos species and their wide distribution in different parts of the world, especially Iran, extensive pharmaceutical research has been conducted on introducing the positive effects of these plants (24). Overall, assessing the medicinal properties and mutagenic potentials of P. crossoptera (particularly native species) is of high importance for introducing new species with health benefits and mutagenic safety. In this study, for the first time, the effects of methanolic extracts from two endemic Prangos species on the growth and proliferation of human lymphocytes were assessed; moreover, their mutagenic potentials were examined by Ames test.

Methods

Species determination and sample preparation: P. crossoptera and P. uloptera were collected at three stages (before blooming, flowering and seed production) during May and June 2014 in the highlands of northern Sanandaj and Saral Divandareh in Kordestan, Iran. The scientific names of the species were determined at the Agriculture and Natural Resources Research Center of Sanandaj. Then, the samples with herbarium No. 1412 (P. crossoptera) and 1536 (P. uloptera) were stored at the herbarium of Agriculture and Natural Resources Research Center at Kurdistan University.

Various parts of the samples including the flowers, leaves, stems, roots and seeds were removed and washed separately; afterwards, they were completely

dried in shade and milled. Then, 50 g of the obtained powder, along with 150 ml of 96% methanol, was placed in the shaker at a speed of 160 rpm at 25 °C for 72 hours. The obtained extracts were condensed at 40°C by the rotary vacuum machine after passing a paper filter (three times); afterwards, the extracts were freeze-dried. The obtained extracts were maintained in sealed sterile plastic containers at 4 °C. To prepare different concentrations of the obtained extracts, 10 mg of each extract was solved in 500 µl of dimethyl sulfoxide 3% (DMSO). Then, by using phosphate-buffered saline (PBS), the samples were diluted to final concentrations of 10, 100, 500, 1000, 1500, 2000 and 2500 µg/ml under sterile conditions in a laminar flow cabinet.

Lymphocyte extraction, maintenance and culture: Blood samples (10 ml) were collected from healthy donors (with no prior history of using antibiotics or other medicines affecting the immune system) in tubes containing heparin. Under sterile conditions, the samples were transferred to 15 ml falcons, containing 5 ml of lymphodex, and were centrifuged for 20 minutes at 1800 rpm. After removing the falcons from the centrifuge, the blood samples were divided into three layers. The upper yellow layer contained platelets, the middle white layer contained lymphocytes and the third layer contained red blood cells. The isolated lymphocytes were separated from other layers under sterile conditions by Pasteur pipettes and were transferred to culture plates, containing RPMI medium (10% bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine and 1 mM pyruvate). The lymphocytes were transferred, incubated and cultured for 48 hours in 5% CO_2.

Investigation of lymphocyte proliferation, using MTT assay: To study the effects of the extracts on the growth and proliferation of lymphocytes, we applied MTT assay, using filtered tetrazolium bromide (a concentration of 5 mg/ml). This assay is a colorimetric test and is based on the revival of yellow tetrazolium crystals [with the molecular and chemical formulae of C18H16BrN and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide, respectively] and formation of insoluble blue formazan crystals by succinate dehydrogenase.

The color intensity caused by the dissolution of crystals is directly related to the number of cells in the culture plate. For performing MTT assay, 180 µl of cellular suspension and 20 µl of different concentrations of plant extracts were added to each well of the plate to reach a final volume of 200 µl. After 72 hours of cell incubation at 37°C, 20 µl of the filtered MTT was added and incubation continued for 4 hours. At the end of the incubation, to solve MTT insoluble crystals, 10 µl of 0.04 M HCL, triton X100 and 2-propanol were used. Finally, the absorption of MTT was measured at 492 nm by ELISA reader. In this study, 1-3% concentrations of DMSO were used as negative controls. To determine the effects of each extract concentration, the process was repeated three times. Finally, the proliferation of lymphocytes was calculated, using the following formula:

Survival percentage = Absorption of each sample/ absorption of negative control × 100

The evaluation of the mutagenicity of extracts by Ames test

Tests confirming TA98 strain: To evaluate the mutagenic potential of extracts with different concentrations, Ames test was used. In this test, different strains of Salmonella were used. These bacteria have different mutations in histidine operon; therefore, they cannot grow in the absence of this amino acid. However, in case of exposure to a mutant substance, the bacteria may regain the ability to synthesize histidine by undergoing another mutation that corrects the original mutation.

In this study, Salmonella typhimurium strain TA98 was used. This Salmonella strain in addition to histidine dependence and ampicillin resistance has two important mutations due to the presence of plasmid pKM101; these features are considered for identifying this strain. The mutations of this strain are as follows: 1) rfa mutation, which is indicated by sensitivity to crystal violet; and 2) UVrB mutation which disables the bacteria to repair the damage caused by UV rays (in case of exposure to UV rays, the cells will be destroyed).

The strains were tested for histidine dependence, ampicillin resistance and UV sensitivity. For this purpose, 1.5×10^8 bacteria, known as 0.5 McFarland, were dissolved in 1.5 ml microtubes, containing 1 ml of PBS solution. Afterwards, they were separately placed in the minimum medium (containing Agar, glucose, citric acid monohydrate, potassium phosphate dibasic, sodium ammonium phosphate and magnesium sulfate) and master medium (containing nutrient agar, sodium chloride, biotin and histidine); then, they were spread evenly using a sterile swab. To confirm ampicillin resistance, we used 10 mg of ampicillin from the standard disk. In order to determine the presence of UVrB mutation, after the complete dispersion of bacteria in the master medium, half of the culture plate

was coated with aluminum foil and placed under a UV lamp for 10 seconds. Finally, histidine-dependence was evaluated by inoculating similar amounts of bacterial suspension in minimum and master media.

The assessment of mutagenicity in different concentrations of plant extracts: After performing strain tests, in order to determine the rate of mutagenesis, the number of returned colonies, the percentage of returned colonies and Qm index were measured and compared. For this purpose, 10 mg of different concentrations of the extracts was dissolved in 100 ml of DMSO. Dilution was performed with sterile PBS to obtain concentrations of 10, 100, 500, 1000, 1500, 2000 and 2500 µg/ml.

To determine the rate of mutagenesis, paper discs immersed in different concentrations of the extracts were placed in the minimum medium containing Salmonella typhimurium TA98. Afterwards, for each concentration of the extracts, Qm index was defined. The mentioned index is able to determine the mutagenicity of the samples. Overall, Qm less than 1.6 indicates lack of mutagenicity, Qm in the range of 1.7-1.9 shows the mutagenic potential of the sample and Qm value≥2 indicates the mutagenicity of the sample (21). In this study, 100 µl of 1 mg/ml sodium azide solution was used as positive control and 1-3% DMSO concentrations were used as negative controls.

For each concentration of the extracts, the procedure was repeated three times. Finally, the number of grown colonies on the minimum culture (as a result of the recovery of histidine synthesis) was compared with the number of colonies grown in the vicinity of positive and negative controls. The following formula was used to determine Qm index:

Quantitative mutagenicity index (Qm)= The number of returned colonies from the sample/ The number of colonies from the negative control

Results

The effects of methanolic extracts on lymphocyte proliferation: The findings showed that the extracts from different parts of the studied species increased the proliferation of lymphocytes. The extent of the effect of extracts was dose-dependent. In fact, in most extracts, 10 and 100 µg/ml concentrations had the least significant effects on the proliferation of lymphocytes and a concentration of 2500 mg had the most significant impact. The comparison of various parts of the extracts showed the greatest impact at a concentration of 2000 µg/ml in P. uloptera leaves. The minimum effect was reported in seed extracts at concentrations of 10-100 µg/ml. Similar results were reported regarding the effectiveness of extracts from different parts of P. crossoptera. In this species, the greatest impact was reported in leaf extracts at a concentration of 2500 µg/ml; the least significant impact was found in seed extracts at a concentration of 10-100 µg/ml (fig 1 & 2). The effectiveness of the extracts from different parts of P. uloptera decreased in the following order: leaves, roots, flowers, stems and seeds, respectively. In P. crossoptera, the effectiveness decreased in the following order: leaves, flowers, roots, stems and seeds. The comparative assessment between the two species also demonstrated the higher effectiveness of P. uloptera, compared to P. crossoptera.

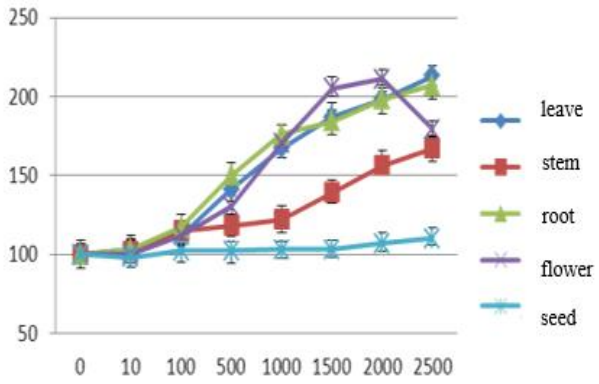

Figure 1. The effects of the extracts from different parts of P. crossoptera on the growth and proliferation of human lymphocytes (each value shows the mean percentage of lymphocyte survival ± standard error of the mean)

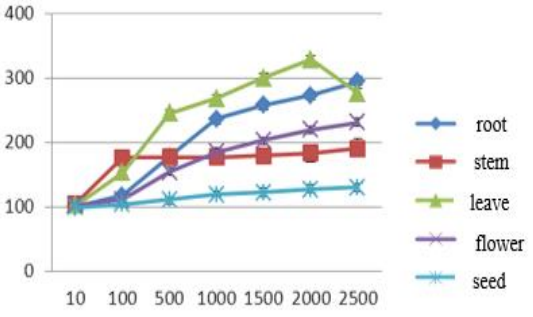

Figure 2. The effects of the extracts from different parts of P.uloptera on the growth and proliferation of human lymphocytes (each value indicates the mean percentage of lymphocyte survival±standard error of the mean)

The assessment of mutagenicity

The results of TA98 strain tests: The results showed that the inoculated bacteria in the minimum culture lacked the minimum growth ability on this medium (fig 3); in fact, they were able to grow only in the presence of histidine and biotin (fig 4). For other mutations in this strain, it was revealed that the bacteria were sensitive to crystal violet (fig 5), and in case of exposure to UV rays, they were unable to grow (fig 6). These results confirm the presence of rfa and UVrB mutations. Moreover, the full growth of bacteria around the ampicillin disk confirmed the presence of plasmid PKM101 (fig 7).

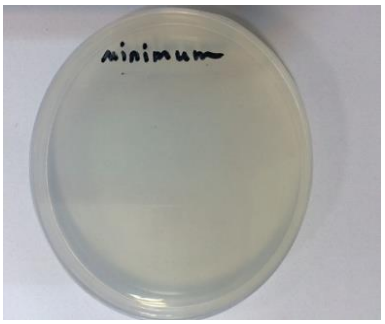

Figure 3. Zero growth of Salmonella typhimurium in the minimum culture without histidine

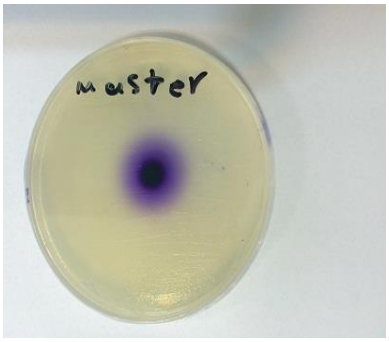

Figure 4. The growth of Salmonella typhimurium in the master culture containing histidine

Figure 5. The sensitivity of Salmonella typhimurium to crystal violet

Figure 6. The resistance of Salmonella typhimurium to ampicillin

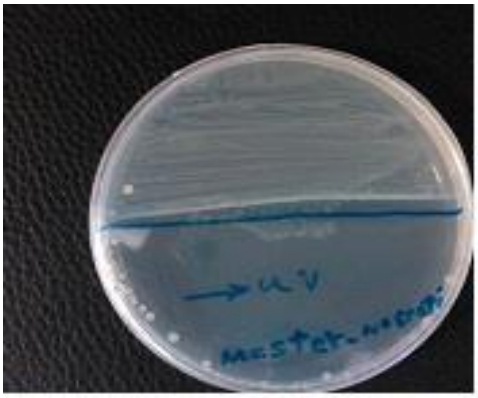

Figure 7. Zero growth of Salmonella typhimurium during UV exposure

The mutagenicity of plant extracts at different concentrations: The number of grown colonies showed that in equal amounts of bacterial suspension, 300 colonies grew on the surface of master medium, 28 colonies grew on the the minimum medium containing negative control (1-3% DMSO) and 75 colonies grew on the surface of the minimum medium containing positive control (sodium azide). This represents spontaneous mutation (9.3%) in the studied strain. The investigation of Qm index at studied concentrations showed that this index gradually increased by amplifying the concentration; however, mutagenicity was not reported in any of the studied concentrations. Qm index was calculated to be less than 1.6 in all studied concentrations of the extracts from different parts of two species. According to the definitions of Qm index, which indicate lack of mutagenicity at a value less than 1.6, the obtained extracts lacked mutagenicity (25). Also, the comparison between the average Qm index of the two species showed that the number of returned colonies from P. crossoptera extracts was lower than P. uloptera extracts (table 1).

Table 1. The results of Qm calculations for different concentrations of P. uloptera and P. crossoptera

The extracted parts	Herbal species	Qm index Mean±SEM					
		500	1000	1500	2000	2500	3000
Leaf	Uloptera	1.23±0.06	1.25±0.03	1.29±0.04	1.35±0.04	1.39±0.1	1.44±0.09
Flower		1.34±0.08	1.36±0.06	1.37±0.2	1.39±0.04	1.46±0.06	1.48±0.1
Root		1.32±0.03	1.34±0.02	1.37±0.03	1.40±0.03	1.47±0.07	1.49±0.04
Stem		1.25±0.07	1.27±0.09	1.29±0.04	1.31±0.04	1.38±0.02	1.42±0.02
Seed		1.20±0.09	1.23±0.06	1.26±0.1	1.28±0.09	1.35±0.06	1.38±0.03
Leaf	Crossoptera	1.25±0.1	1.28±0.09	1.31±0.04	1.33±0.07	1.37±0.09	1.40±0.04
Flower		1.27±0.03	1.30±0.04	1.33±0.05	1.37±0.1	1.42±0.03	1.47±0.07
Root		1.34±0.04	1.34±0.06	1.37±0.02	1.41±0.02	1.44±0.04	1.45±0.02
Stem		1.20±0.02	1.24±0.05	1.25±0.02	1.28±0.03	1.32±0.03	1.35±0.03
Seed		1.24±0.03	1.26±0.06	1.30±0.08	1.35±0.02	1.37±0.02	1.40±0.06

Discussion

The results showed that methanolic extracts from different parts of the studied Prangos species enhanced the growth and proliferation of human lymphocytes and lacked mutagenic potential. According to the findings, the increased growth was dose-dependent. In other words, the highest rate of growth was observed at the highest concentrations of most herbal extracts. Also, the comparative assessment showed that the effectiveness of the extracts followed a similar pattern; in fact, in both species, the leaves and seeds had the most and least significant effects on the growth and proliferation of lymphocytes, respectively. Generally, the methanolic extracts of P. uloptera showed more lymphocyte growth and proliferation, compared to P. crossoptera.

The outbreak of infectious diseases, genetic disorders and acquired deficiencies, affecting the immune system, has led to significant developments in pharmaceutical research in recent years to identify new drug compounds, especially herbal compounds which are capable of modulating immune responses (26). In this regard, herbal compounds have particular importance by stimulating the growth and proliferation of lymphocytes as one of the main components of immune system in increasing immune responses in a variety of infectious diseases (27).

Sumardi and colleagues showed that the methanolic extract of Myrmecodia tuberosa increased the proliferation of TCD4+ cells in studied rats, whereas it had no significant effects on the number of TCD8+ cells (28). Also, a study by Wang et al. showed that Allium sativum extracts increased the activity of natural killer cells, elevated the secretion of interferon-alpha, interleukin-2 and TNF-α and reduced IL-4 volume (29). Moreover, Punturee and colleagues showed that the aqueous extract of Centella asiatica increased the proliferation of lymphocytes, while the ethanolic extract resulted in the suppression of lymphocyte proliferation (30). Despite the positive properties of medicinal plants, numerous studies have shown that these plants may contain toxic, mutagenic and health-threatening compounds. Therefore, in recent years, several studies have been conducted to ensure the safety of most important medicinal plants by considering the absence of mutagenicity in medicinal plants used in different communities. Some studies have confirmed the safety and some have demonstrated the potential hazards of these plants (31).

Eren et al. studied the mutagenicity and toxicity of the aqueous extracts of Limonium globuliferum. The results showed that despite the medicinal use of this plant, its extract led to chromosomal damages in the meristematic cells of onions; the results of Ames test showed the mutagenic potential of this plant (32). In a similar study by Ali and colleagues, the extracts of Limonium sokotranum, which is traditionally used for the treatment of fungal diseases, were capable of destroying human amniotic epithelial cells at a concentration of 615.1 mg/ml (33).

Many studies have indicated the safety of many medicinal plants in terms of mutagenic potential. Thepouyporn et al. showed that the aqueous extracts of Peperomia Pellucida, Colocasia esculenta and Brachiaria mutica, which are widely used as medicinal plants in Thailand, had no mutagenicity according to Ames test (34). In another study by Resende et al., the ethyl acetate extract of Baccharis dracunculifolia, which

is used as a medicinal herb in Brazil, not only lacked mutagenic potential, but also possessed anti-mutagenic effects (35). Considering the multiple applications of Prangos species in traditional medicine, substantial research has been conducted to confirm the therapeutic effects of these plants. Two Prangos species, examined in this study (uloptera and crossoptera), are endemic to Iran. The evaluation of therapeutic effects and mutagenic potential is of high significance due to the numerous medicinal properties of these species (especially for a variety of bacterial and fungal infections) and their wide distribution in most parts of Iran. Studies on the health benefits of the constituents of Prangos species have shown medicinal effects such as anti-microbial, antioxidant, anti-cancer and anti-diabetic properties (36). In a study on the antioxidant effects of Prangos ferulacea, secondary metabolites such as alkaloids, flavonoids and terpenoides in the extracts had more antioxidant characteristics, compared to vitamin E; this plant also affected the enzyme activity of glutathione S-transferases (37).

Studies on the antibacterial properties of the mentioned species have shown that among methanolic, ethanolic, aqueous and hexane extracts, the methanolic extracts of these plants have the most significant antibiotic property against Gram-positive/negative bacteria such as Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Klebsiella pneumonia (38). In a survey conducted by Ulubelen et al. on the anti-fungal and anti-bacterial activities of Prangos platychlaena, it was revealed that the extracts of this plant had anti-microbial effects against Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis and Candida albicans (39). Also, Fukuzawa et al. showed that Prangos pabularia and Prangos tschimganica contain compounds with significant antioxidant properties and possess lipid oxidation inhibitory effects (40).

Phytochemical studies on compounds, found in different parts of Prangos species, introduced different alkaloids, flavonoids, coumarins and terpenoids (41). A study by Alikhah-Asl et al. on compounds found in the aerial parts of P. uloptera showed that 35 compounds were present in the oil of this plant in both fresh and dry states. The most important compounds in the fresh state were as follows: alpha-pinene, trans-beta-ocimene, beta-caryophyllene, delta-3-carene, germacrene D and beta-myrcene. In the dry state, these compounds included beta-caryophyllene, alpha-pinene, caryophyllene oxide, spathulenol and germacrene D (42). In a survey conducted by Razavi and colleagues on coumarins found in the hexane extracts from the aerial parts of P. uloptera, the results showed that these compounds possess significant antioxidant properties (43). In a similar study, which focused on coumarins in roots, the results showed three new coumarin derivatives, which attributed a significant antioxidant property to this plant (44).

The assessment of the medicinal properties of P. uloptera root also showed that the extracts had a destructive effect on HeLa cancer cell line and contained a significant amount of antioxidant and anti-bacterial compounds (45). Also, a study by Zahri and colleagues on the induction of apoptosis by P. uloptera extracts showed that dichloromethane extracts from different parts of P. uloptera could induce apoptosis in McCoy cancer cell lines (46).

Unlike P. uloptera, which has been extensively assessed regarding its numerous therapeutic properties, no studies have been conducted on the benefits of P. crossoptera. The current research is one of the few studies on this medicinal herb. In fact, most related studies are concerned with the botanical properties of this species and its place of origin (47). Therefore, considering the favorable effects of these two species on the growth and proliferation of lymphocytes and the significant accumulation of secondary metabolites (particularly alpha-pinene and coumarin), these compounds seem to contribute to the effectiveness of these plants.

Finally, considering the positive effects of methanolic extracts from different parts of the studied Prangos species on lymphocyte proliferation and their non-mutagenicity, these species can be used as safe medicinal plants for patients with immune deficiencies and different bacterial infections.

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