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New Prospective Materials for Chemoprevention: Three *Phlomis*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AU, EG and YD designed the study and prepared the manuscript. Author HC managed the literature searches. Authors CS and MCU identified the species of plant and prepared plant extracts. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Plants are permanent sources of biologically active compounds, used by about 80% of the world population as manufactured drugs. The aims of this paper are to determine the mutagenic and antimutagenic activities of methanol, ethyl acetate and water extracts from *Phlomis nissoli* (PNM, PNE, PNW) *Phlomis pungens* var. *pungens* (PPM, PPE, PPW) and *Phlomis armeniaca* (PAM, PAE, PAW) against well-known mutagens.
Methodology: Mutagenic/antimutagenic activities were determined by Ames test.
Results: Extracts of *P. nissoli, P. pungens and P. armeniaca* were not mutagenic against TA98

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and TA100 in the condition both with and without S9 mix. The strongest antimutagenic action was revealed by PNE extract at a dose of 5000 µg/plate against 2-amino-flourene with 96% inhibition for TA98 with S9 mix. PNM extract alleviated the mutagenic action of 2-amino-anthracene and exhibited strong inhibition rates (93%, 78%, 57%, respectively) with S9 mix at all doses for TA100 strain. While PAE extract manifested 95%, 91% and 84% inhibition against 2-amino-flourene for TA98, it revealed 90%, 84%, and 87% inhibition ratios, respectively, making them as strongly antimutagenic against 2-amino-anthracene for TA100 with S9 mix.

Conclusion: *P. nissoli, P. pungens* and *P. armeniaca* extracts have significant antimutagenic capacities and they can represent a good model for the development of new drug formulations in pharmaceutical industry and they could be used in food industry as chemoprevention agent.

Keywords: Phlomis nissoli; Phlomis pungens var. pungens; Phlomis armeniaca; antimutagenicity; chlorogenic acid.

1. INTRODUCTION

In the midmost of 20th century, the act of the mutation in carcinogenesis was not well known and the certain evidence could not be supported [1]. Recently, it is known that mutation and especially gene mutations have a key role in the formation of carcinogenesis. It may be thought that decreasing of the mutation rates can reduce the frequency of cancer constitution. Our nature permanently encounters with mutagenic and carcinogenic agents and extermination of these chemicals seems to be very demanding process. To prevent consuming of mutagens and carcinogens is the best way for humans for decreasing the mutation ratio [2]. Nowadays it is the fact that plants and their products present one of the main sources for compounds with antimutagenic action and, indeed, several secondary plant metabolites have demonstrated chemo preventive activity against to genotoxic agents. These antimutagens and anticarcinogens may inhibit one or more stages of the carcinogenic process and prevent or delay the formation of cancer [3]. So plant metabolites are in the center of the researches aiming to discover new antimutagenic substances from various sources including plants [4].

Screening of carcinogens and other chemicals for mutagenicity *in vitro*, starting with the use of a base-substitution strain of *Escherichia coli* [5] and then the new set of tester strains modified to detect different types of gene mutations in *Salmonella typhimurium* [6]. The Salmonella mutagenicity assay, which was developed initially as a spot test then as a plate-incorporation test [7] using strains of *Salmonella* bacteria is a deceptively simple tool that can be used to detect the mutagenicity of environmental chemicals, environmental mixtures, body fluids, foods, drugs, and physical agents. Although more complex tests can be applied to confirm and characterize further the mutagenic activity of the agent [6], the *Salmonella*/microsome assay (Salmonella test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations [8]. The Ames test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs. For these reasons *Salmonella* mammalian microsome mutagenicity assay has been central to the field of genetic toxicology since the 1970s. There are some published data on the antimutagenic activity of plants and some plant metabolites against well-known mutagens as determined by this test system [9].

The genus *Phlomis* as perennial herbs of Lamiaceae family consists of more than 100 species distributed in Europe, Asia, and Africa. In Turkish flora, 52 taxa include 6 varieties, 12 natural hybrids, 34 endemic taxa which are distributed [10]. Many species of this genus have usage for medicinal and aromatic purposes. The aerial parts of some species including *Phlomis* have distinctive taste and is used for the herbal tea in traditional medicine as stimulants, tonics, carminative, appetizer, and diuretics [11]. It has been shown that *Phlomis* species have exhibited the various biological properties such as in treatment of ulcer and hemorrhoids, anti-inflammatory, antimicrobial, antiulcerogenic, immunosuppressive, and free radical scavenging activities [12]. Pytochemical investigations of *Phlomis* species were the subject to several studies, and, consequently, iridoid and phenyl propanoid glycosides were isolated [13]. Some parts of Phlomis species such as *P. crinita* was evaluated by Ames test for antimutagenic action [14]. Also mutagenicity and antimutagenicity of *P. fruticosa* essential oils were investigated by this

test system [15]. DNA protecting activity of *P. armeniaca* [16] and antitumor activity of *P. pungens* were studied [17].

The individual phenolic components and their concentrations have been already assessed in our previous studies [18,19]. In this context, the detected profiles were also given to further evaluating in this article in order.

In view of the above, the goal of this study was to thoroughly examine the mutagenic and anti-mutagenic activities of methanol, ethyl acetate and water extracts from *Phlomis nissoli* (PNM, PNE, PNW) *Phlomis pungens* (PPM, PPE, PPW) and *Phlomis armeniaca* (PAM, PAE, PAW) wild plants distributing in Turkey. This is the first report on mutagenic and antimutagenic activities of these *Phlomis* species.

2. MATERIALS AND METHODS

2.1 Plant Material

The aerial parts of *Phlomis* species were collected at the flowering stage from different regions of Turkey. Taxonomic identification of the plant materials were confirmed by the senior taxonomist Dr. Olcay Ceylan, in Department of Biology, Mugla University. The voucher specimens have been deposited at the Herbarium of the Department of Biology, Mugla University, Mugla-Turkey.

Localities and collection periods of *Phlomis* species are as following:

1. *P. armeniaca* WILLD.: Kemerkaya town, Tömsö castle, Bolvadin-Afyonkarahisar-Turkey on 23 June 2013 (38° 53' 59"N 31° 06' 13"E, 1520 m, Voucher No: MUH1877)
2. *P. nissolii* L.: Cumra-Bozkır highway, Konya-Turkey on 20 July 2013 (10-15 km, 37° 32' 46"N 32° 40' 22"E, 1026 m, Voucher No: MUH2445).
3. *P. pungens* WILLD. var. *pungens* WILLD.: Karagöl-Çubuk highway, Ankara-Turkey on 13 July, 2013 (2 km, 40° 16' 27''N 33° 00' 52"E, 1080 m; Voucher No: MUH3544)

2.2 Preparation of the Solvent Extracts

To produce solvent extracts the air-dried samples (20 g) of the aerial parts of *P. armeniaca* were separately extracted with a Soxhlet extractor for 5 h with 250 ml of solvents (ethyl acetate and methanol). For water extract, the air-dried samples (20 g) were extracted by boiling deionized water (200 mL) for 15 min. Ethyl acetate and methanol were then removed by a rotary evaporator. The water extract was freeze–dried. All extracts were stored at +4℃ until analyzed [20]. Yields of the ethyl acetate, methanol, and water extracts were determined as 2.45%, 15.50% and 14.05 (w/w) from *P. armeniaca*, 3.78%, 20% and 14.80% (w/w) for *P. nissolii,* 2.93%, 15.45% and 17.25% (w/w) for *P. pungens* var. *pungens*, respectively.

2.3 Bacterial Strains

Salmonella typhimurium strains TA98 and TA100, histidine-requiring mutants, were kindly provided by Microbiology Research Laboratory, Science Faculty, Selcuk University and are maintained as described by Maron and Ames [21]. These strains were grown overnight from frozen cultures for 12–14 h in Oxoid Nutrient Broth No. 2. The genotype analysis of strains (histidine requirement, the presence of the plasmid pKM101, rfa mutations, and uvrB mutation) were checked routinely. They were stored at −80°C for further use. TA98 strain is competent for determination of frame shift mutations while TA100 strain is capable for revealing of base pair exchange mutations.

2.4 S9 Preparation

S9 is the mitochondrial enzyme mix required for metabolic activation of indirect acting mutagens like 2-Aminoflourene and 2-Aminoanthracene. The metabolic activation mixture (S9 fraction), prepared from livers of male Sprague–Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg/kg), was purchased from Molecular Toxicology Inc. (Boone, NC, USA). The metabolic activation system consisted of 4% S9 fraction, 1% 0.4 M MgCl2, 1% 1.65 M KCl, 0.5% 1 M D-glucose-6-phosphate disodium and 4% 0.1 M NADP, 50% 0.2 M phosphate buffer and 39.5% sterile distilled water [21] and this mixture prepared fresh for each experiment.

2.5 Determination of Toxic Concentration of Extract

The extract concentrations were selected on the basis of a preliminary toxicity test performed according to Dean, Brooks [22]. Non toxic doses of the plant extracts were determined as follows:

For *P. nissolii* water extract (PNW): 10000, 5000, and 1000 µg/plate; for ethyl acetate

extract (PNE): 5000, 1000, and 500 µg/plate; for methanol extract (PNM): 10000, 5000, and 1000 µg/plate.

For *P. pungens* water extract (PPW): 10000, 5000, and 1000 µg/plate; for ethyl acetate extract (PPE): 1000, 500, and 100 µg/plate; for methanol extract (PPM): 10000, 5000, and 1000 µg/plate.

For *P. armeniaca* water (PAW), ethyl acetate (PAE) and methanol (PAM) extract: 10000, 5000, and 1000 µg/plate

2.6 Mutagenicity Test

In our study, mutagenicity was assayed by the Ames test (*Salmonella*/microsome assay) according to Maron and Ames [21]. The test was performed without and/or with metabolic activation.

To assay mutagenic activity, three different concentrations of each extract were tested by plate incorporation method. Briefly, the bacterial strains were incubated in Nutrient Broth for 16 h at 37℃ in an orbital shaker to get a turbidity of 1-2 × 10^9 colony forming units (CFU/ml). 0.1 ml of an overnight culture of bacteria, 0.5 ml of sodium phosphate buffer (0.2 M, pH 7.4 for assay without S9) and 0.1 ml of different concentrations of each extract added into the 2.5 ml molten top agar supplemented with (0.5 mM L-histidine/D-biotin solution). They were mixed using vortexer for 8 seconds. Then complete mixture was poured on minimal agar plates. The plates were incubated at 37℃ for 48-72 h and the revertant bacterial colonies of each plate were counted. Data were collected with a mean ± standard deviation of two experiments (n = 3). In each assay positive and negative controls were included. The known mutagens 4-nitro-*O*-phenylenediamine (4-NPDA, 5 µg/plate) for *S. typhimurium* TA98 and sodium azide (SA) (5 µg/plate) for *S. typhimurium* TA100 were used as positive controls in the absence of S9 metabolic activation enzymes. 2-aminofluorene (2-AF, 7.5 µg/plate) and 2-aminoanthracene (2-AA, 5 µg/plate) were used as positive controls with S9, respectively. Dimethyl sulfoxide (DMSO, 100 µL/plate) and sterile distilled water served as negative (solvent) control (plates were prepared as: 100 µl DMSO +100 µl bacteria+ 500 µl S9/buffer).

The possible mutagenicity was investigated for each concentration tested. The sample was considered mutagenic when a dose–response relationship was detected and a two-fold increase in the number of revertants was observed for at least one concentration.

2.7 Antimutagenicity Test

Only extracts considered as non toxic and non-mutagenic were subject to this experiment employing the method of plate incorporation developed by Maron and Ames [21]. Three different concentrations of plant extract (10000-1000 µg/plate for PNW, 5000-500 µg/plate for PNE, 10000-1000 µg/plate for PNM; 10000-1000 µg/plate for PPW, 1000-100 µg/plate for PPE, 10000-1000 µg/plate for PPM; 10000-1000 µg/plate for PAW, PAE and PAM) were associated with known mutagens in tests in the absence and presence of metabolic activation, using *S. typhimurium* tester strains TA98 andTA100. Two and a half milliliters of top agar (45℃) were added with 0.1 mL of bacterial culture grown in nutrient broth overnight, 0.5 mL of S9 mix (or buffer solution – in the absence of metabolic activation), 0.1 mL of positive mutagen solutions and 0.1 mL of tested extracts. The content of each tube was poured onto a plate of minimal glucose agar. After solidification of the top agar, the plates were incubated for 48-72 h at 37℃, and the number of revertant colonies per plate was counted. Triplicate plates were run for each assay.

The number of revertant colonies grown on plates containing the mutagen without plant extract was defined as 100% with 0% inhibition. The antimutagenic potential (Inhibition) was determined by equation: [(A-B)/(A-C)]× 100, where A = No. of his. revertants in the absence of sample, B = No. of his. revertants in the presence of sample, C = spontaneous revertants [3,9]. Results were interpreted as no antimutagenic effect when the inhibition was lower than 25%, a moderate effect for a value between 25% and 40% and strong antimutagenicity for values greater than 40% [23].

2.8 Statistical Analysis

Except for inhibition rates, all values obtained are expressed as the mean ± S.D. The evaluation of statistical significance was determined by one-way ANOVA (SPSS 13.0 for Windows). The level of significance was set at $P = .05$.

3. RESULTS

3.1 The Earlier Chemical Fingerprints of the Phlomis Extracts

The identification and quantification of individual phenolic components of the studied extracts have been evaluated in our previous studies [18,19]. These results are listed in Table 1. Apparently, chlorogenic acid was detected as the predominant phenolic in all extracts and ranged from 1.35 to 17.38 mg/g extract. The content might be attributed to the observed biological effects for the *Phlomis* extracts.

3.2 Evaluation of Mutagenicity

Table 2 shows the mean number of revertants/plate, the standard deviation after the treatments with the plant extracts, observed in *S. typhimurium* strains TA98, TA100, in the presence (+S9) and absence (−S9) of metabolic activation. The results showed that all extracts did not induce an increase in the number of revertant colonies relative to the negative control, indicating mutagenic activity. In order to investigate whether there was a dose relationship between doses, 3 different doses of nine *Phlomis* extracts were examined and the extracts did not induced two-fold increase of spontaneous revertants at all test concentrations. Therefore extracts of *P. nissolii, P. pungens and P. armeniaca* were not found to be mutagenic for TA98 and TA100 in the condition both with and without S9 mix. These results suggest that these plant extracts may be safe for use in humans and should be considered for further medical development studies.

3.3 Evaluation of Antimutagenicity Test

3.3.1 *P. nissolii*

P. nissoli water extract (PNW) can be considered as strong antimutagenic at a dose of 10000 µg/plate for TA98 with a rate of 59% against 4-NPDA and it manifested moderate antimutagenicity (40%) at a dose of 5000 µg/plate in the absence of S9 (Figs. 1a, Table 3). For TA100 strain only 10000 µg/plate dose of extract showed moderate antimutagenic activity both in the presence and absence of metabolic activation against SA and 2-AA, respectively (30% and 37%) (Figs. 2a, Table 3). Other doses were decided to be weak antimutagenic for two tester strains against mutagens in the presence and absence of S9 mix. Although *P. nissolii* ethyl acetate extract (PNE) revealed moderate antimutagenicity (25%) against 4-NPDA and SA (33%) at a dose of 5000 µg/plate in the absence of S9 mix for TA98 and TA100 respectively, the same extract exhibited excellent antimutagenic action against 2-AF with a rate of 96% for TA98 and against 2-AA with a rate of 84% for TA100 in the presence of S9 enzymes. Also 1000 µg/plate doses showed strong antimutagenic activity (44% and 66%) with S9 mix for TA98 and TA100 strains, respectively (Figs. 1a, 2a and Table 3). A moderate antimutagenic activity of PNE was observed at a concentration of 500 µg/plate in the presence of S9 for TA100 while no antimutagenic activity was determined at the same dose for TA98. It can be stated that S9 enzymes ameliorated the action of mutagens at higher doses. Methanol extract of *P. nissolii* revealed very strong antimutagenicity (62%, 58%, 78%) against 4-NPDA at all doses in the absence of S9 mix for TA98 (Figs. 1a, Table 3). Except for 1000 µg/plate dose, the inhibition of 2-AF was increased by S9 mix with rates of 73% and 72% (strong antimutagenicity). PNM extracts alleviated the mutagenic action of 2-AA and exhibited excellent inhibition rates (93%, 78%, 57%, respectively) with S9 mix at all doses for TA100 strain while it revealed moderate antimutagenicity (37%) at a concentration of 10000 µg/plate without S9 mix (Figs. 2a, Table 3).

3.3.2 *P. pungens*

When the *P. pungens* water (PPW) extract were evaluated, it was determined that only 10000 µg/plate dose of extract showed moderate antimutagenic activity with a rate of 32% in the absence of S9 for TA98 (Figs. 1b, Table 3) and it exhibited a moderate action with a rate of 29% for TA100 strain in the presence of S9 metabolic enzymes (Figs. 2b, Table 3). PPE extract alleviated the actions of mutagens at 10000 µg/plate dose as strong antimutagenic (42%) agent for TA98 and TA100 (76%) without S9 mix. At the same dose extract showed moderate (37%) antimutagenicity for TA 98 and strong antimutagenicity (57%) for TA100 by the addition of S9 (Figs. 1b, 2b and Table 3). Extract of PPM can be described as strong antimutagenic at 10000 µg/plate dose against 4-NPDA (48% inhibition) and 2-AF (58% inhibition) in the presence and absence of S9 mix, although 5000 µg/plate dose can be considered as moderately antimutagenic, as they inhibited 33% (with S9) and 37%(without S9) of revertants, respectively for TA98 strain (Figs. 1b, Table 3). Also 1000

µg/plate dose of extract had moderately antimutagenic capacity (36%) in the absence of S9 for TA98. When combined with SA and 2-AA, the extract at a concentration of 10000 µg/plate showed 76% and 77% inhibition, respectively and can be considered strong antimutagenic for TA100. While the dose of 5000 µg/plate revealed moderate antimutagenicity with a rate of 33% without S9, the extract induced the inhibition of revertants and were determined as strong antimutagenic with a ratio of 48% by adding S9 mix for TA100 (Figs. 2b and Table 3).

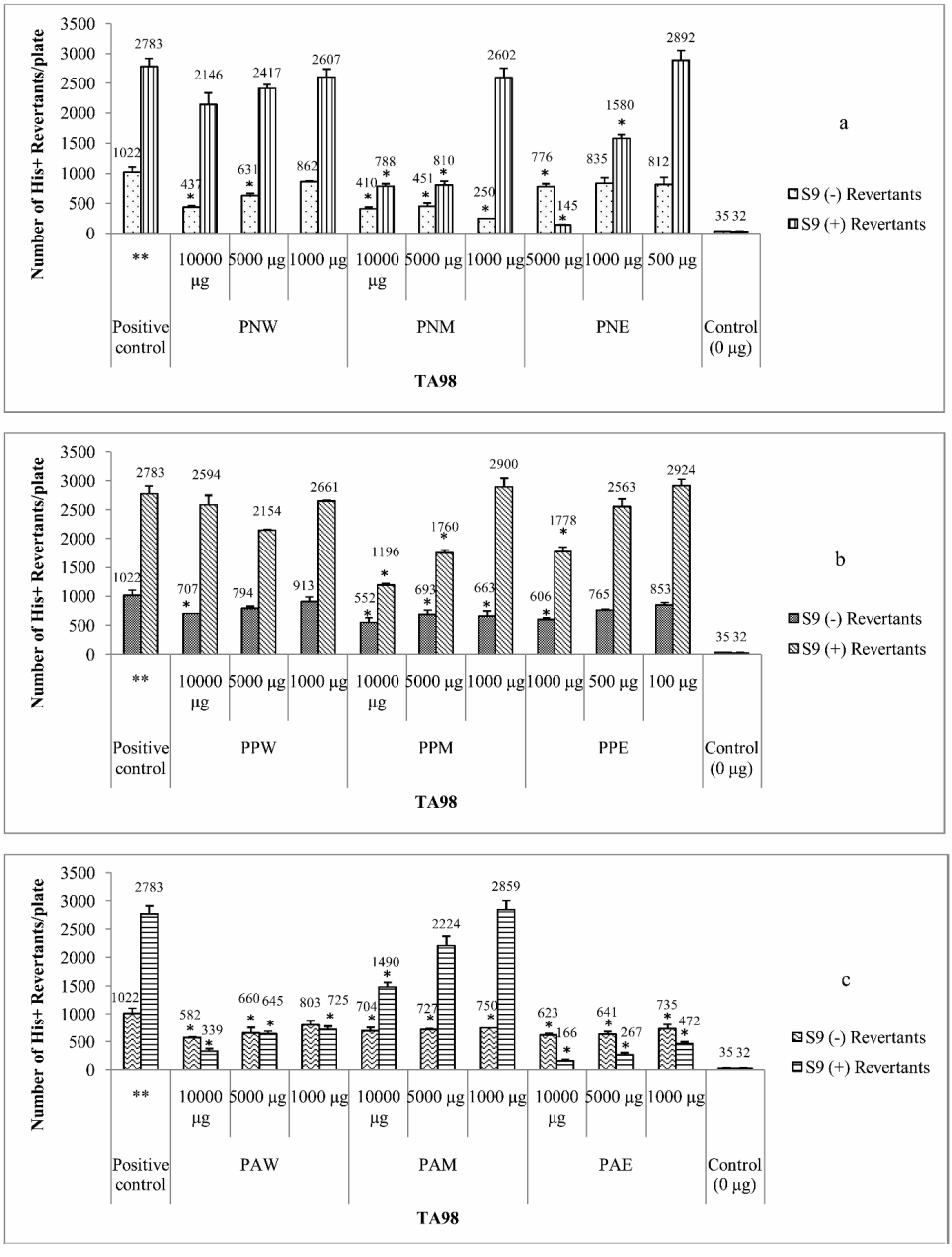

Fig. 1. Decreasing number of TA98 revertants/plate with addition of a) *P. nissoli* extracts b) *P. pungens* extracts c) *P. armeniaca* extracts against direct and indirect mutagens

** P<0.05 (ANOVA)*

*** Positive controls: 2-Aminofluorene (7.5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; 4-nitro-O-phenilendiamine (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA98 strain*

Table 1. Phenolic components in the solvent extracts from *P. armeniaca* [18], *P. nissolii, P. pungens* var. *pungens* [19]

No	Phenolic components	Concentration (mg/g extract)								
		P. armeniaca			*P. nissolii*			*P. pungens* var. *pungens*		
		Ethyl acetate	Methanol	Water	Ethyl acetate	Methanol	Water	Ethyl acetate	Methanol	Water
1	Protocatechuic acid	0.37±0.022	0.26±0.005	0.28±0.005	0.17±0.004	0.18±0.004	0.10±0.017	0.15±0.003	0.18±0.005	0.20±0.004
2	(+)-Catechin	2.00±0.070	0.77±0.016	0.98±0.018	1.34±0.060	0.69±0.013	0.74±0.001	0.68±0.010	0.81±0.016	0.33±0.014
3	p-Hydroxybenzoic acid	0.57±0.020	nd	0.16±0.001	0.56±0.020	nd	0.24±0.101	0.19±0.007	0.19±0.001	0.29±0.010
4	Chlorogenic acid	3.21±0.060	11.95±0.919	8.58±0.125	1.53±0.060	9.18±0.713	6.39±0.012	1.35±0.060	17.38±0.906	11.72±0.188
5	Caffeic acid	0.43±0.010	0.15±0.011	0.39 ±0.012	0.36±0.010	0.11±0.009	0.29±0.012	0.20±0.007	0.13±0.011	0.80±0.014
6	(-)-Epicatechin	0.13±0.007	nd	nd	nd	nd	nd	nd	nd	nd
7	Ferulic acid	nd	0.29±0.011	0.21±0.012	0.10±0.007	0.10±0.009	0.12±0.012	0.09±0.007	nd	0.26±0.010
8	Benzoic acid	0.65±0.020	nd	6.14±0.089	nd	0.26±0.025	3.63±0.084	nd	nd	nd
9	Rutin	0.98±0.040	0.85±0.016	1.09±0.018	0.66±0.010	4.66±0.050	3.94±0.068	0.46±0.010	0.49±0.016	nd
10	Rosmarinic acid	nd	nd	nd	0.92±0.030	4.56±0.088	nd	0.19±0.002	4.68±0.113	nd
11	Apigenin	0.59±0.010a	nd	nd	0.41±0.010	0.81±0.013	nd	0.18±0.010	0.32±0.016	nd

nd: Not detectable

Table 2. Mutagenicity of water, ethyl acetate and methanol extracts of three *Phlomis* species towards *S. typhimurium* TA98 and TA100 strains with and without S9

	Concentration (mg/plate)	Number of his$^+$ revertants/plate											
		Water extracts				Ethyl acetate extracts				Methanol extracts			
		TA 98		TA 100		TA 98		TA 100		TA 98		TA 100	
		S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)
†Negative control	100 µl/plate	35±2	32±4	103±6	132±13	35±2	32±4	103±6	132±13	35±2	32±4	103±6	132±13
®Positive control		2626±29*	2866±57*	1952±169*	2915±56*	2626±29*	2866 ±57*	1952±169*	2915±56*	2626±29*	2866±57*	1952±169*	2915±56*
	0	36±5	37±7	116±18	144±13	36±5	37±7	116±18	144±13	36±5	37±7	116±18	144±13
Phlomis nissolii	10000	21±3	32±3	121±11	162±6					34±5	56±6	167±8	17 3±2
	5000	30±4	39±2	142±10	128±12	28±3	41±7	182±6	159±8	25±6	50±2	146±8	173±12
	1000	27±1	35±3	156±13	148±4	35±1	43±2	147±4	157±15	25±1	51±3	150±21	186±21
	500					41±3	40±2	72±3	133±1				
Negative control	100 µl/plate	35±2	32±4	103±6	132±13	35±2	32±4	103±6	132±13	35±2	32±4	103±6	132±13
Positive control		2626±29*	2866±57*	1952±169*	2915±56*	2626±29*	2866 ±57*	1952±169*	2915±56*	2626±29*	2866±57*	1952±169*	2915±56*
	0	36±5	37±7	116±18	144±13	36±5	37±7	116±18	144±13	36±5	37±7	116±18	144±13
Phlomis pungens	10000	26±5	50±1	84±19	182±4					30±6	40±2	129±5	153 ±10
	5000	39±4	45±3	104±10	193±11					28±5	42±6	78±10	14 7±7
	1000	26±3	43±1	104±13	175±2	28±1	39±7	121±18	146±2	20±0	49±7	90±3	168±11
	500					26±5	55±4	71±1	148±1				
	100					21±1	50±5	81±2	144±1				

	Concentration (mg/plate)	Number of his$^+$ revertants/plate											
		Water extracts				Ethyl acetate extracts				Methanol extracts			
		TA 98		TA 100		TA 98		TA 100		TA 98		TA 100	
		S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)
Negative control	100 µl/plate	35±2	32±4	103±6	132±13	35±2	32±4	103±6	132±13	35±2	32±4	103±6	132±13
Positive control		2626±29*	2866±57*	1952±169*	2915±56*	2626±29*	2866 ±57*	1952±169*	2915±56*	2626±29*	2866±57*	1952±169*	2915±56*
	0	36±5	37±7	116±18	144±13	36±5	37±7	116±18	144±13	36±5	37±7	116±18	144±13
	10000	21±1	39±3	120±12	198±4	22±3	45±4	79±2	199±2	2 3±1	45±0	107±14	177±11
Phlomis armeniaca	5000	31±4	45±7	97±21	193±17	25±0	32±1	101±14	176±8	24±3	51±5	111±4	187±7
	1000	24±1	46±2	99±11	173±12	24±5	34±5	107±5	183±14	21±1	37±5	107±5	189±17

** P<0.05(ANOVA), † Negative control: DMSO (100 µl/plate) was used as negative control for S. typhimurium TA98 and TA100 both in the presence and absence of S9*

® Positive controls: 2-Aminofluorene (7.5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; 4-nitro-O-fenilendiamine (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA98 strain, 2-Aminoanthracene (5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; Sodium azide (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA100

Table 3. Antimutagenicity and inhibition ratios of *Phlomis* extracts towards *S. typhimurium* TA98 and TA100 strains with and without metabolic activation (S9) against direct and indirect mutagens

	Concentration (mg/plate)	% Inhibition rates of extracts against positive mutagens											
		Water extracts				Ethyl acetate extracts				Methanol extracts			
		TA 98		TA 100		TA 98		TA 100		TA 98		TA 100	
		S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)
Phlomis nissolii	10000	59	23	30	37					62	73	37	93
	5000	40	13	17	22	25	96	33	84	58	72	24	78
	1000	16	6	6	0	19	44	26	66	78	7	13	57
	500					21	0	21	38				
Phlomis pungens	10000	32	7	18	29					48	58	76	77
	5000	23	23	9	0					33	37	33	48
	1000	11	4	4	0	42	37	76	57	36	0	12	12
	500					26	8	29	0				
	100					17	0	19	0				
	10000	45	89	21	74	40	95	42	90	32	47	29	58
Phlomis armeniaca	5000	37	78	13	71	39	91	33	84	30	20	24	32
	1000	22	75	0	63	29	84	20	87	28	0	14	0

Negative control: DMSO (100 µl/plate) was used as negative control for S. typhimurium TA98 and TA100 both in the presence and absence of S9

Positive controls: 2-Aminofluorene (7.5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; 4-nitro-O-fenilendiamine (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA98 strain, 2-Aminoanthracene (5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; Sodium azide (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA100

3.3.3 *P. armeniaca*

Associated with 4-NPDA, water extract of *P. armeniaca* (PAW) was described as strong antimutagenic (45%) at a dose of 10000 µg/plate while 5000 µg/plate dose of extract was found to be moderately antimutagenic with the ratio of 37% for TA98 strain without S9 (Figs. 1c, Table 3). When combined with 2-AF, the PAW extracts induced the inhibition greater than 40%, reaching 75%, 78%, and 89%, respectively in the presence of S9 for TA 98 and making them as strongly antimutagenic (Figs. 1c, Table 3). Although PAW extract was revealed no antimutagenicity at all doses tested without S9 for TA100, by the addition of S9 mix, the extract showed inhibition of revertants exceeding 63%, reaching 71% and 74% at two concentrations,

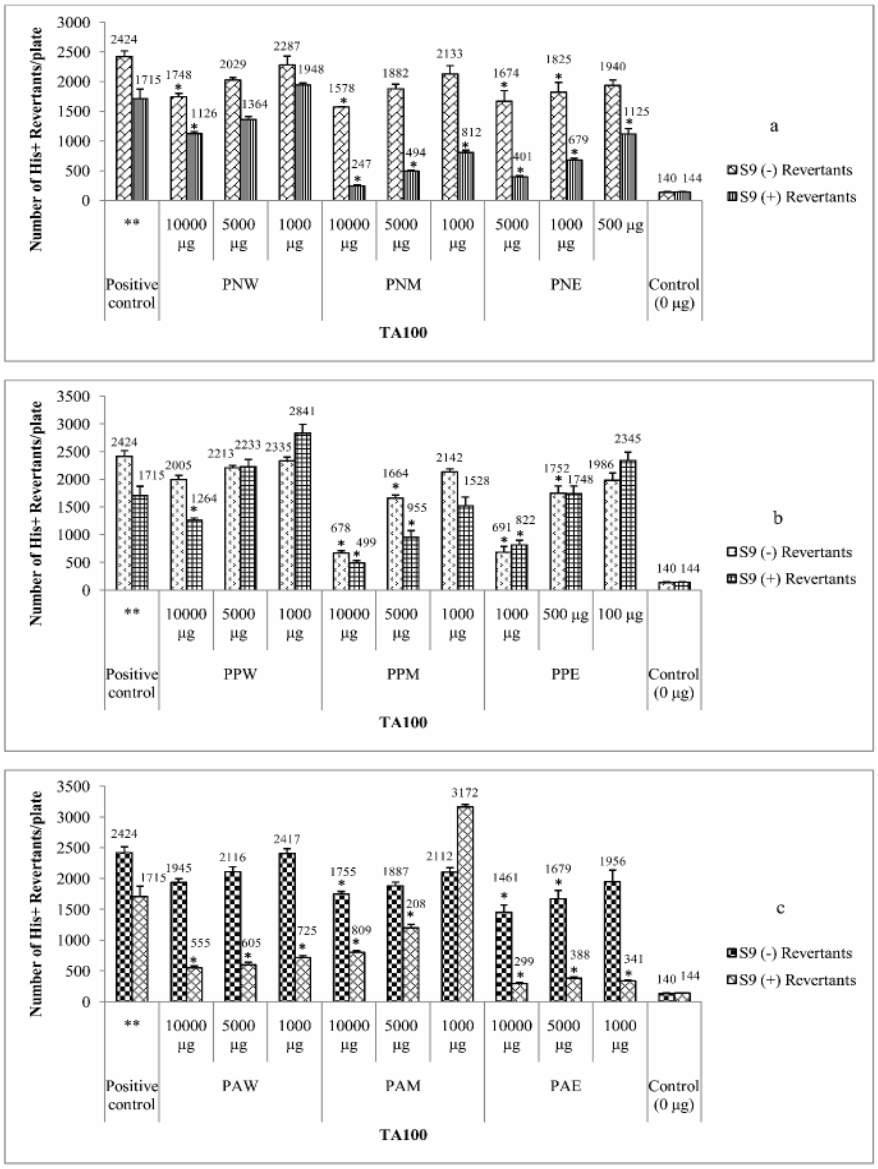

Fig. 2. Decreasing number of TA100 revertants/plate with addition of a) *P. nissoli* extracts b) *P. pungens* extracts c) *P. armeniaca* extracts against direct and indirect mutagens

* *P<0.05* (ANOVA)

*** Positive controls: 2-Aminoanthracene (5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; Sodium azide (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA100*

representing very high antimutagenicity (Figs 2c, Table 3). Although ethyl acetate extract of *P. armeniaca* (PAE) manifested moderately antimutagenic action against 4-NPDA at all test doses (40%, 39%, 29%, respectively) without S9, all tested concentrations showed more than 40% inhibition and concentrations in the range of 10000-1000 µg/plate achieved 95%, 91% and 84% inhibition, respectively, making the extract a very strong antimutagen in the presence of metabolic activation system for TA98 (Figs. 1c, Table 3). For TA100, PAE extract showed strong antimutagenic activity (42%) at a dose of 10000 µg/plate, while 5000 µg/plate dose revealed moderate antimutagenic capacity with a ratio of 33% against SA without S9 (Figs. 2c, Table 3). Against 2-AA in the presence of S9 mix, the three concentrations of PAE extract showed more than 40% inhibition and the highest concentration attained 90%, the others were determined as 84% and 87%, respectively, making them as strongly antimutagenic. When PAM extract were evaluated it demonstrated a potential for significant reduction in the numbers of revertants and it was found to be moderate antimutagenic at all test doses without S9 mix for TA98 against 4-NPDA (Figs. 1c, Table 3). But the highest dose of extract manifested strong antimutagenicity with inhibition rate reaching 47% against 2-AF in the presence of S9. While 10000 µg/plate dose of extract showed moderate antimutagenicity (29%) against SA without S9, it had strong antimutagenic capacity against 2-AA with S9. Also 5000 µg/plate dose of extract was considered to be moderate antimutagenic agent for TA100 (Figs. 2c, Table 3).

4. DISCUSSION

Traditional medicinal plants have received considerable attention because their bioactive components may lead to new drug discoveries. The *Phlomis* genus has been instrumental in the discovery of natural medicinal products [12]. According to some published data *Phlomis* species revealed antitumor activity. In a study conducted by Turker and Yildirim [17] alcoholic extracts of *P. pungens* manifested 85% tumor inhibition on potato disc method against *Agrobacterium tumefaciens* tumors. Also, strong antibacterial activity was determined against *Streptococcus pyogenes* by ethanolic extract. Similarly ethyl acetate, chloroform, and methanol extracts of *P. crinita* flowers strongly alleviated the mutagenicity induced by 2-aminoanthracene with a rate of higher than 40%, reaching 86%, on *S. typhimurium* TA102 and 104 in the presence of S9 mix [14]. In our study, *Phlomis* species tested, revealed strong antimutagenic actions with ratios of 96% (PNE), 77% (PPM), and 95% (PAE) against mutagens requiring metabolic activation by addition of S9 mix both for TA98 and TA100 strains. In another study performed by Limem-Ben Amor, Skandrani [24], SOS chromotest with Escherichia coli PQ37 was used for screening of genotoxicity and antigenotoxicity of *P. crinita* ssp*. mauritanica* extracts. Authors found that methanol and ethyl acetate extracts were genotoxic although total oligomer flavonoids (TOF) and infusion extracts were not genotoxic. Each extract inhibited the mutagenicity induced by aflatoxin B1 but the ethyl acetate extract showed the strongest level of protection toward the genotoxicity induced by both directly and indirectly genotoxic NF and AFB1. In Ames assay conducted by us, no mutagenicity were determined for each extracts of three *Phlomis* species in test doses. Also they were protective against well-known mutagens such as 4-NADP and SA, direct mutagens, 2-AF and 2-AA, requiring metabolic activation enzymes. Sokovic, Marin [15] exhibited that essential oil and crude extract of *P. fruticosa* had no antimutagenicity but only high doses of the extracts and essential oil cause an increase of cell viability in *Escherichia coli* K12 reversion assay. In addition, Yumrutas and Saygıdeger [16] revealed that all extracts of *P. armeniaca* (20-40 µg/mL) exhibited DNA protecting activities in DNA nicking assay. Our results were consistent with the results of Turker and Yildirim [17], Dellai, Mansour [14], Yumrutas and Saygıdeger [16] and Limem-Ben Amor, Skandrani [24] that *Phlomis* species had chemoprotective effects against mutagenic agents.

In our previous studies, major phenolic compounds of *P. nissoli, P. pungens* var. *pungens* [19] and *P. armeniaca* [18] were evaluated by RP-HPLC. It was seen that main component was chlorogenic acid. The rates of this compound was determined as 1.53±0.060, 9.18±0.713, 6.39±0.10 mg/g extract for *P. nissolii*; 1.35±0.060, 17.38±0.90, 11.72±0.188 mg/g extract for *P. pungens*; 3.21±0.06, 11.95±0.919, and 8.58±0.125 mg/g extract for *P. armeniaca* in ethyl acetate, methanol and water extracts, respectively. The high amount of the chlorogenic acid in all extract suggested that this compound may be the responsible from the high antimutagenic activity. Yamada and Tomita [25] proved that chlorogenic acid and caffeic acid strongly alleviated the mutagenic activity of some

mutagens such as Trp-P-I, Glu-P-2, 4-NQO and 2-AF with rates of >40%, reaching 96%, on *S. typhimurium* TA98 strain. Similarly Belkaid, Currie [26] reported that chlorogenic acid, the most potent functional inhibitor of the microsomal glucose-6- phosphate translocase (G6PT), is thought to possess cancer chemo preventive properties. The impact of chlorogenic acid, to which have been attributed possible cancer chemoprevention properties, is well understood with the inhibition of endoplasmic reticulum (ER) glucose-6-phosphate (G6P) transport on brain tumor cells. Tanaka, Kojima [27] described the inhibition of 4-nitroquinoline-1-oxide- induced rat tongue carcinogenesis by the caffeic, ellagic, chlorogenic and ferulic acid. In mice, chlorogenic acid afforded significant protection against gamma-radiation induced micronucleus formation in bone marrow [28]. The ability of chlorogenic acid to alter hepatic and intestinal xenobiotic phase I and phase II enzyme activities was also tested in mice [29]. Our suggestion revealing chlorogenic acid is the antimutagenic agent in *Phlomis* species tested is strongly supported by the findings of the authors mentioned above.

5. CONCLUSION

In this study it was determined that PNE extract revealed the strongest antimutagenic action at a dose of 5000 µg/plate against 2-amino-flourene with 96% inhibition for TA98 with S9 mix. PNM extract alleviated the mutagenic action of 2-amino-anthracene and exhibited strong inhibition rates (93%, 78%, 57%, respectively) with S9 mix at all doses for TA100 strain. While PAE extract manifested 95%, 91% and 84% inhibition against 2-amino-flourene for TA98, it revealed 90%, 84%, and 87% inhibition ratios, respectively, making them as strongly antimutagenic against 2-amino-anthracene for TA100 with S9 mix.

As far as our literature survey could ascertain, there are no published data about the antimutagenic actions of Phlomis species tested and this is the first report on this activity. As a result, it was determined that *P. nissolii, P. pungens* var. *pungens* and *P. armeniaca* extracts had significant antimutagenic capacities and they could be used in the pharmaceutical and food industries.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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