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Full Length Research Paper

In vitro antiviral activities under cytotoxic doses against *herpes simples* type-1 and *parainfluensa*-3 viruses of *Cicer arietinum* L. (Chickpea)

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The objective of this study was to evaluation antiviral activities of the extracts from the seed, fruit skin and aerial parts of ten registered varieties *Cicer arietinum* (Chickpea) against *Herpes simplex* type 1 (*HSV*-1) and *Parainfluenza-3* (*PI*-3) viruses. Madin-Darby Bovine Kidney and Vero cell lines were employed for antiviral assessment of the *Cicer arientinum L.* extracts, in which acyclovir for *HSV*-1 and oseltamivir for *PI*-3 were tested as reference drugs. This is the first report showing that *C. arietinum* seed extracts of Cse-5 possesses significant antiviral activity both of DNA (32 - 4 µg ml^{-1}) and RNA (32 - 16 µg ml^{-1}) viruses compared to the fruit skin and aerial part extracts as well as the controls. Besides, the extracts of fruit skin (Cfs-4) and aerial parts (Cap-5) showed remarkable activity against DNA viruses at 32 - 1 µg ml^{-1}.

Key words: Antibacterial, antifungal, antiviral activity, *Cicer arietinum*, chickpea.

INTRODUCTION

Traditional healers have long used plants to prevent or cure infectious disease. Many of screening efforts have been made to find new antimicrobial and antiviral agents from these plants for a variety of newly active compounds with different molecular targets that control infectious caused by bacteria, fungi and viruses. Many of these plants have been investigated scientifically and summarized by reviewers for their activity. A number of these agents appear to have structures and modes of action that distinct from those of the antibiotics in current use. So, it is worthwhile to study plants and plant products for activity against microorganisms. One approach that has been used for the discovery of antimicrobial agents from plants is based on the evaluation of traditional medicinal plant extracts. Plants are also well-known to be the rich sources of biologically active compounds. Since not many people have access to benefit professional health services, particularly in rural areas of undeveloped and developing countries, plants used in traditional folk medicine for antimicrobial properties are still widely used to treat infections and, therefore, one approach that has been used for the discovery of antimicrobial agents from natural sources is based on the evaluation of traditional plant extracts (Özçelik et al., 2005; 2006; 2008, 2009, Koca et al., 2009; Uysal- Gökçe et al., 2004, Esquenazi et al. 2002 and Orhan et al., 2009).Food legumes are crops of the family *Leguminosae* also called *Fabacae*. They are mainly grown for their edible seeds and thus are also named grain legumes (Iqbal et al., 2006). *Cicer arietinum* L. (Chickpea) is the third most important cool-season food legume after common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.), based on world production estimates. Chikpea is generally consumed as a seed food, being a good source of protein and other essential human nutrients. However, young chickpea leaves are also eaten as a cooked vegetable green in certain parts of the world and could be a useful source of dietary nutrients, especially in malnourished populations (Ibrikci et al., 2003). In this study, we aimed to investigate the antiviral properties of edible and non-edible parts of *Cicer*

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Table 1. Percentage yields (w/w) of dried extracts, from different botanical parts of ten Turkish chickpea varieties.

Examined Cicer arietinum L.		***Seed***	***Fruit Skin***	*Aerial part*
(Chickpea) varieties		**Extract (Cse) %**	**Extract (Cfs) %**	**Extract (Cap) %**
1	Damla 89	0.86	8.01	7.03
2	Ça atay	1.21	10.15	9.23
3	Gülümser	1.06	11.49	11.38
4	Menemen 92	1.47	9.56	11.76
5	Aydın 92	1.06	8.53	7.35
6	Izmir 92	1.76	10.74	11.91
7	Cevdetbey	1.52	9.10	9.10
8	Sarı 98	1.36	9.54	10
9	Akçin 91	0.87	10.15	8.53
10	Gökçe	1.64	9.70	10.61
	Mean ± SD	1.28 ± 0.32	9.70 ± 1.02	9.69 ± 1.75

arietinum L. (Chickpea). On this purpose, extracts of the seed, fruit skin and other aerial part of plant were tested for their antiviral activity both DNA virus *Herpes simplex virus* Type-1 (*HSV*-1) and RNA virus *Parainfluenza virus-3* (*PI*-3) were employed.

MATERIALS AND METHODS

Plant material

Ten registered varietes (Damla 89, Ça atay, Gülümser, Menemen 92, Aydın 92, Izmir 92, Cevdetbey, Sarı -98, Akçin 91, Gökçe) of chickpea were grown at Bahri Da da International Agricultural Research Institute in Central Anatolian Region of Turkey. Chickpea seeds were sowed in 30 March, 2005 and were harvested 10 July 2005. Seed, fruit skin and aerial part herb were dried at room temperature under shade and were used as the material for the study.

Preparation of extracts

6.00 g of powdered plant material (seed, fruit skin and aerial parts) were mixed with 50 ml methanol and extracted two times 1 h in a 100 ml erlenmeyer by magnetic stirrer at 40°C. The extracts were combined and evaporated to dryness under a temperature not exceeding 40°C. Dried and methanol free extracts were used for testing the antibacterial, antifungal and antiviral activities. Yields of the crude extracts obtained are given in Table 1.

Microbiological studies

The extracts from the seed, fruit skin and aerial parts of ten registered varieties *Cicer arietinum* (Chickpea) were prepared in dimethylsulphoxide (DMSO) at a final concentration of 512 μ g ml^{-1} and sterilized by filtration using 0.22 μm Millipore (MA 01730; USA) and used as the stock solutions (CLSI, Formerly NCCLS).

Cytotoxicity and antiviral activities

Cell line and growth conditions: Vero cell line (African green monkey kidney) used in this study was obtained from Department of Virology, Faculty of Veterinary, Ankara University (Ankara-Turkey). The culture of the cells were grown in EMEM (Eagle's Minimal Essential Medium; Seromed; Biochrom; Berlin; Germany) enriched with 10% fetal calf serum (Biochrom, Germany), 100 mg mL^{-1} of streptomycin and 100 IU mL^{-1} of penicillin in a humidified atmosphere of 5% carbondioxide (CO_2) at 37ºC. The cells were harvested using Trypsin solution (Bibco Life Technologies, UK).

Test viruses: In order to determine the antiviral activity of the extracts, *Herpes simplex* virus Type-1 (*HSV*-1), as representative of DNA viruses and *Parainfluenza*- 3 virus (*PI*-3), as representative of RNA viruses, were used. The test viruses were obtained from Department of Virology, Faculty of Veterinary, Ankara University.

Antiviral activity: Media (EMEM) were placed into each 96 wells of the microplates (Greiner^R; Essen, Germany). Stock solutions of the extracts were added into first raws of microplates and two- fold dilutions of the extracts (51.2 - 0.012 µg/mL) were made by dispensing the solutions to the remaining wells. Two-fold dilution of each material was obtained. Acyclovir (Biofarma Co.) and oseltamivir (Roche Co.) was used as the control agents. Strains of *HSV*- 1 and *PI*-3 titers were calculated as tissue culture infecting dose and inoculated into all the wells. The sealed microplates were incubated in 5% CO_2 at 37°C for 2 h to detect the possible antiviral activities of the samples. Following incubation, 50 µL of the cell suspension of 300.000 cells mL^{-1} which were prepared in EMEM together with 5% fetal bovine serum were put in each well and the plates were incubated in 5% CO_2 at 37°C for 48 h. After the end of this period, the cells were evaluated using cell culture microscope by comparison with treated-untreated control cultures and with acyclovir and oseltamivir. Consequently, maximum Cytopathic Effect (CPE) concentrations as the indicator of antiviral activities of the extracts were determined. All organisms and samples were tested in triplicate in each run of experiments (Özçelik et al., 2009 and Ufuk et al., 2009).

Cytotoxicity: The maximum non-toxic concentrations (MNTCs) of each sample were determined by the method described previously by Özçelik et al. based on cellular morphologic alteration. Several concentrations of each sample were placed in contact with confluent cell monolayer and incubated in 5% CO_2 at 37°C for 48 h. After the incubation period, drug concentrations that are not toxic to viable cells were evaluated as nontoxic and also compared with nontreated cells for confirmation. The rows that cause damage in all cells were evaluated as toxic in the present concentration. In addition, maximum drug concentrations that did not affect the cells

Table 2. Cytotoxicity and Antiviral activity results of the Chickpea seed extracts (Cse-1 to 10), Chickpea fruit skin (Cfs -1 to 10), and aerial part extracts (Cap-1 to 10).

	MDBK cells			Vero cells		
	MNTC (µg ml^{-1})	CPE inhibitory concentration (µg ml^{-1})		MNTC (µg ml^{-1})	CPE inhibitory concentration (µg ml^{-1})	
		HSV-1			*PI*-3	
Extracts		**Max.**	**Min.**		**Max.**	**Min.**
Cse-1	64	-[c]	-	32	-	-
Cse-2	32	-	-	32	-	-
Cse-3	32	-	-	16	-	-
Cse-4	64	32	16	16	-	-
Cse-5	64	32	4	32	32	16
Cse-6	32	-	-	32	-	-
Cse-7	64	32	16	16	-	-
Cse-8	32	-	-	64	-	-
Cse-9	128	-	-	64	-	-
Cse-10	16	8	2	64	-	-
Cfs -1	32	-	-	16	-	-
Cfs -2	64	-	-	32	-	-
Cfs -3	32	32	8	32	32	4
Cfs -4	32	32	1	32	32	16
Cfs -5	64	-	-	32	-	-
Cfs -6	64	32	16	32	-	-
Cfs -7	32	32	2	32	-	-
Cfs -8	64	-	-	32	-	-
Cfs -9	64	32	4	32	32	16
Cfs -10	32	-	-	16	-	-
Cap -1	64	-	-	32	16	8
Cap -2	32	32	16	64	-	-
Cap -3	64	-	-	32	-	-
Cap -4	32	32	16	32	-	-
Cap -5	32	32	1	32	-	-
Cap -6	64	64	32	32	32	16
Cap -7	32	-	-	32	-	-
Cap -8	64	-	-	32	32	8
Cap -9	16	16	2	32	-	-
Cap -10	64	-	-	32	-	-
Acyclovir	16	16	<0.25	-	-	-
Oseltamivir	-	-	-	32	32	<0.25

MNTC, maximum non-toxic concentration, CPE, cytopathogenic effect, -: No activity observed.

were evaluated as non-toxic concentration. MNTCs were determined by comparing treated and controlling untreated cultures (Özçelik et al. 2005; Özçelik et al. 2006)

RESULTS AND DISCUSSION

Seed extracts of Cse-5 demonstrate good antiviral activity against both of DNA (*HSV*-1; 32 - 4 µg ml^{-1}) and RNA (*PI*-3; 32 - 16 µg ml^{-1}) viruses compared among to the seed (Cse), fruit skin (Cfs) and aerial part (Cap) extracts as well as the controls. The rest of the seed extracts (Cse-4, Cse-7 and Cse-10) were observed selective inhibition against *HSV*-1 at concentrations ranges of 32 - 16 µg ml^{-1} and 8 - 2 µg ml^{-1} respectively (Table 2).

As shown in Table 2. Antiviral activity against *HSV*-1 were seen on Cfs- 6 (32 - 16 µg ml^{-1}), and Cfs -7 (32 - 2 µg ml^{-1}). This effect are observed of aerial part (Cap) extracts; as Cap-2, 4 (32 - 16 µg ml^{-1}), Cap-5 (32 - 1 µg ml^{-1}), and Cap-9 (16 - 2 µg ml^{-1}). Only one of them (Cap-6) were active against both of *HSV*-1 and *PI*-3. Cap-1 and 8 were

selective active against *PI*-3 at concentrations ranges of 16 - 8 µg ml^{-1}, 32 - 8 µg ml^{-1} respectively.

The antimicrobial effect of *Cicer arietinum* L were determined in many studies, but the activity against commonly confronted human pathogen of this plant has not been mentioned yet, as our knowledge. In a previous study, several proteins including a glucanase, a chitinase, an antifungal cyclophyllin-like protein and three antifungal peptides designated cicerin, arietin, and cicearin have been isolated from the chickpea (*Cicer arietinum* L.) (Chu et al., 2003). The antifungal protein designated chickpea cyclophilin-like antifungal protein, isolated from seeds of the chickpea, possessed a molecular weight of 18 kDa and an N-terminal sequence with resemblance to cyclophilin. It displayed an antifungal action which was evident against a number of fungi including *Mycos-phaerelle arachidicola*, *Botrytis cinerea*, and *Rhizoctonia solani*, they different somewhat in antifungal activity. It is reported that probable structural differences further along the protein sequence account for these functional discrepancies so as to different activity results. On the other hand, the protein was also capable of inhibiting human immunodeficiency virus-1 (*HIV*-1; RNA viruses) reverse transcriptase (Ye et al., 2002a).

We searched the antiviral activities of phenolic constituents (gallic, ferulic, chlorogenic, cinnamic and salicylic acids) of chickpea using methanol extraction against human pathogens (Udai et al., 2003; Sarma et al., 2002). Methanolic extracts of different parts (seed, fruit skin and aerial parts) of Chickpea varieties used for activity studies should contain different amount of phenolic compounds. That is why this shows different activity. It is reported by Ye et al. (2002b), that two antifungal peptides with novel N-terminal sequences have been isolated from chickpea. Although the two chickpea peptides cicerin and arietin were similar in molecular weight (5 - 8 kDa), they differed somewhat in antifungal activity. Arietin was more potent against *M. arachidicola*, *B. cinerea*, and *F. oxysporum*. Cicerin also exhibited a higher cell-free translation-inhibiting activity than arietin (Aslam et al., 2009). In chickpea, the role of isoflavonoid phytoalexins in fungal resistance to *A. rabiei* and *F. oxysporum* f.sp *ciceri* was well defined in several reports. Based on previous reports showing effect of flavonoids on fungal resistance in other species and the differential patterns of flavanone 3-hydroxlase in chickpea upon fungal infection, some degree of antifungal activity of flavonoids in chickpea is expected (Cho el al., 2005). The main phenolic constituents of chickpea seed are reported the formononetin-7-O-glucoside- 6''-malonate, biochanin A-7- O-glucoside-6''-malonate and biochanin A-7-O-glucoside (Kebmann and Barz, 1987; Summer et al., 1996). *Cicer arietinum* contains only one major saponin, belonging to the soyasaponin group B, which is characterized by a reducing sugar 2, 3-dihydro-2, 5 dihydroxy-6-methyl-4H-pyran-4-one (DDMP) moiety on C-22. The pure chickpea saponin exhibited significant inhibitory activity against *Penicillum digitatum* and filamentous fungi (Kerem et al., 2005).

In our study, seed extracts of Cse-5 demonstrate good antiviral activity against both of DNA (*HSV*-1; 32 - 4 µg ml^{-1}) and RNA (*PI*-3; 32 - 16 µg ml^{-1}) viruses compared among to the seed (Cse), fruit skin (Cfs) and aerial part (Cap) extracts as well as the controls. The rest of the seed extracts (Cse-4, Cse- 7, and Cse-10) were observed selective inhibition against HSV at concentrations ranges of 32-16 µg ml^{-1}, and 8-2 µg ml^{-1}, respectively (Table 2).

Among the tested fruit skin (Cfs; 1 - 10) extracts, three of them (Cfs-3, Cfs- 4, Cfs-9) appeared active against both of *HSV*- 1 and *PI*-3. Given, maximum non-toxic concentration, the remarkable antiviral activities were observed against HSV, in Cfs-4 (32 - 1 µg ml^{-1}), Cfs-3 (32 - 8 µg ml^{-1}), and Cfs-9 (32 - 4 µg ml^{-1}), respectively. On the other hand, the activities against PI-3 were similar in Cfs-4 and Cfs-9 (32-16 µg ml^{-1}), while on Cfs-3 (32 - 4 µg ml^{-1}) as seen in Table 2.

Selective antiviral active against *HSV*-1 were seen on Cfs-6 (32 - 16 µg ml^{-1}), and Cfs-7 (32 - 2µg ml^{-1}). This effect were observed of aerial part (Cap) extracts; as Cap-2, 4 (32 - 16 µg ml^{-1}), Cap-5 (32 - 1 µg ml^{-1}), and Cap-9 (16 - 2 µg ml^{-1}). Only one of them (Cap-6) were active against both of *HSV*-1 and *PI*-3. Cap-1 and 8 were selective active against *PI*-3 at concentrations ranges of 16 - 8 and 32 - 8 µg ml^{-1} respectively.

It may be concluded that peptides and proteins contribute to the antimicrobial activity of the chickpea with the phenolic compounds, which were shown antiviral activity. To our knowledge, our study is the first report about the antiviral activities of chickpea phenolic extracts against human pathogens. Further research is under investigation in our laboratuvary.

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