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Antimicrobial and Antiviral Activities of Two Seed Oil Samples of *Cucurbita pepo* L. and Their Fatty Acid Analysis

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This paper is dedicated to Professor Yoshinori Asakawa for his 65th birthday.

Pumpkin seeds are used in the treatment of benign prostatic hyperplasia and the active constituents have been reported to be the sterols and fatty acids. In this study, the oils extracted from the two seed samples of pumpkin (*Cucurbita pepo* L. ssp. *pepo*) growing in Turkey were analyzed for their fatty acid contents. Fatty acids were determined by capillary gas chromatography-mass spectrometry (GC-MS) equipped with a flame ionization detector. The results showed that both samples, which had very similar fatty acid compositions, were quite rich in linoleic acid (43.3 and 43.8%), followed by oleic (36.8 and 35.8%), palmitic (11.9 and 10.9), and stearic acids (6.7 and 7.6%). Saponification, esterification, iodine, peroxide, acidification indexes, and unsaponifiable matter of the oils were also calculated. In addition, the two oil samples were tested for their antibacterial, antifungal, and antiviral activities. Both of the oils displayed the same activity profile, having a good antibacterial activity against *Klebsiella pneumoniae* and *Acinobacter baumannii* (16 μg/mL), potent antifungal activity against *Candida albicans* (8 μg/mL), as well as a moderate antiviral effect towards *Parainfluenza* virus type-3 (16-8 μg/mL).

Keywords: *Cucurbita pepo*, Cucurbitaceae, pumpkin seed, fatty acid, GC-MS, antimicrobial activity.

Of all medicines today, 30-40% contain one or more active ingredients of herbal origin. One such herbal drug, *Cucurbita pepo* L. (Cucurbitaceae) has become popular in phytotherapy for its beneficial effects against benign prostatic hyperplasia (BPH) [1-4]. Pumpkin seed oil has been also reported to have a modifying effect on the potency of the calcium antagonist felodipine and angiotensin-converting enzyme inhibitor captopril in retarding the progression of hypertension [5].

Cucurbitae Semen has been approved by German Pharmacopoeia, German Standard License Monographs, British Herbal Pharmacopoeia, and American Pharmacopoeia and has also been included in Commission E monographs for its use in irritable bladder and prostate complaints [6]. In the present study, the oils obtained from two samples of *C. pepo* ssp. *pepo* growing in Turkey (coded as CP-I and CP-II, respectively) were examined with respect to fatty acid composition, and several chemical indexes (acidification, saponification, iodine, and peroxide indices, as well as unsaponifiable matter) in an attempt to determine the composition and quality characteristics of the oils. The oils were also screened *in vitro* for their antibacterial, antifungal, and antiviral activities.

The oils coded as CP-I and CP-II obtained from two *C. pepo* seed samples, upon Soxhlet extraction with

n-hexane, yielded 41.9±0.5 and 43.7±0.8% of oils (% seed weight) that were brownish-yellow in color. These yields were lower than that of the European variety of *C. pepo* (60%), but higher than that reported for the African variety (22-35%) [7,8]. The fatty acid compositions of both CP-I and CP-II oils were very similar (Table 1).

Table 1: Relative fatty acid percentages of seed oils of two *C. pepo* samples.

Fatty acids detected	Retention times (Min)	Relative percentages	
		CP-I	CP-II
Palmitic (C16:0)	24.9	11.9±0.9	10.9±0.6
Stearic (C18:0)	35.8	6.7±0.2	7.6±0.5
Oleic (C18:1)	36.7	36.8±0.7	35.8±1.5
Linoleic (C18:2)	38.5	43.3±1.0	43.8±0.5
Linolenic (C18:3)	41.1	0.1±0.02	0.4±0.08
Eicosanoic (C20:0)	43.1	0.5±0.9	0.5±0.01
Total	-	99.3	99.0

The fatty acid composition of any fixed oil depends on several factors, such as variety, locality of cultivation, climate, maturity, soil nature, and humidity [9]. There have been several studies on the elucidation of the fatty acid content of *C. pepo* seed oils of different origins. These studies have shown that the dominating fatty acids are palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids. In a study by Murkovic et al. [7], the dominant fatty acids in the seed oil samples of *C. pepo* growing in Austria and Slovenia, as well as Hungary (known as "Styrian oil"), have been found to be palmitic (9.5-14.5%), stearic (3.1-7.4%), oleic (21.0-46.9%), and linoleic acids (35.6-60.8%), which is consistent with our data.

On the other hand, the seed oil of *C. pepo* growing in Africa was found to contain palmitic (13.3%), stearic (8.0%), oleic (29.0%), and linoleic (47.0%) acids [8]. These data resemble those of our sample (Table 1), and are also similar to those of Egyptian seed oil, except for the amount of oleic acid [10]. The Egyptian sample had 20.4% oleic acid, whereas the content of our samples ranged between 37.8-35.8%. Moreover, the chemical index values of our oil samples were dissimilar to those of African origin; only the iodine values were similar [8].

In Applequist et al.'s study [11], fatty acid contents of *C. moschata*, *C. maxima*, and *C. argyrosperma*, as well as *C. pepo* were studied by GC using a flame ionization detector (FID). Considerable variation in total lipid content and composition was found and *C. pepo* was stated to possess the lowest saturated fatty acid amount, while *C. maxima* had the highest. The fatty acid composition of *C. pepo* in that study was quite similar to our results presented here.

Table 2: Chemical indices calculated for the oil samples of *C. pepo*.

Indexes	**CP-I**	**CP-II**
Acid value (%)	2.4±0.07	2.7±0.01
Saponification value	204.2±1.4	206.7±0.5
Iodine value	109.7±1.5	105.8±1.1
Peroxide value	3.4±0.2	3.6±0.03
Unsaponifiable matter	1.0±0.02	1.1±0.04

The oil samples exerted a selective inhibitory effect on *Parainfluenza* virus (Type-3), while they were completely inactive against *Herpes simplex* (Table 3). However, the range of activity on *Parainfluenza* virus (Type-3) was quite narrow (16-8 μg/mL) when compared to oseltamivir (32-<0.25 μg/mL). The MNTC values of the two oil samples were higher (16 μg/mL) than that of oseltamivir on Vero cells, while they appeared to be less toxic against MDBK cells (32 μg/mL).

Table 3: Antiviral activity of the pumpkin seed oils.

	MDBK Cells (μg/mL)			Vero Cells (μg/mL)		
Oils	MNTC (μg/mL)	CPE Inhibitory Concentration HSV		MNTC (μg/mL)	CPE Inhibitory Concentration PI-3	
		Max.	Min.		Max.	Min.
CP-I	32	-*	-	16	16	8
CP-II	32	-	-	16	16	8
Acyclovir	16	16	<0.25	-	-	-
Oseltamivir	-	-	-	32	32	<0.25

-* : No activity observed; MNTC: Maximum non-toxic concentration; CPE: Cytopathogenic effect.

Both of the oil samples were also screened against the standard strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinobacter baumannii*, *Staphylococcus aureus*, and *Bacillus cereus* for their antibacterial activity using the microdilution method, while their antifungal activity was tested against the yeast *Candida albicans* and the results compared to those obtained with ketoconazole and fluconazole (Table 4). The oils were highly active against *K. pneumoniae* and *A. baumannii* at a concentration of 16 μg/mL, whereas they displayed insignificant inhibition against the rest of the bacteria.

In conclusion, the yields of *C. pepo* seed oil obtained from plants grown in Turkey and its fatty acid composition were very similar to those of pumpkin seed oils from other countries. To the best of our knowledge, this is the first report of antiviral and

Table 4: Antimicrobial activity, as minimum inhibitory concentrations (MICs) (μg/mL), of the pumpkin seed oils.

Microorganisms Oils	*E. coli*	*P. aeruginosa*	*P. mirabilis*	*K. pneumoniae*	*A. baumannii*	*S. aureus*	*B. subtilis*	*C. albicans*
Cucurbita pepo ssp. *pepo*-I (CP-I)	64	128	64	16	16	128	128	8
Cucurbita pepo ssp. *pepo*-II (CP-II)	64	128	64	16	16	128	128	8
Ampicillin	2	-*	2	2	2	<0.12	0.12	-
Oflaxocin	0.12	1	<0.12	0.12	0.12	0.5	0.5	-
Ketoconazole	-	-	-	-	-	-	-	2
Fluconazole	-	-	-	-	-	-	-	4

-*: No activity observed

antimicrobial activities of pumpkin seed oil. The results also point out that these seeds may be beneficial as a dietary source.

Experimental

Plant materials: The pumpkin seeds used in this study were obtained from two separate fields in Kirsehir (Turkey) during the summer of 2005. The seeds were hand selected to eliminate those that were damaged in any way.

Oil extraction: Oil contents of the seed samples were determined using Soxhlet extraction of powdered samples with *n*-hexane for 8 hrs at 80°C. The extraction procedure was triplicated for each sample, each of which consisted of approximately 40 seeds. The solvent was removed under vacuum, and the percentage oil yields (w/w) were calculated. The values are the mean of three determinations.

Saponification and methylation of the oils: The two oils were weighed independently in 50 mL volumetric flasks, and then saponified by adding 12 mL 0.5 N methanolic NaOH to the mixture [12]. Later, the flasks were heated on a steam bath until the fat globules went into solution (approximately 5 minutes). BF_3/MeOH (Sigma Co.; 20 mL) was added to each flask and the mixtures were boiled for 2 minutes. After the samples had cooled, they were made up to 50 mL with saturated NaCl solution. These mixtures were then transferred to a separating funnel, and extracted with 30 mL light petroleum. The petroleum phases were taken and evaporated on a water bath at 60°C. The obtained methyl esters of the fatty acids were dissolved in 5 mL *n*-hexane and 1μL of each sample was injected and analyzed by GC-MS.

GC-MS: Gas **c**hromatographic-mass spectrometric analysis (GC-MS) was carried out on an Agilent 6890N Network GC system combined with an Agilent 5973 Network Mass Selective Detector. The capillary column used was an Agilent 19091N-136 (Hewlett Packard Innowax Capillary; 60.0 m x 0.25 mm x 0.25 μm). Helium was used as carrier gas at a flow rate of 0.8 mL/min with 1 μL injection volume. Samples were analyzed with the column held initially at 60ºC after injection with 10 min hold time, then increased to 220ºC with 4ºC/min heating ramp and kept at 220ºC for 10 min. Then the final temperature was increased to 240ºC, with 1ºC/min heating ramp. The injection was performed in split mode (50:1). Detector and injector temperatures were 230°C and 280°C, respectively. Run time was 80 min. MS scan range was (*m/z*): 35-450 atomic mass units (AMU) under electron impact (EI) ionization (70 eV).

Identification of fatty acids: Identification of peaks was accomplished using Wiley and Nist Libraries, comparison of retention times (Rt), and comparison of standard compounds with mass spectra. Relative content of % fatty acids was determined with area under peaks using Agilent software. The results are expressed as an average of three determinations in all cases.

Microbiological studies: The oils were dissolved in ethanol:*n*-hexane (1:1) by using 51% tween 80 solution at a final concentration of 1024 μg/mL, sterilized by filtration using 0.22 μm Millipore filters (MA 01730, USA), and used as the stock solutions. Standard antibacterial powders of ampicilline (AMP, Fako), ofloxacine (OFX, Hoechst Marion Roussel), and also standard antifungal powders of ketoconazole (KET, Bilim) and fluconazole (FLU, Pfizer) were obtained from their respective manufacturers and dissolved in phosphate buffer solution (AMP**,** pH: 8.0, 0.1 mol/L), DMSO (KET), in water (FLU). The stock solutions of the agents were prepared in media according to the NCCLS recommendations as referred in our previous studies [13-16].

Standard strains of the following bacteria, namely *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 10145), *Proteus mirabilis* (ATCC 7002), *Klebsiella pneumoniae* (RSKK 574),

Acinetobacter baumannii (RSKK 02026), *Staphylococcus aureus* (ATCC 25923), and *Bacillus subtilis* (ATCC 6633) for antibacterial activity, as well as a standard strain of *Candida albicans* (ATCC 10231) were employed. Mueller-Hinton Broth (Difco) and Mueller-Hinton Agar (Oxoid) for the bacteria, Sabouraud liquid medium (Oxoid) and Sabouraud dextrose agar (SDA) (Oxoid) for fungi and culture suspensions were prepared as described in our earlier publications using the microdilution method [13-17].

Antiviral activity: African green monkey kidney (Vero) and Madin-Darby bovine kidney (MDBK) cell lines, as well as the test viruses *Herpes simplex* virus (HSV) and *Parainfluenza*-3 virus (PI-3) used in this study were obtained from the Department of Virology, Veterinary Faculty, Ankara University (Turkey). Cell culture was as explained elsewhere [15]. Acyclovir (Biofarma) and oseltamivir (Roche) were used as the references.

Strains of HSV and PIV titers were calculated by the Frey and Liess method as $TCID_{50}$ [18]. The assay was performed as reported previously and maximum cytopathogenic effect (CPE) concentrations, as the indicator of antiviral activities of the extracts, were determined [13-16].

Cytotoxicity: The maximum non-toxic concentration (MNTC) of each sample was determined by the method described previously, based on cellular morphological alteration [13-16].

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