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Antiviral activity and cytotoxicity of the lipophilic extracts of various edible plants and their fatty acids

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ABSTRACT

The lipophilic extracts of a number of edible plants, including *Anthemis tinctoria* var. *tinctoria*, *A. austriaca*, *Carduus acanthoides*, *C. nutans*, *Cirsium hypoleucum*, *Cynara scolymus* (Asteraceae), *Silene vulgaris* (Caryophyllaceae), *Ajuga chamaepitys* ssp. *chia* var. *ciliata* (Lamiaceae), *Lappula barbata* (Boraginaceae), *Rumex obtusifolius* ssp. *subalpinus* (Polygonaceae), *Digitalis lamarckii* (Scrophulariaceae), and *Sedum hispanicum* (Crassulaceae) were tested against DNA virus *Herpes simplex* (HSV) and RNA virus *Parainfluenza* (PI-3) using Madin-Darby bovine kidney and Vero cell lines, and also analysed by capillary gas chromatography–mass spectrometry (GC–MS). Most of the extracts exerted quite appreciable antiviral effect against both viruses, as compared to acyclovir and oseltamivir. Cytotoxicity of the extracts was also determined as maximum non-toxic concentrations (MNTCs). Most of the extracts were found to be rich in saturated fatty acids. No correlation was found between antiviral activity and fatty acid contents of the extracts.

1. Introduction

Frequent human infections of viral origin could be exemplified as common cold, influenza, chickenpox, AIDS, avian influenza, etc. Because viruses use the host cell to reproduce and reside, it is rather difficult to exterminate them without killing the host cell. The most effective treatment strategies for viral diseases are vaccination, which provides resistance to infections, as well as antiviral drugs, such as nucleoside analogues (acyclovir, ribavirin, etc.). However, there is still great need to develop new antiviral drugs, as new and complex types of viral infections and resistance are emerging day by day.

In this study, we gathered a number of edible plants for antiviral screening: *Anthemis tinctoria* L. var. *tinctoria*, *A. austriaca* Jacq., *Carduus acanthoides* L., *C. nutans* L. sensu lato, *Cirsium hypoleucum* DC, *Cynara scolymus* L. (Asteraceae), *Silene vulgaris* (Moench) Garcker (Caryophyllaceae), *Ajuga chamaepitys* (L.) Schreber ssp. *chia* (Schreber) Arcangeli var. *ciliata* Briq. (Lamiaceae), *Lappula barbata* (Bieb.) Gürke (Boraginaceae), *Rumex obtusifolius* L. ssp. *subalpinus* (Schur) Celak. (Polygonaceae), *Digitalis lamarckii* Ivan. (Scrophulariaceae), *Sedum hispanicum* L. (Crassulaceae). Among them, *Anthemis* species are used as tea in Anatolia (Baytop, 1999). *Carduus* species are used as an appetiser and anti-diabetic, in the form of a decoction, by the local people (Baytop, 1999). Different species of the genus *Cirsium* are known to be an appetiser and tonic; besides the young flowers, stems, and roots are cooked and eaten as vegetable (Baytop, 1999; Facciola, 1990; Kunkel, 1984). *C. scolymus* is well-known as artichoke and consumed as a vegetable all over the world. Before flowering, the leaves of *S. vulgaris* are collected and cooked in the southern part of Anatolia (Baytop, 1999). *A. chamaepitys* (*syn. Teucrium chamaepitys*) is drunk as a tea in Anatolia (Baytop, 1999). *Lappula* species are used as an herbal remedy in Chinese and Mongolian traditional medicines (Fu et al., 2000; Wiedenfeld, Amarsanaa, Altanchimeg, & Narantuya, 2005). The leaves of *Rumex* species are eaten as a vegetable in salads in Turkey (Baytop, 1999). *Digitalis* species, well-known for their medicinal properties, are used as an infusion in Anatolia (Baytop, 1999). *Sedum* leaves are consumed raw or cooked (Tanaka, 1976). The main interest in the edible quality of this plant is as a survival food, since it grows wild in the deserts (Phillips & Foy, 1990). Therefore, the present investigation was carried out to assess antiviral activities of the lipophilic extracts of these native edible plants against DNA virus *Herpes simplex* (HSV-1) and RNA virus *Parainfluenza* (PI-3), using Madin-Darby bovine kidney and Vero cell lines. The lipophilic extracts prepared with *n*-hexane were also analysed by capillary gas chromatography–mass spectrometry (GC–MS) and their fatty acid compositions were characterised.

2. Materials and methods

2.1. Plant materials

The collection sites, duration and herbarium numbers of the plant materials are given in Table 1. The plants were identified by Prof. Dr. Mecit Vural of Department of Biology, Faculty of

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Table 1
Collection sites, durations and herbarium numbers of the plants.

Plant species	Collection site	Month of collection	Herbarium number
Asteraceae			
Anthemis tinctoria var. *tinctoria*	Adaören village, Beypazari, Ankara	June, 2003	GUE 2369
A. austriaca	Kaman, Ankara	June, 1997	GUE 2370
Carduus nutans	Gölbasi, Ankara	June, 1997	GUE 2371
C. acanthoides	Ilgaz Mountain, Kastamonu	June, 1997	GUE 2372
Cirsium hypoleucum	Ilgaz Mountain, Kastamonu	June, 1997	GUE 2291
Cynara scolymus	Purchased from public bazaar	June, 1997	-
Caryophyllaceae			
Silene vulgaris	Akdagmadeni, Yozgat	June, 2004	GUE 2373
Lamiaceae			
Ajuga chamaepitys ssp. *chia* var. *ciliata*	Akdagmadeni, Yozgat	June, 2004	GUE 2374
Boraginaceae			
Lappula barbata	Akdagmadeni, Yozgat	June, 2004	GUE 2375
Polygonaceae			
Rumex obtusifolius ssp. *subalpinus*	City center, Ankara	June, 2004	GUE 2376
Scrophulariaceae			
Digitalis lamarckii	Adaören village, Beypazari, Ankara	June, 2003	GUE 2377
Crassulaceae			
Sedum hispanicum	Akdagmadeni, Yozgat	June, 2004	GUE 2378

Science and Art, Gazi University, Ankara, Turkey, and are preserved in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkey (GUE).

2.2. Preparation of the lipophilic extracts

Each plant part used in this study was dried under shade, ground in a mechanic grinder to fine powder and weighed accurately. The powdered plant parts were macerated with 100 ml of *n*-hexane (Merck, Darmstadt, Germany) at room temperature for 2 days and shaken occasionally by hand. After extraction, the *n*-hexane phase of each extract was filtered through a filter paper and evaporated under reduced pressure until dryness. The obtained lipophilic extracts were employed in the antiviral activity tests and GC–MS analysis.

2.3. Test viruses used in the antiviral assays

The test viruses, *Herpes simplex* virus type-1 (HSV-1) and *Parainfluenza*-3 virus (PI-3), were obtained from Department of Virology, Faculty of Veterinary Studies, Ankara University.

2.4. 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 Studies, Ankara University (Turkey). The culture of the cells was grown in EMEM (Eagle's Minimal Essential Medium; Biochrom, Berlin, Germany) enriched with 10% foetal calf serum (Biochrom), 100 mg ml^{-1} of streptomycin and 100 IU ml^{-1} of penicillin in a humidified atmosphere of 5% carbon dioxide (CO_2) at 37 °C. The cells were harvested using trypsin solution (GibcoLife Technologies, Gaithersburg, MD).

2.5. Antiviral activity assays

Media (EMEM) were placed into 96-well microplates. Stock solutions of the samples were added to the first row of microplates and two-fold dilutions of the samples (512–0.25 μg ml^{-1}) were made by dispensing the solutions to the remaining wells. Two-fold dilution of each material was obtained according to Log_2 on the microplates. Acyclovir (Biofarma Co.) and oseltamivir (Roche Co.) were used as the references. Strains of HSV-1 and PI-3 titres were calculated as tissue culture infecting dose ($TCID_{50}$) 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% foetal 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 cytopathogenic effect (CPE) concentrations as an indicator of the antiviral activities of the extracts were determined (Özcelik, Orhan, & Toker, 2006).

2.6. Cytotoxicity

The maximum non-toxic concentrations (MNTCs) of each sample were determined by the method described previously by Özcelik, Deliorman-Orhan, Karaoglu, and Ergun (2005b), based on cellular morphological 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 were not toxic to viable cells were evaluated as non-toxic and also compared with non-threatened cells for confirmation. The rows that caused damage in all cells were evaluated as toxic at the present concentrations. In addition, maximum drug concentrations that did not affect the cells were evaluated as non-toxic concentrations. MNTC were determined by comparing treated and control untreated cultures.

2.7. Derivatisation of the extracts for GC–MS analysis

The lipophilic extracts were independently saponified with 0.5 N methanolic NaOH solution, by heating on a steam bath until fat globules disappeared on the surface of the solution, an approximately five-minute step, then boiled for 2 min. Twenty millilitres of boron trifluoride-methanol complex reagent (20%, Merck) were subsequently added into the solutions and kept for another 2 min again in a boiling water bath. After cooling, each solution was added to saturated NaCl solution in 25 ml measuring flasks. The mixtures were left for 30 min to allow the oily part to gather on the surface of the solution; this was then converted to methyl esters prior to GC–MS analysis (Morrison & Smith, 1964).

2.8. Conditions of GC–MS analysis

Chromatographic analysis was carried out on a Hewlett–Packard HP 6890 series GC–MS apparatus combined with a mass selective detector. The capillary column used was an HP-5MS (5% phenyl methylsiloxane, 30.1 m × 250 μm × 0.25 μm). Helium was used as carrier gas at a flow rate of 1.0 ml min^{-1} with 5 μl injection volume. Samples were analysed with the column held initially at 180 °C for 0.5 min after injection, then increased to 240 °C with an 8 °C/min heating ramp with a 1 min hold time. The temperature was then increased to 300 °C with 2 °C/min heating ramp for 10 min. The injection was performed in split mode (split ratio: 10:1). Interface and injector temperatures were 250 °C and 280 °C, respectively. Run time was 49 min. MS scan range was *m*/*z* 20–440 using electron impact (EI) ionisation (70 eV) and an ion source temperature of 250 °C. All injections were done in triplicate.

2.9. Identification of the fatty acids

The fatty acid components of the lipophilic extracts were determined by comparing their mass fragmentation patterns with those of mass spectra from the Wiley and NIST databases as well as comparison of the retention times and mass spectra with authentic samples of fatty acids.

3. Results

3.1. Antiviral activity of the lipophilic extracts

The extracts had lower cytotoxicity than those of references as expressed by their MNTC values (Table 2). After determining cytotoxicity of the extracts, antiviral activity against HSV-1 and PI-3 was investigated by CPE inhibitory test. All of the lipophilic extracts showed a very high inhibition against both virus types (Table 2). Acyclovir, the reference drug against HSV-1 showed a CPE between 0.16 and <0.012 mg ml^{-1}, while the extracts of *L. barbata*, *D. lamarckii*, *S. hispanicum*, *C. acanthoides*, and *A. austriaca* displayed a comparably close inhibition to that of acyclovir against HSV-1 ranging between 0.1 and 0.05 mg ml^{-1}. On the other hand, *S. vulgaris* (stem and flower), *A. chamaepitys*, *C. scolymus* (fruit, leaves with branch), *C. hypoleucum*, *C. nutans* (leaf and stem) and *D. lamarckii* were the most potent extracts (0.05–0.025 mg ml^{-1}) towards PI-3, where oseltamivir, the reference drug for *Parainfluenza*, had a CPE between 0.16 and <0.012 mg ml^{-1}.

3.2. Fatty acid composition of the extracts by GC–MS

The proximate analysis of fatty acid compositions (mean and standard error values) of the lipophilic extracts by GC–MS is presented in Table 3. According to the results, a large variation in the amounts of palmitic (C16:0), myristic (C14:0), linoleic (C18:2), and linolenic (C18:3) acids was observed in the extracts, while palmitoleic acid (C16:1) was only detected in *C. nutans* in a minor amount. Most of the extracts seemed to be rich in terms of saturated fatty acids. *C. hypoleucum* had the highest palmitic acid (26.35%), followed by *C. acanthoides* (12.86%), the bract of *C. scolymus* (11.69%), *C. nutans* (9.54%), *R. obtusifolius* ssp. *subalpinus* (7.68%), the aerial parts of *A. tinctoria* var. *tinctoria* (6.72%) and its flowers (4.70%). *A. chamaepytis* ssp. *chia* var. *ciliata* was low in palmitic acid (1.56%). The fruits of *C. scolymus* contained myristic acid in the highest amounts (20.38%), while cerotic acid (26:0) was dominant in the aerial parts of *A. tinctoria* var. *tinctoria*.

The fruits of *C. scolymus* and *C. hypoleucum* were remarkably rich in linoleic acid (12.77 and 9.85%, respectively), whereas the fruits of *R. obtusifolius* ssp. *subalpinus* possessed the highest amount of linolenic acid (9.16%).

4. Discussion

Plant extracts are known to consist of many chemicals and among them, a few compounds could be acting synergistically. Sometimes, isolation of the compounds from the extract may cause a decrease in desired activity, which underlines the importance of extract screening. On the other hand, antiviral drugs are expected to have effect against a virus without producing cytotoxicity in the host cells. Therefore, as the MNTC values of the extracts varied in the range of 0.2–0.025 μg ml^{-1} the cytotoxicity of the extracts was comparable to that of the references (0.16 μg ml^{-1}) (Table 2).

In our earlier study, we had found that the lipophilic extracts of different parts of *Pistacia vera* tree displayed great antiviral activity (Özcelik, Aslan, Orhan, & Karaoglu, 2005a). The results of that study prompted us to examine the lipophilic extracts of the abovementioned edible plants. Accordingly, the hexane extracts screened were strongly active against both viruses, which made us consider that non-polar compounds could be responsible for the antiviral activity. In consistent with our present data, numerous researchers have found the hexane extracts from various plants to possess antiviral activity against *H. simplex* and other viruses (Akanitapichat, Wangmaneerat, Wilairat, & Bastow, 2006; Binns, Hudson, Merali, & Arnason, 2002; Huerta-Reyes et al., 2004; Maregesi et al., 2008).

We tried to characterise the composition of the hexane extracts by GC–MS, which led us to identify their fatty acids (Table 3). *C. hypoleucum* having a high amount of palmitic acid was previously

Table 2
Antiviral activity and cytotoxicity of the lipophilic extracts and references as minimum inhibitory concentration (MIC) values (μg ml^{-1}).

MDBK cells				Vero cells		
Extracts	MNTCs (μg ml^{-1})	HSV-1		MNTCs (μg ml^{-1})	PI-3	
		CPE inhibitory concentration			CPE inhibitory concentration	
		Maximum	Minimum		Maximum	Minimum
AA	0.8	0.1	0.05	0.4	0.1	0.025
AC	0.4	0.2	0.1	0.8	0.05	0.025
AT (flower)	0.8	0.2	0.1	0.8	0.2	0.025
AT (aerial)	0.4	0.2	0.1	0.2	0.2	0.1
CA	0.8	0.1	0.05	0.2	0.1	0.025
CH	0.8	0.2	0.1	0.2	0.05	0.025
CN	0.8	0.2	0.1	0.2	0.05	0.025
CS (bract)	0.4	0.2	0.1	0.2	0.2	0.1
CS (receptaculum)	0.8	0.2	0.1	0.4	0.05	0.025
CS (leaf, stem)	0.8	0.2	0.1	0.2	0.05	0.025
DL (flower)	0.8	0.1	0.05	0.4	0.1	0.025
DL (leaf, stem)	0.8	0.2	0.1	0.2	0.05	0.025
LB	0.4	0.1	0.05	0.2	0.1	0.025
SH	0.8	0.1	0.05	0.4	0.1	0.025
SV (flower)	0.8	0.2	0.1	0.8	0.05	0.025
SV (stem)	0.4	0.2	0.1	0.2	0.05	0.025
RO (fruit)	0.8	0.2	0.1	0.8	0.2	0.025
Acyclovir	0.16	0.16	<0.012	-	-	-
Oseltamivir	-	-	-	0.16	0.16	<0.012

MNTCs: Maximum non-toxic concentrations, CPE: Cytopathogenic effect, –: Not activity observed.
AT: *Anthemis tinctoria* var. *tinctoria*, AA: *A. austriaca*, AC: *Ajuga chamaepitys* ssp. *chia* var. *ciliata*, CA: *Carduus acanthoides*, CH: *Cirsium hypoleucum*, CN: *C. nutans*, CS: *Cynara scolymus*, DL: *Digitalis lamarckii*, LB: *Lappula barbata*, RO: *Rumex obtusifolius* ssp. *subalpinus*, SH: *Sedum hispanicum*, SV: *Silene vulgaris*.

Table 3
Fatty acid composition of the lipophilic extracts.

Fatty acids identified	Relative composition %																
	AT (flower)	AT (aerial)	AA	CN	CA	CH	CS (bract)	CS (receptaculum)	CS (leaf, stem)	SV (flower)	SV (stem)	AC	LB	RO (fruit)	DL (flower)	DL (leaf,stem)	SH
Saturates																	
Myristic (14.0)	0.38 ± 0.02	0.83 ± 0.03	–	0.42 ± 0.01	0.66 ± 0.06	0.51 ± 0.02	–	20.38 ± 1.17	–	–		–	–	–	–	–	0.05 ± 0.00
Palmitic (16.0)	4.70 ± 0.12	6.72 ± 0.28	2.96 ± 0.50	9.54 ± 0.09	12.86 ± 1.37	26.35 ± 0.53	11.69 ± 1.27	–	10.37 ± 0.78	–	0.47 ± 0.05	1.56 ± 0.01	1.79 ± 0.15	7.86 ± 0.77	0.23 ± 0.02	5.07 ± 1.01	0.39 ± 0.33
Stearic (18.0)	2.41 ± 0.02	1.33 ± 0.08	0.89 ± 0.10	0.88 ± 0.01	1.94 ± 0.08	2.00 ± 0.13	1.63 ± 0.10	1.60 ± 0.02	1.80 ± 0.13	–	0.05 ± 0.01	–	–	0.63 ± 0.03	0.93 ± 0.07	0.92 ± 0.11	0.03 ± 0.01
Arachidic (20.0)	1.17 ± 0.06	–	–	0.51 ± 0.02	1.43 ± 0.25	0.90 ± 0.05	–	–	1.94 ± 0.08	–	0.07 ± 0.00	–	1.23 ± 0.15	–	–	0.58 ± 0.19	–
Behenic (22.0)	–	0.92 ± 0.01	–	0.42 ± 0.01	1.96 ± 0.20	1.27 ± 0.01	–	–	0.61 ± 0.02	–	–	–	2.95 ± 0.13	–	0.90 ± 0.05	0.30 ± 0.08	–
Lignoseric (24:0)	–	4.54 ± 1.46	1.28 ± 0.08	0.35 ± 0.01	1.81 ± 0.15	1.73 ± 0.02	0.85 ± 0.03	–	0.97 ± 0.01	–	0.39 ± 0.01	–	3.15 ± 0.07	1.29 ± 0.08	1.59 ± 0.05	–	0.12 ± 0.01
Cerotic (26:0)	–	8.28 ± 0.34	2.24 ± 0.28	0.11 ± 0.02	1.81 ± 0.32	–	–	–	1.65 ± 0.01	Trace	0.56 ± 0.04	–	1.55 ± 0.14	–	0.61 ± 0.07	–	0.09 ± 0.01
Δ9-desaturates																	
Palmitoleic (16:1)	–	–	–	0.28 ± 0.02		–	–	–	–		–	–	–	–	–	–	–
Oleic (18:1)	5.82 ± 0.03	4.60 ± 0.82	–	2.35 ± 0.03	3.95 ± 0.20	–	–	0.73 ± 0.01	–	–	–	–	–	–	–	3.42 ± 1.10	0.21 ± 0.01
ω3-Fatty acids																	
Linolenic (18:3)	–	–	–	–	–	–	–	2.52 ± 0.09	3.17 ± 0.15	–	–	–	–	9.16 ± 0.77			
ω6-Fatty acids																	
Linoleic (18:2)	4.62 ± 0.15	2.29 ± 0.01	–	4.20 ± 0.22	5.41 ± 0.15	9.85 ± 0.05	–	12.77 ± 0.71	2.25 ± 0.11	–	0.16 ± 0.02	–	–	–	–	–	–
Total	19.10	29.51	7.37	19.06	31.83	42.61	14.17	38.00	22.76	–	1.70	1.56	10.67	18.76	4.26	10.29	0.89

AT: *Anthemis tinctoria* var. *tinctoria*, AA: *A. austriaca*, AC: *Ajuga chamaepitys* ssp. *chia* var. *ciliata*, CA: *Carduus acanthoides*, CH: *Cirsium hypoleucum*, CN: *C. nutans*, CS: *Cynara scolymus*, DL: *Digitalis lamarckii*, LB: *Lappula barbata*, RO: *Rumex obtusifolius* ssp. *subalpinus*, SH: *Sedum hispanicum*, SV: *Silene vulgaris*.

reported to have a powerful antiviral activity against *H. simplex* (HSV) and *Parainfluenza* viruses (PIV) (Özcelik et al., 2005a). However, no direct correlation was apparent between antiviral activity and the extract compositions.

According to the outcomes we obtained, the plants belonging to the Asteraceae family look to have a better fatty acid composition, as compared to the rest. Above all, *C. scolymus* and *R. obtusifolius* ssp. *subalpinus*, two edible crop plants, may have a high nutritional value. There has been so far no report on the fatty acid composition of the plants selected in this study. For that reason, to the best of our knowledge, this is the first report on antiviral activity (except for *C. hypoleucum*) and the fatty acids of *A. tinctoria* var. *tinctoria*, *A. austriaca*, *C. acanthoides*, *C. nutans*, *C. hypoleucum*, *C. scolymus*, *S. vulgaris*, *A. chamaepitys* subsp. *chia* var. *ciliata*, *L. barbata*, *R. obtusifolius* ssp. *subalpinus*, *D. lamarckii*, and *S. hispanicum*.

5. Conclusion

Search on antiviral activity of medicinal plants is pertinent both in terms of promoting the conventional use of plants and in the development of novel antiviral based on their active ingredients. The extracts that exhibited a great activity could be considered as a source of potential antiviral substances. The phytochemical characterisation of the extracts and identification of the compounds responsible for activity are necessary.

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