OPEM
www.opem.org

Oriental Pharmacy and Experimental Medicine 2008 **8(3)**, 222-227
DOI 10.3742/OPEM.2008.8.3.222

KHUP

Effect of *Citrus aurantium* var *amara* on weight change in mice

Satyajit Dey Sarker[1,*], Bohlol Habibi[2], Tohid Sharifi[2], Solmaz Asnaashari[3], Lutfun Nahar[1] and Abbas Delazar[4]

[1]School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Co. Londonderry, N. Ireland, UK; [2]Department of Pharmacology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran; [3]Pharmacognosy Laboratory, Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [4]Faculty of Pharmacy and Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Received for publication August 22, 2006; accepted April 11, 2008

SUMMARY

Citrus aurantium var. *amara* L., commonly known as 'bitter orange' or 'sour orange', of the family Rutaceae, has traditionally been used in the treatment of various ailments, and it possesses different types of pharmacological properties. As a part of our on-going studies on the plants from the Iranian flora, the extract of *C. aurantium* var. *amara* has been studied for its weight loss properties using the mice model. While the Sep-Pak fraction, 20% methanol (MeOH) in water, of the hydro-methanolic extract of the peels of *C. aurantium* var. *amara* fruits, when injected intraperitoneal (i.p.) at a dose of 10 mg/kg, significantly decreased the level of weight gain of the mice in comparison with control the group ($P < 0.01$), the Sep-Pak fraction 80% MeOH in water decreased the initial weight of mice by 0.44% in six weeks. The administration of the total extract (10 and 20 mg/kg, i.p.), and the Sep-Pak fractions, 40% and 60% MeOH in water (10 mg/kg, i.p.) did not show any significant change of weight of the test mice. Of the two active fractions, the 80% MeOH in water fraction did not show any noticeable adverse effects on mice, and was therefore analysed by reversed-phase preparative high performance liquid chromatography resulting in the isolation and identification of four major components, two coumarins, meranzin hydrate (1) and bergamottin (2), and two flavonoids, xanthomicrol 5,4'-di-methyl ether (tangeritin, 3) and hymenoxin 5,7-di-methyl ether (nobiletin, 4).

Key words: Rutaceae; *Citrus aurantium* var. *amara*; Coumarin; Flavonoid; Weight reduction

INTRODUCTION

Citrus aurantium var. *amara* L., commonly known as 'bitter orange' or 'sour orange', of the family Rutaceae is native to the countries of tropical Asia and also widely distributed in many tropical and sub-tropical countries (GRIN database, 2007). This plant has traditionally been used in the treatment of various ailments, and it possesses different types of pharmacological properties (Dr. Duke's Phytochemical and Ethnobotanical Databases, 2007). In Chinese traditional medicine, this plant is known as 'zhi shi' and used for weight loss (Firenzuoli *et al.*, 2005). Antioxidant and genotoxicity modulatory activities of *C. aurantium* var. *amara* have been reported (Hosseinimehr and Karami, 2005). Recently, immature bitter orange fruit and

*Correspondence: Satyajit Dey Sarker, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Co. Londonderry, N. Ireland, UK. Tel: +442870324302; Fax: +44287032 4965; E-mail: s.sarker @ulster.ac.uk

its extracts have been introduced into the market as an alternative to *Ephedra* in weight loss products (Mattoli *et al.,* 2005). A great variety of secondary metabolites including alkaloids, coumarins, flavonoids, terpenoids, organic acids, and cinnamic acid derivatives have previously been reported from this plant (Ganzera *et al.,* 2005; Dr. Duke's Phytochemical and Ethnobotanical Databases, 2007).

As a part of our on-going phytochemical and pharmacological evaluation of medicinal plants from the Iranian flora (Delazar *et al.,* 2004, 2005, 2006a,b), we have evaluated the weight loss effect of the extract and Sep-Pak fractions of *C. aurantium* var. *amara* in mice model. Isolation of two coumarins, meranzin hydrate (**1**) and bergamottin (**2**), and two flavonoids, xanthomicrol 5,4'-di-methyl ether (tangeritin, **3**) and hymenoxin 5,7-di-methyl ether (nobiletin, **4**) is also reported.

MATERIALS AND METHODS

General

Nuclear Magnetic Resonance (NMR) spectra were recorded in CD_3OD on a Bruker 200 MHz NMR Spectrometer (200 MHz for ^1H and 50 MHz for ^{13}C) using residual solvent peak as internal standard. High performance liquid chromatography (HPLC) separation was performed in a Shimadzu HPLC system. A Shim-Pak ODS column 20 mm, 250 mm × 20 mm was used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractionation.

Plant materials

The fruits of *C. aurantium* var. *amara* L. were collected from Sari in Mazandaran province (Iran) and the identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the School of Pharmacy, Tabriz University of Medical Sciences, Iran. A voucher specimen (TUM-ADA 118) for this collection has been retained in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Science.

Extraction

Dried ground fruit peels of *C. aurantium* var. *amara* L (500 g) were macerated in water: MeOH (3: 7) for 24 h (three times), and the hydro-methanolic solution was subsequently concentrated to dryness by rotary evaporator at a temperature not exceeding 50°C.

Sep-Pak fractionation

The hydro-methanolic (70%) extract of *C. aurantium* var. *amara* L. was fractionated on a Sep-Pak C_{18} (10 g), cartridge using a step gradient of water-MeOH mixture: 20, 40, 60 and 80% MeOH in water, and 100% MeOH (200 ml each).

Isolation of compounds

The preparative reversed-phase HPLC analysis (mobile phase: 0 to 50 min gradient 70 to 90% MeOH in water; flow-rate: 20 ml/min, detection at 245 nm) of the 80% methanolic Sep-Pack fraction resulted in the isolation of compounds **1** (4.7 mg, t_R = 6.05 min), **2** (3.7 mg, t_R = 8.12 min), **3** (3.8 mg, t_R = 7.89 min) and **4** (5.1 mg, t_R = 15.86 min). The structures of the isolated compounds (**1** - **4**) were determined by spectroscopic means.

Merzanzin hydrate (**1**). White amorphous solid; UV λ_{max} (MeOH): 226, 264, 302; ESIMS *m/z* $[M+H]^+$ 279; ^1H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD): Table 1 (Müller *et al.,* 1993; Dondon *et al.,* 2006).

Bergamottin (**2**). Pale yellow amorphous solid; UV λ_{max} (MeOH): 224, 244, 248, 261, 268, 312; ESIMS *m/z* $[M+H]^+$ 337; ^1H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD): Table 1 (Girennavar *et al.,* 2006).

Xanthomicrol 5,4'-di-methyl ether (Tangeritin **3**). Yellow amorphous solid; UV λ_{max} (MeOH): 280, 296 sh, 334; ESIMS *m/z* $[M+H]^+$ 373; ^1H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD): Table 1 (Mabry *et al.,* 1970; Piattelli and Impellizzeri, 1971; Jahaniani *et al.,* 2005; Nagase *et al.,* 2006).

Hymenoxin 5,7-di-methyl ether (Nobiletin, 4). Yellow amorphous solid; UV λ_{max} (MeOH): 254, 282,

Table 1. Weight changes resulting from administration of *C. aurantium* hydromethanolic (70%) extract, and Sep-Pak fractions 20, 40, 60 and 80% MeOH in water (the results represent mean ± S.E.M.)

Groups	Weight in grams		Variation in weights (%)	*P*-value
	At start	After 6 w		
Control	28.250 ± 0.45	35.143 ± 1.32	24.400%	NA
Extract 10 mg/kg i.p.	28.375 ± 0.5	35.500 ± 1.35	25.110%	ns
Extract 20 mg/kg i.p.	28.000 ± 0.38	32.375 ± 1.22	15.625%	ns
80% Methanolic fraction 10 mg/kg i.p.	28.500 ± 0.46	28.375 ± 0.82	- 0.440%	*
60% Methanolic fraction 10 mg/kg i.p.	27.625 ± 0.50	34.125 ± 0.77	23.530%	ns
40% Methanolic fraction 10 mg/kg i.p.	27.750 ± 0.45	33.625 ± 0.65	21.171%	ns
20% Methanolic fraction 10 mg/kg i.p.	28.875 ± 0.52	29.125 ± 1.39	0.865%	*

*P < 0.01; NA: Not applicable; ns: Non-significant; -: Weight loss

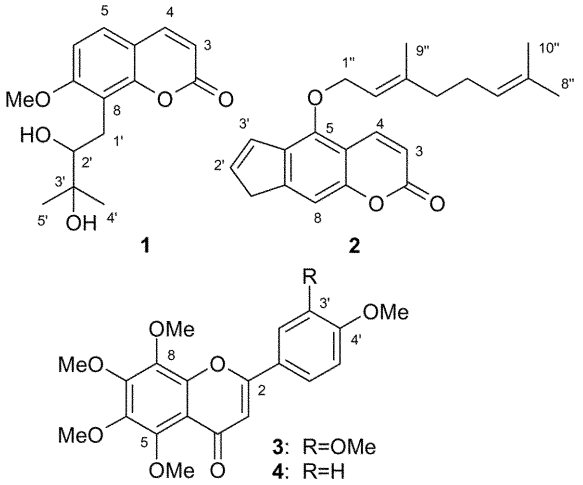

Fig. 1. Structures of compounds (**1** - **4**) isolated from the Sep-Pak fraction 80% MeOH in water.

344; ESIMS *m/z* $[M+H]^+$ 403; ^1H NMR (200 MHz, CD_3OD) and ^{13}C NMR (50 MHz, CD_3OD): Table 1 (Mabry *et al.,* 1970; Piattelli and Impellizzeri, 1971; Waddell, 1973; Nagase *et al.,* 2006).

Preparation of the extract solutions for bioassays

The hydro-methanolic (70%) extract and four fractions obtained by Sep-Pack method were dissolved in mixture of dimethylsulphoxide (DMSO)-water (1: 3). Each dose was dissolved in 0.1 ml of DMSO-water (1: 3).

Animals

In this study 56 male mice (25 - 30 g) were selected and divided to 7 groups (n = 8).

Evaluation of weight change in mice

In this study 56 male mice (25 - 30 g) were selected and divided to 7 groups (n = 8). Seven groups of animals received during 42 days: mixture of DMSO-water (1: 3) (0.l ml/day as control group), the hydro-methanolic (70%) dried extract (10 and 20 mg/kg i.p.) dissolved in 0.1 ml of DMSO-water (1: 3), and four different dried Sep-Pack fractions (10 mg/kg, i.p.) in 0.1 ml of DMSO-water (1: 3).

RESULTS

In this study, while the Sep-Pak fraction, 20% MeOH in water, of the hydro-methanolic extract of the peels of *C. aurantium* var. *amara* fruits, when injected intraperitoneal (i.p.) at a dose of 10 mg/kg, significantly decreased the level of weight gain of the mice in comparison with control the group (P < 0.01), the Sep-Pak fraction 80% MeOH in water decreased the initial weight of mice by 0.44% in six weeks (P < 0.01) (Table 1, Figs. 2 and 3). However, the administration of the total extracts (10 and 20 mg/kg, ip), 40% and 60% methanolic fractions (10 mg/kg, i.p.) did not show any statistically significant change of weight of the test mice.

Although the Sep-Pak fraction, 20% MeOH in water, was active, it also displayed considerable adverse effects on mice, including hyperactivity and increased excitements. The other active fraction, 80% MeOH in water, did not exhibit such adverse effects at all.

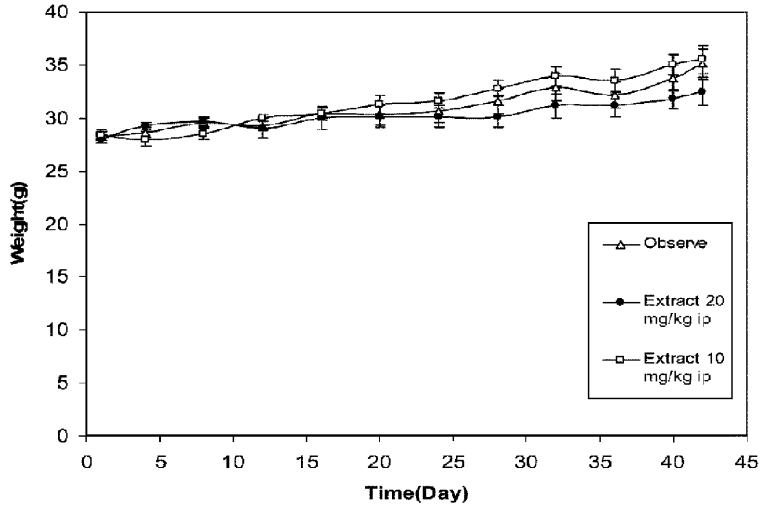

Fig. 2. The comparison of the weight changes resulting from hydromethanolic extract of *C. aurantium* (10 and 20 mg/kg, i.p.) with control group (DMSO-water) (the results represent mean ± S.E.M.).

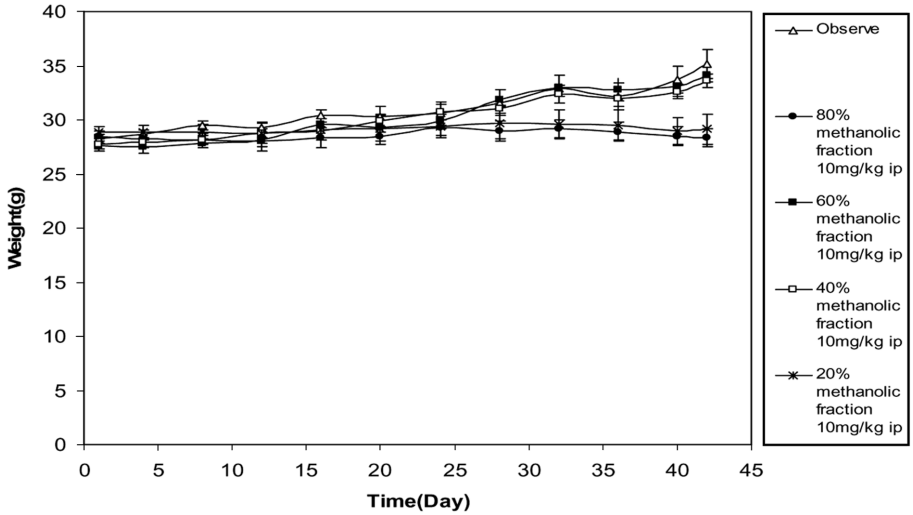

Fig. 3. The comparison of the weight changes resulting from the Sep-Pak fractions 20, 40, 60 and 80% MeOH in water (10 mg/kg, i.p.) with control group (DMSO-water) (the results represent mean ± S.E.M.).

As 80% MeOH in water fraction had significant weight loss activity, but no adverse effects on mice, this fraction was analysed for isolation and identification of major components. The reversed-phase preparative HPLC analysis of this fraction afforded four main compounds, two coumarins, meranzin hydrate (**1,** Müller *et al.,* 1993; Dondon *et al.,* 2006) and bergamottin (**2,** Girennavar *et al.,* 2006), and two flavonoids, xanthomicrol 5,4'-di-methyl ether (tangeritin, **3**, Mabry *et al.,* 1970; Piattelli and Impellizzeri, 1971; Jahaniani *et al.,* 2005; Nagase *et al.,* 2006) and hymenoxin 5,7-di-methyl ether (nobiletin, **4**, Mabry *et al.,* 1970; Piattelli and Impellizzeri, 1971; Waddell, 1973; Nagase *et al.,* 2006) (Fig. 1). The structures of these compounds were determined by UV, MS, ^{1}H and ^{13}C NMR (Table 1) spectroscopic data analyses. The experimental data were in good agreement with respective

Table 2. ^{1}H (200 MHz, CD_3OD, coupling constant J in Hz in parentheses) and ^{13}C NMR (50 MHz, CD_3OD) data of compounds **1- 4**

Carbon no.	Chemical shifts in ppm							
	H				C			
	1	2	3	4	1	2	3	4
1	-	-	-	-	-	-		-
2	-	-	-	-	160.5	160.9	160.72	163.4
3	6.28 d (9.5)	6.33 d (9.8)	6.73 s	6.71 s	112.8	112.1	106.8	105.7
4	7.92 d (9.5)	8.31 d (9.8)	-	-	144.1	140.6	176.2	177.3
5	7.54 d (8.7)	-	-	-	126.9	149.8	144.0	144.7
6	7.08 d (8.7)	-	-	-	107.4	119.5	138.1	138.5
7	-	-	-	-	161.4	159.2	151.4	152.2
8	-	7.24 s	-	-	115.7	93.8	138.1	131.4
9	-	-	-	-	153.3	152.3	148.0	148.4
10	-	-	-	-	113.1	107.7	114.7	126.0
1’	2.98-3.18 m	-	-	-	25.5	105.3	119.8	123.5
2’	3.86 dd (9.2, 3.6)	7.84 d (2.4)	7.57 d (2.16)	8.02 d (8.8)	78.2	145.9	109.7	114.7
3’	-	7.22 d (2.4)	-	7.15 d (8.8)	73.1	-	149.5	114.7
4’	1.31 s	-	-	-	26.5	-	152.3	162.9
5’	1.32 s	-	7.16 d (8.57)	7.15 d (8.8)	26.5	-	112.5	114.7
6’	-	-	7.70 dd (8.6, 2.2)	8.02 d (8.8)	-	-	123.7	127.7
1”	-	5.09 d (7.0)	-	-	-	69.9	-	-
2”	-	5.72 t (7.0)	-	-	-	114.7	-	-
3”	-	-	-	-	-	143.6	-	-
4”	-	2.42 m	-	-	-	39.2	-	-
5”	-	2.23 m	-	-	-	26.2	-	-
6”	-	3.96 t (6.9)	-	-	-	123.6	-	-
7”	-	-	-	-	-	132.2	-	-
8”	-	1.19 s	-	-	-	24.8	-	-
9”	-	1.73 s	-	-	-	15.7	-	-
10”	-	1.15 s	-	-	-	23.9	-	-
OCH_3-5	-	-	4.15 s	4.14 s	-	-	61.5	61.7
OCH_3-6	-	-	3.96 s	3.93 s	-	-	61.1	61.2
OCH_3-7	3.98 s	-	4.07 s	4.06 s	56.2	-	61.6	61.6
OCH_3-8	-	-	3.98 s	3.96 s	-	-	61.2	61.2
OCH_3-3’	-	-	3.95 s	-	-	-	54.8	-
OCH_3-4’	-	-	3.92 s	3.93 s	-	-	55.0	55.1

published data.

DISCUSSION

All these compounds (**1 - 4**) are known to possess a variety of biological properties (ISI database, 2008). Thus it is reasonable to assume that these compounds, owing to their presence in significant amounts in the extract as well as in the Sep-Pak 80% MeOH in water fraction, and their bioactive nature, might be responsible for weight loss activity in mice.

Previous reports have indicated that octopamime and synephrin, two amines of *C. aurantium*, possess weight loss properties as well as cardio-vascular

side effects. In our study, as Sep-Pak 20% MeOH in water fraction showed adverse effects, and this fraction might contain these amines. However none of these amines could be detected or isolated from the Sep-Pak 80% MeOH in water fraction, which did not show any adverse effect.

The significant weight loss property of the Sep-Pak 80% MeOH in water fraction of the hydromethanolic extract of the peels of the fruits of *C. aurantium* var. *amara* in mice model provides scientific evidence in favour of its use as an alternative to Ephedra in weight loss products.

REFERENCES

Delazar A, Celik S, Gokturk RS, Unal O, Nahar L, Sarker SD. (2005) Two acylated flavonoids from *Stachys bombycina* and their free radical scavenging activity. *Pharmazie* **60**, 878-880.

Delazar A, Byres M, Gibbons S, Kumarasamy Y, Nahar L, Modarresi M, Shoeb M, Sarker SD. (2004) Iridoid glycosides from *Eromostachys glabra*. *J. Nat. Prod.* **67**, 1584-1587.

Delazar A, Modarresi M, Shoeb M, Nahar L, Reid RG, Majinda RRT, Sarker SD. (2006a) Eremostachiin: A new furanolabdane diterpene glycoside from *Eromostachys glabra*. *Nat. Prod. Res.* **20**, 167-172.

Delazar A, Biglari F, Nazemiyeh H, Talebpour A, Imani Y, Nahar′ L, Sarker SD. (2006b) GC-MS analysis of the essential oils, and the isolation of phenylpropanoid derivatives from the aerial parts of *Pimpinella aurea*. *Phytochemistry* **67**, 2176-2181.

Dondon R, Bourgeois P, Fery-Forgues S. (2006) A new bicoumarin from the leaves and stems of *Triphasia trifolia*. *Fitoterapia* **77**, 129-133.

Firenzuoli F, Gori L, Galapai C. (2005) Adverse reaction to an adrenergic herbal extract (*Citrus aurantium*). *Phytomedicine* **12**, 247-248.

Ganzera M, Lanser C, Stuppner H. (2005) Simultaneous determination of *Ephedra sinica* and *Citrus aurantium* var. *amara* alkaloids by ion-pair chromatography. *Talanta* **66**, 889-894.

Girennavar B, Poulose SM, Jayaprakasha GK, Bhat NG, Patil, BS. (2006) Furanocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes. *Bioorg. Med. Chem.* **14**, 2606-2612.

GRIN database. (2007) USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network - (GRIN), National Germplasm Resources Laboratory, Beltsville, Maryland, USA. Available on-line at: http://www.ars-grin.gov/cgi-bin/npgs/html/genform.pl.

Hosseinimehr SJ, Karami M. (2005) Citrus extract modulates genotoxicity induced by cyclophosphamide in mice bone marrow cells. *J. Pharm. Pharmacol.* **57**, 505-509.

ISI Database. (2008) ISI Web of Knowledge Service for UK Education, Thompson Scientific, UK. Available on-line at: http://wok.mimas.ac.uk/

Jahaniani F, Ebrahimi SA, Rahbar-Roshandel N, Mahmoudian M. (2005) Xanthomicrol is the main cytotoxic component of *Dracocephalum kotshyii* and a potential anti-cancer agent. *Phytochemistry* **66**, 1581-1592.

Mabry TJ, Markham KR, Thomas MB. (1970) The Systematic Identification of Flavonoids, Spinger-Verlag, New York.

Mattoli L, Cangi F, Maidecchi A, Ghiara C, Tubaro M, Traldi P. (2005) A rapid liquid chromatography electrospray ionization mass spectrometry(n) method for evaluation of synephrine in *Citrus aurantium* L. samples. *J. Agric. Food Chem.* **53**, 9860-9866.

Muller AH, Degaspari LRO, Vieira PC, Da Silva MFDGF, Fernandes JB, Pitani JR. (1993) 3-Methoxyfurano-coumarins from *Pilocarpus riedelianus*. *Phytochemistry* **34**, 585-586.

Nagase H, Omae N, Omori A, Nakagawasai O, Tadano T, Yokosuka A, Sashida Y, Mimaki Y, Yamakuni T, Ohizumi Y. (2005) Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities. *Biochem. Biophys. Res. Comm.* **337**, 1330-1336.

Phytochemical and Ethnobotanical databases. (2007) USDA, ARS, Beltsville Agricultural Research Center, Beltsville, Maryland, USA. Available on-line at: http://www.ars-grin.gov/cgi-bin/duke/ethnobot.pl.

Piattelli M, Impellizzeri G. (1971) Fungistatic flavones in the leaves of citrus species resistant and susceptible to *deuterophoma tracheiphila*. *Phytochemistry* **10**, 2657-2659.

Waddell TG. (1973) Hymenoxin: A flavone from *Helianthus angustifolius*. *Phytochemistry* **12**, 2061.