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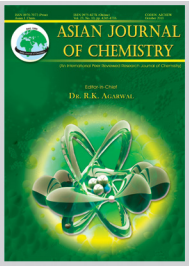

Phytochemical Study and *in vitro* Cytotoxic Effect of *Ajuga chamaecistus* ssp. *tomentella*

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In this study total methanolic extract (80 %), *n*-hexane, diethyl ether, hydroalcoholic fractions and three major compounds from aerial parts of *Ajuga chamaecistus* ssp. *tomentella* were investigated for *in vitro* cytotoxic effect against cancer (HT-29, Caco-2, T47D) and normal (NIH 3T3) cell lines by the MTT assay. The *n*-hexane fraction showed cytotoxicity against all cell lines with $IC_{50} \geq 200$ µg/mL and the diethyl ether fraction exhibited medium cytotoxic effect against HT-29 cell line (IC_{50} 311.01 ± 9.0 µg/mL). The diethyl ether fraction was chromatographed on silica gel using a chloroform-ethyl acetate-methanol gradient system to give compound **1**, **2** and **3**. The structure of compound **1**, **2** and **3** were determined to be 20-hydroxyecdysone, cyasterone and 8-acetylharpagide, respectively, by means of spectroscopic analysis. These three major compounds were inactive in cytotoxicity evaluation (IC_{50} > 400 and 800 µg/mL), suggesting little correlation between the degree of cytotoxic effect of the diethyl ether fraction and the isolated compounds.

Key Words: *Ajuga chamaecistus* ssp. *tomentella*, Cytotoxic effect, 20-Hydroxyecdysone, Cyasterone, 8-Acetylharpagide.

INTRODUCTION

The genus *Ajuga* (Lamiaceae), are distributed over the world and used as medicinal plant in traditional medicine of several countries as anthelmintic, antifungal, antifebrile, antitumor, antimicrobial and diuretic agents[1]. Five species of this genus have been known in the flora of Iran, of which *Ajuga chamaecistus* has several endemic subspecies including ssp. *tomentella*[2]. The species belonging to this genus are used for treatment of joints pains, gout and jaundice in Iranian traditional medicine[3]. Several biological studies have been achieved on many species of the genus *Ajuga*, which confirmed its ethnopharmacological effects as hypoglycemic[4], treatment of joint disease[5], antiinflammatory[6] and antimalarial[7]. Many phytochemicals such as phytoecdysteroids, diterpenes and iridoids have been isolated from *Ajuga* species[8], several of which showed antitumor activity[9-11]. The aim of this study is to determine cytotoxic activities of total methanolic extract and partition fractions and a phytochemical investigation of the diethyl ether fraction obtained from aerial parts of *Ajuga chamaecistus* ssp. *tomentella*, collected in Tehran (Iran), which has not been previously reported. Furthermore, we examined cytotoxicity of two major phytoecdysteroids and one iridoid glycoside, isolated from the diethyl ether fraction of this plant, against colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), breast ductal carcinoma (T47D) and Swiss mouse embryo fibroblast (NIH 3T3) by MTT assay.

EXPERIMENTAL

^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY and HSQC spectra were measured in DMSO-d_6 and TMS as internal standard at Bruker Avance spectrometer (500 MHz). ESI-MS were recorded on ESI-TOF, Agilent 6210 ESI-TOF, Agilent Technologies, in Department of Chemistry, Free University of Berlin. Melting points were recorded on a Reichert-Jung apparatus. FT-IR spectra were determined using a Nicolet 550-A spectrometer (KBr disks). Column chromatography was performed on silica gel 60 (230-400 mesh, Merck).

Plant material: Aerial parts of *Ajuga chamaecistus* Ging. ssp. *tomentella* (Boiss.) Rech. F. were collected from Tehran, Iran, in June 2008 and verified by Prof. Gholamreza Amin. A voucher specimen (THE-6697) has been deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical sciences, Tehran, Iran.

Extraction and isolation: The air-dried and ground aerial parts of *A. chamaecistus* ssp. *tomentella* (1 kg) were extracted with methanol 80 % (7 × 2.5 L) at room temperature and concentrated under reduced pressure to give a dark brown extract

(180 g). The extract (150 g) was defatted through repeated extraction with *n*-hexane. The defatted extract was partitioned between methanol 80 % and diethyl ether. The diethyl ether layer (10 g) was selected for phytochemical studies. Thus, it was chromatographed on silica gel (mesh 230-400) eluting with a gradient of chloroform-ethyl acetate and methanol to afford 7 fractions. Fraction 7 (6 g) was subjected to silica gel and eluted with chloroform-methanol (9:1.5, v/v) to give compound **1** (400 mg) and compound **3** (100 mg). Compound **2** (160 mg) was given through chromatography of fraction 6 on silica gel eluting with chloroform-methanol (9:0.75, v/v).

Spectroscopic data

20-Hydroxyecdysone (1): Yellow amorphous powder, m.p. 240-245 ºC. FT-IR, ν_{max}, cm^{-1}: 3403, 3070, 1654. ^1H and ^{13}C NMR (DMSO-d_6), Table-1; ESI-MS m/z 503.298 $[M + Na]^+$, 983.607 $[M_2 + Na]^+$.

Cyasterone (2): White amorphous powder, m.p. 160-165 ºC, FT-IR, ν_{max}, cm^{-1}: 3439, 2939, 1751 and 1654. ^1H and ^{13}C NMR (DMSO-d_6), Table-1.

8-Acetylharpagide (3): White amorphous powder, m.p. 154-156 ºC, ^1H NMR (DMSO-d_6, 500 MHz) δ:1.35 (3H, s, Me-10), 1.75 (1H, dd, Hα-7), 1.92 (3H, s, OAc), 2.04 (1H, brd, Hβ-7), 2.63 (1H, brs, H-9), 2.96 (1H, m, H-2'), 3.06 (1H, m, H-5'), 3.11-3.14 (2H, m, H-3', H-4'), 3.67 (1H, brd, H-6'), 4.36 (1H, d, H-1'), 4.86 (1H, brd, H-4), 5.86 (1H, brs, H-1), 6.34 (1H, d, H-3); ^{13}C NMR (DMSO-d_6, 500 MHz) δ: 22.02 (Me-Ac), 22.07 (Me-10), 44.50 (CH_2-7), 54.40 (CH-9), 60.67 (CH_2-6'), 70.12 (CH-4'), 71.16 (C-5), 73.02 (CH-2'), 75.67 (CH-3'), 76.13 (CH-6), 77.09 (CH-5'), 86.36 (C-8), 92.44 (CH-1), 97.30 (CH-1'), 107.34 (CH-4), 141.16 (CH-3), 170.10 (CO); ESI-MS m/z 429.139 $[M + Na]^+$, 835.28 $[M_2 + Na]^+$.

Cell culture: The colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2) and breast ductal carcinoma (T47D) cell lines were maintained as exponentially growing cultures in RPMI 1640 cell culture medium (PAA, Germany) supplemented with 10 % fetal bovine serum (FBS; Gibco, USA) for HT-29 cells and 15 % FBS for Caco-2 and T47D cells. The Swiss mouse embryo fibroblast (NIH 3T3) cell line was kept in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Germany) supplemented with 10 % FBS. 100 IU/mL penicillin and 100 µg/mL streptomycin (Roche, Germany) were added to the media. All cell lines were cultured at 37 ºC in air/carbon dioxide (95:5) atmosphere.

Determination of cell viability by MTT assay: The concentration of 5, 50, 150, 450 and 900 µg/mL from all samples including total methanolic extract and partition fractions were tested for each cell line. Samples were dissolved in DMSO (dimethyl sulfoxide) and further diluted with cell culture medium. The final DMSO concentration used was 1 % of total volume of medium in all treatments, including the control group. Cells with no treatment and methanolic (80 %) extract of *Vinca rosea* treatment were examined as negative and positive control respectively.

For mitochondrial tetrazolium test (MTT) assay, 1×10^4 cells/well were plated into 96-well plates (Nunc, Denmark) and incubated for 24 h before addition of extracts. After 72 h of incubation for HT-29 cells, 96 h for T47D and NIH 3T3 cells and 120 h for caco-2 cells, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT; Merck, Germany) reagent (5 mg/mL) in phosphate buffered serum (PBS) was added to each well. The incubation time for each cell line was assigned according to the normal growth curve of that cell line and was determined twice as long as the doubling time of each cell line. The plates were incubated at 37 ºC for 4 h. At the end of the incubation period, the medium was removed and 100 µL cell culture grade DMSO was added to each well. The formazan salts were quantified by reading the absorbance at 550 nm on a microplate reader (Anthos, Austria)[12]. Cell viability in MTT assays was calculated as a percentage of untreated cells (control value). The cytotoxicity value was presented as IC_{50} (the median growth inhibitory concentration) of the reagents compared to control. IC_{50} values were calculated by Sigmaplot (10) software.

RESULTS AND DISCUSSION

Bioassay of plants which are used in traditional medicine could be the first step in discovery of new drugs. Regarding to this, we studied *in vitro* cytotoxicity of the methanolic extract and some partition fractions of *A. chamaecistus* ssp. *tomentella* on cancer and normal cell lines. Percolation of the aerial parts of *A. chamaecistus* ssp. *tomentella* with MeOH 80 % yielded the crude extract. The defatted extract was partitioned between methanol 80 % and diethyl ether led to the 10 g diethyl ether layer. We selected the diethyl ether fraction for isolation and characterization of active compounds because it contains a wide variety of compounds from low to medium polarity. The diethyl ether layer was subjected to open column chromatography on silica gel, resulting in isolation of three major compounds. These compounds were identified by spectroscopic data (^{13}C and ^1H NMR, ^1H-^1H COSY, HSQC and ESI-MS) and compared with literature values[13-15]. ^1H and ^{13}C NMR data of these compounds run in DMSO-d_6 reported for the first time. δ_H and δ_C (ppm) of compound **1** and **2** were noted in Table-1.

TABLE-1
^1H NMR AND ^{13}C NMR DATA OF COMPOUND **1** AND **2** (500 MHz IN DMSO-d_6)

Compound **1**			Compound **2**		
Position	δ_H	δ_C	Position	δ_H	δ_C
1 eq	1.60	36.35	2 ax	3.74(m)	66.7
2 ax	3.62(m)	66.53	3 eq	3.76(brs)	66.5
3 eq	3.76(brs)	66.34	5	2.20(dd)	50.09
4 eq	1.57(m)	31.3	6		202.67
5	2.19(dd)	49.86	7	5.63(d)	120.5
6		205	8		165.1
7	5.62(brs)	120.2	9	3.00(brt)	33.18
8		166.1	10		37.6
9 ax	3.00(brt)	32.91	12 ax	2.02(td)	30.82
11 eq	1.62(brd)	19.82	13		46.95
12 ax	2.01(td)	30.61	16		20.18
15	1.86(m)	30.07	18	0.77(s)	17.1
17	2.25(brt)	48.4	19	0.84(s)	23.8
18	0.76(s)	16.9	20		75.7
19	0.83(s)	23.6	21	1.07(s)	20.6
21	1.05(s)	20.7	22	3.6(brd)	73.3
22	3.11(dd)	75.95	23		33.18
23 a	1.23(m)	25.84	25	2.44(m)	41.48
26	1.06(s)	28.7	27	1.17(d)	15.1
27	1.07(s)	29.7	28	4.16(m)	79.3
			29	1.32(d)	19.0

TABLE-2
IC_{50} VALUES (μg/mL) OF METHANOLIC EXTRACT AND PARTITION FRACTIONS OF *A. chamaecistus* ssp. *tomentella* AND METHANOLIC EXTRACT OF *Vinca rosea* (AS POSITIVE CONTROL) AGAINST TUMOR AND NORMAL CELL LINES. RESULTS ARE EXPRESSED AS MEAN ± SD

Samples	Cell Lines[a] (MTT assay)			
	T47D	Caco-2	HT-29	NIH3T3
Total extract	780.24 ± 531	> 800	> 800	> 800
n-Hexane fraction	239.57 ± 5.0	253.28 ± 50.1	244.26 ± 137.4	175.21 ± 214.
Diethylether fraction	544.08 ± 5.0	> 800	311.01 ± 9.0	270.84 ± 195.4
Hydroalcoholic fraction	502.78 ± 5.0	> 800	754.39 ± 168	540 ± 5.0
20-Hydroxyecdysone	> 400	> 800	> 800	> 800
Cyasteron	> 800	> 800	> 800	> 800
8-Acetylharpagide	> 400	> 400	> 800	> 800
Total extract of *Vinca rosea*	195.78 ± 17.96	571.32 ± 5.0	412.09 ± 43.55	> 900

[a]Key to cell lines employed: HT-29 and Caco-2 (colon adenocarcinoma), T47D (breast carcinoma), NIH 3T3 (Swiss embryo fibroblast).

Compounds **1**, **2** and **3** were identified as 20-hydroecdysone, cyasterone (phytoecdysteroid) and 8-acetylharpagide (an iridoid glycoside), respectively (Fig. 1). The IC_{50} values of total extract and fractions with different polarity are shown in Table-2.

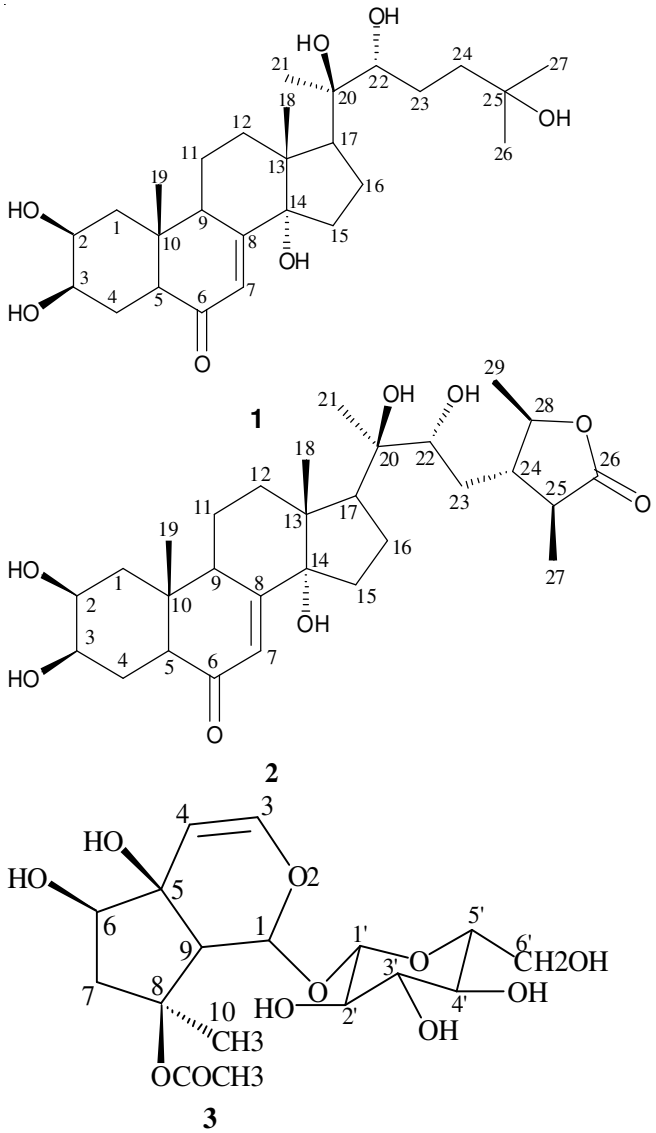

Fig. 1. Molecular structures of isolated compounds **1** (20-Hydroxyecdysone), **2** (Cyasterone) and **3** (8-Acetylharpagide) from the diethyl ether fraction of *Ajuga chamaecistus* ssp. *tomentella*

Phytoecdysteroids are mainly C_{27}-C_{29} molecules derived from phytosterols with A/B-*cis* ring, an α, β-unsaturated ketone in ring-B and multiple hydroxyl groups attached to the structure. Phytoecdysteroids are similar to insect steroidal hormones, found in plants. This group of natural products produce a wide range of pharmacological activities in mammals including adaptogenic, anabolic[16], antidiabetic[17], hepato-protective, immunoprotective, wound-healing[18], antioxidant and free radical scavenging activities[19]. In plants of genus *Ajuga*, a variety of phytoecdysteroids have been identified among them, 20-hydroxyecdysone (β-ecdysone) and cyasterone are the most abundant[9].

Iridoids are monoterpenoid in origin and contain a cyclopentane ring, which is usually fused to a six-membered oxygen heterocycle, they are found usually as glycosidic forms in nature and rarely exist as aglycone. Iridoids are found in many medicinal plants and produce a broad range of pharmaceutical and biological activities. A number of studies showed anti-inflammatory, antioxidant, cytotoxic activity[20], chemoprotective[10], cardiovascular, hypoglycemic and hypolipidemic effects of iridoids[21].

In comparison with total methanolic extract of *Vinca rosea*, that comprises antineoplastic compounds (vinblastine and vincristine), total extract of this plant exhibited weak cytotoxic effect against T47D cell line and was inactive on other cell lines up to 800 μg/mL. Among the all fractions, *n*-Hexane fraction, which includes non polar constituents, showed more cytotoxicity on all cell lines than the other fractions. The diethyl ether fraction indicated moderate cytotoxicity against HT29 and T47D as well as in NIH 3T3 cell lines. The pure compounds (**1**, **2** and **3**) were actually inactive, showing IC_{50} values higher than 400 and 800 μg/mL.

Conclusion

The results of present study show that phytoecdysteroids and iridoid glycoside could be considered as major components of *Ajuga chamaecistus* ssp. *tomentella*. According to these results it can be suggested that medium cytotoxic activity of the diethyl ether fraction could be related to other compounds or as a result of a synergic effect of these compounds.

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REFERENCES

1. N. Sh. Ramazanov, *Chem. Nat. Compd.*, **41**, 361 (2005).
2. V. Mozaffarian, A Dictionary of Iranian Plant Names, Tehran, p. 21 (2007).
3. F. Naghibi, M. Mosaddegh, S.M. Motamed and A. Ghorbani, *Iran. J. Pharm. Res.*, **2**, 63 (2005).
4. J. Hilaly and B. Lyoussi, *J. Ethnopharmacol.*, **80**, 109 (2002).
5. Y. Ono, Y. Fukaya, S. Imai and T. Yamakuni, *Biol. Pharm. Bull.*, **31**, 1199 (2008).
6. R. Gautam, S.M. Jachak and A. Saklani, *J. Ethnopharmacol.*, **133**, 928 (2011).
7. K.A.M. Kuria, S. Coster, G. Muriuki, W. Masengo, I. Kibwage, J. Hoogmartens and G.H. Laekeman, *J. Ethnopharmacol.*, **74**, 141 (2001).
8. M.-Pia Calcagno, F. Camps, J. Coll, E. Mele and F. Sanchez-Baeza, *Tetrahedron*, **51**, 1219 (1995).
9. P. Akbay, J. Gertsch, I. Calis, J. Heilmann, O. Zerbe and O. Sticker, *Helv. Chim. Acta*, **85**, 1930 (2002).
10. T. Konoshima, M. Takasaki, H. Tokuda and H. Nishino, *Cancer Lett.*, **157**, 87 (2000).
11. M. Takasaki, H. Tokuda, H. Nishino and T. Konoshima, *J. Nat. Prod.*, **62**, 972 (1999).
12. T. Mosmann, *J. Immunol. Methods*, **65**, 55 (1983).
13. M. Wesner, B. Champion, J. Girault, N. Kaouadji, B. Saidi and R. Lafont, *Phytochemistry*, **31**, 3785 (1992).
14. C. Boros and F.R. Stermiyz, *J. Nat. Prod.*, **53**, 1066 (1990).
15. S.D. Sarker, V. Sik, H.H. Rees and L. Dinan, *Phytochemistry*, **49**, 2305 (1998).
16. L. Dinan, *Arch. Insect. Biochem. Physiol*., **72**, 126 (2009).
17. K. Hamden, F. Ayadi, K. Jamoussi, H. Masmoudi and A. Eifeki, *Bio. Factors*, **33**, 165 (2008).
18. L. Dinan, *Phytochemistry*, **57**, 325 (2001).
19. Y.-J. Cai, J.-Q. Dai, J.-G. Fang, L.-P. Ma, L.-F. Hou, L. Yang and Z.-L. Liu, *Can. J. Physiol. Pharmacol.*, **80**, 1187 (2002).
20. K.A.M. Kuria, H. Chepkwony, C. Govaerts, E. Roets, R. Busson, P. Witte, I. Zupko, G. Hoornaert, L. Quirynen, L. Maes, L. Janssens, J. Hoogmartens and G. Laekeman, *J. Nat. Prod.*, **65**, 789 (2002).
21. S. Bouderbala, J. Prost, M.A. Lacaille-Dubois and M. Bouchenak, *Nutr. Res.*, **30**, 358 (2010).