ORIGINAL ARTICLE

PHYTOCOMPONENTS IDENTIFICATION IN MISTLETOE (*VISCUM ALBUM*) YOUNG LEAVES AND BRANCHES, BY GC-MS AND ANTIPROLIFERATIVE EFFECT ON HEPG2 AND MCF7 CELL LINES

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Abstract

In the present work, aqueous and alcoholic extracts of *Viscum album* young leaves and branches were evaluated, in order to identify the most relevant constituents that confirm its biological activity. GC-MS was the employed technique used for separation and identification of the extracted components. Both extracts revealed the presence of important terpenoids, fatty acids and natural antioxidants such as vitamin E. The antiproliferative potential of the obtained extracts was also investigated on neoplastic cell lines, HepG2 and MCF-7. The alcoholic extract was more efficient and proved a significant cytotoxic effect at a concentration of 75 mg/mL, but inhibition of cell proliferation was also observed when the aqueous extract was used.

Rezumat

În lucrarea de față, au fost evaluate extractele apoase și alcoolice de *Viscum album* (frunze tinere și ramuri), pentru a identifica elementele constitutive cele mai relevante care confirmă activitatea sa biologică. Pentru separarea și identificarea compușilor extrași, s-a folosit o metodă GC-MS. Ambele extracte au evidențiat prezența terpenoidelor, a acizilor grași, și a antioxidanților naturali precum vitamina E. Potențialul antiproliferativ al extractelor obținute a fost de asemenea investigat pe linii celulare neoplazice, HepG2 și MCF-7. Extractul alcoolic a fost mai eficient, prezentând efect citotoxic semnificativ la concentrația de 75 mg/mL, dar inhibarea proliferării celulare a fost de asemenea observată și în cazul extractului apos.

Keywords: *Viscum album*, mistletoe, GC-MS, cell proliferation, cytotoxicity

Introduction

The high biological properties of plant's extracts have been the subject of intense studies. Chemical investigations of plant material are of great interest in medicine, because of their antioxidant and antimicrobial potentials [10, 12]. Mistletoe (*Viscum album*), also known as iscidor, helixor, isorel in European countries, is a semi-parasitic plant, that is regarded as an effective medication in treating cancer [3]. Numerous studies regarding the identification of biological potential of the plant were reported so far [7-9, 16]. Antioxidant properties were assessed in different host plants proving the neuroprotective effect of the semi- parasitic plant [13]. The antitumor effect was also evaluated in several carcinoma cell lines and the results evidenced cytotoxic, apoptosis – inducing and immunostimulatory activity [3, 15]. Various clinical studies reported the improvement in survival and quality of life, after using mistletoe extracts, underling the ability of the plant to support the conventional medicine [5].

The present study followed the identification of the phytocomponents of mistletoe (*Viscum album*) young leaves and branches in both aqueous and ethanolic extract, after acid hydrolysis and derivatization with bis-(trimethylsilyl)-trifluoro-acetamide. Gas chromatography was used for separation of volatile compounds and mass spectrometry was used for identification of the resulted peaks. The study also aimed to highlight the antiproliferative potential of

Viscum album extracts in neoplastic cell lines: HepG2 and MCF-7.

Materials and Methods

Materials. Ethanol, GC purity, was purchased from Sigma – Aldrich. Ultrapure water (Type I in house made) was used for the extraction procedure. Derivatization agent, bis (trimethylsilyl) trifluoro-acetamide (BSTFA) with the addition of 1% trimethylchlorosilane (TMCS) was obtained from Cerriliant. Hydrochloric acid (HCl) 37%, sodium sulphate (Na_2SO_4), and hexane (GC purity) were purchased from Sigma – Aldrich.

Plant material. The raw material was harvested in March, in apple trees from spontaneous flora of Bihor County and was identified by two specialists (a gardener engineer, Phd. Ivan Pauliuc and a biologist, Phd. Filimon Nicoleta). After harvesting, young leaves and branches were cleaned and left to dry naturally at room temperature, protected from light. Young leaves and branches were powdered and the obtained powder was maintained at room temperature, in dark, until required for the extraction procedure.

Extraction procedure of plant material. 20 g of dried powder were extracted using 95% ethanol, 5% methanol and Type I deionized water in dark for 24 hours under magnetic stirring. The two extracts were evaporated to dryness under reduced pressure. All extracted components were stored in amber bottles at 4°C.

Acid hydrolysis. Acid hydrolysis was performed with 3.5 M HCl under reflux, at 100°C, for an hour, followed by extraction with a nonpolar solvent, in order to release the algycones from the plant material. Before the derivatization procedure, samples were concentrated under nitrogen stream.

Derivatization was performed using bis-(tri-methylsilyl)-trifluoroacetamide reagent. The reaction was maintained at 70°C for 60 min. In order to increase the trimethylsilyl donor potential, silylation was catalysed by the addition of 1 % TMCS.

Gas chromatography. Plant extracts were analysed on a 450 gas chromatography system (Varian), using helium as carrier gas, at a constant flow rate of 1.1 mL/min, and a linear velocity of 39.8 cm/sec. The separation was achieved on BR – 5 ms capillary column (30 m x 0.25 mm ID, 0.25 µm film) (Bruker). Column program was set as following: 50°C, 2 min, raised to 200°C with 10°C/min, 2 min, followed by 250°C, with 5°C/min, 2 min, and the final temperature was set at 300°C, with a stationary time of 10 min. Injection port temperature was set at 250°C. The injection volume was 1 µL, using split mode ratio for 3 min, followed by splitless mode for the rest of the analysis. Total run time was achieved in 60 min, under the chromatographic conditions described above.

Mass spectrometry. A 240 MS Ion Trap (Varian) instrument was used for the identification of chemical compounds extracted from *Viscum album*. Electron impact ionization energy was set at 70 eV. The ionization parameters were set as follows: ion trap temperature at 170°C and transfer line temperature at 240°C. For the identification of the eluate, nominal mass was used and total ion current (TIC, mass range 50 - 650 atomic mass unit) technique was applied. For the confirmation of the identity of the separated compounds, the obtained MS spectra were integrated in the spectral database: National Institute for Standard and Technology (NIST), Wiley and Pfleger, Maurer and Weber (PMW).

Cell culture. HepG2 cell line (Human Hepato-cellular Carcinoma cell line) (CLS Cell Lines Service Germany) and MCF-7 cell line (Human Breast adenocarcinoma cell line) (CLS Cell Lines Service Germany) were cultured in specific medium: DMEM (Dulbecco's Modified Eagle Medium) (Invitrogen, Carlsbad, CA, USA) for MCF-7 cell line, and DMEM: F12 for HepG2 cell. The medium was supplemented with 1% penicillin streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% (v/v) FBS (heat-inactivated fetal bovine serum) (Invitrogen). Then, the cell lines were incubated at 37°C in 5% CO_2, in order to achieve the proposed goals after seeding cells in a concentration of $1x10^5$ cells well in 96 wells plates. At 24 hours of cultivation, *Viscum album* extracts were added. Aqueous *Viscum album* (A) and alcohol (B) extracts were resuspended in both specific culture medium and dimethyl-sulfoxide at the concentrations of 15 mg/mL, 25 mg/mL, 50 mg/mL and 75 mg/mL. The extracts were applied on cell lines and, after 48 hours of incubation, the cell proliferation assay was performed.

*Cell proliferation w*as achieved using *Vybrant MTT Cell Proliferation Assay Kit* (Invitrogen), which is a simple method for determination the number of cells using standard absorbance reader. The cells suspension was centrifuged and after removing the supernatant in 96-well plates the fresh medium in a volume of 100 µL was added. Following that, 10 µL MTT (2H-Tetrazolium-2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-bromide) (12 mM) was added in each well and incubated at 37°C for a period of 4 hours. After incubation, the medium was removed while preserving only 25 µL in each well. 50 µL DMSO was added and incubated at 37°C for 10 minutes. The absorbance was read at 540 nm. Sample results were expressed as a relative percentage to the control value.

Results and Discussion

In the present study, the phytochemical profile of *Viscum album* young leaves and branches aqueous and alcoholic extracts was evaluated using the GC-MS technique. The cytotoxic effect was also

evaluated *in vitro* on tumorigenic breast cancer oestrogen positive cell (MCF-7) and HepG2 using the tetrazolium assay. After isolation and separation by gas chromatography, the eluate was introduced into the mass selective detector for the identification of chemical constituents found in both ethanolic and aqueous extracts of *Viscum album*. The relative percentage area of each constituent was calculated by comparing its average peak area to the total area. Cytotoxicity results were expressed as IC_{50}, defined as the sample concentration that caused the inhibition of 50% cells growth.

Following derivatization with bis-(trimethylsilyl)-trifluoroacetamide, components were separated on a BR – 5 ms capillary column. The mass spectrometry analysis of the alcoholic extract (Table I) evidenced the presence of phytosterols, like β-sitosterol, a powerful antioxidant that lowers the serum cholesterol levels in humans [4] campesterol, stigmasterol and has stabilizing effects on phospholipids bilayer and possess a protective effect against cardiovascular diseases, and also on colon and breast cancer [6]. Olean-12-en-3-yl acetate, a terpenoid with powerful antimicrobial, anti-diabetic and antiamylase inhibitory activities [14], was the major compound identified in both extracts.

Table I

GC-MS analysis of *Viscum album* young leaves and branches alcoholic extract

Retention time, min	Compound name	Area, %
16.3	3-isopropenyl-2-methylcyclohexanol	0.18
16.8	1,1,4a-trimethyl-3,4,4a,5,6,7-hexahydro-2(1H)-naphthalenone	0.13
17.8	2,4-decadienal	0.17
18.6	syringol	0.14
18.8	1,1,6-trimethyl-1,2-dihydronaphthalene	0.24
20.1	4-[2,6,6-trimethyl-5-cyclohexen-1-yl)-2-butanone	0.17
22.6	4,4,5,8-tetramethylchroman-2-ol	0.15
22.7	cendran-8,13-diol	0.16
22.9	1,2,4-cyclopentanetrione	0.24
23.0	(-)-calamenene	0.19
23.2	4,47a-trimethyl-5,6,7,7a-tetrahydro-1-benzofuran-2(4H)-one	0.30
24.0	16-heptadecen-2,5,8-trione	0.18
24.2	4-(2,3,6-trimethylphenyl)2-butanone	0.22
24.3	4-(2,4,4-trimethyl-cyclohexa-1,5-dienyl) but-3-en-2-one	0.25
24.8	3,3-dimethyl-1,2,3,4-tetrahydro-1,2-naphthalenediol	0.33
25.0	3,3,5,6-tetramethyl-1-indanone	1.42
25.4	2-methyl-4-(2,6,6-trimethylcyclohex-1-enyl) but-2-en-1-ol	0.29
25.6	3-oxo-7,8-dihydro-α-ionol	0.23
26.1	8,9-dehydro-neoisolongifolene	0.68
28.5	Z-9-pentadecenol	0.53
29.4	oleic acid	0.65
29.9	(2E)-3,7,11,15-tetramethyl-2-hexadecen-1-ol	1.25
30.0	hexahydrofarnesyl acetone	0.50
31.3	9-tetradecenoic acid trimethylsilyl ester	0.24
31.7	pentadecanoic acid 13-methyl methyl ester	0.30
33.2	palmitic anhydride	3.59
34.7	3-deoxyestradiol	0.51
35.6	α-glycerol linolenate	0.40
37.4	methyl 5,11,14-eicostrienoate	5.33
40.1	6,9,12,15-docosatetraenoic acid methyl ester	0.70
41.2	1-heptatriacotanol	0.75
43.5	behenic alcohol	1.57
47.2	17-pentatriacontene	1.37
49.0	cis-11-eicosenoic acid	0.53
52.4	4,4,6a,6b,8a,11,14b-octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	1.09
54.0	α-tocopherol	2.00
55.3	campesterol	0.89
55.7	stigmasterol	3.14
56.6	γ-sitosterol	8.14
57.2	β-amyrin	9.00
57.4	β-sitosterol	3.00
58.0	lupeol	8.57
58.9	olean-12-en-3-yl acetate	22.00
59.7	lupenyl acetate	17.92

β-amyrin (3β-hydroxy-olean-12-en-3-ol), a pentacylic triterpene of considerable interest for its pharmacological properties, was also identified in the ethanolic extract. Lupeol, lupenyl acetate, terpenoids that possess neuroprotective, antioxidant and anti-inflamatory effects [11] were also identified in the ethanolic extract. Tetralin derivatives such as (1S,4S)-1,6-dimethyl-4-propan-2-yl-1,2,3,4-tetrahydro-naphthalene, 3,3-dimethyl-1,2,3,4-tetrahydro-1,2-naphthalene diol that possess antimicrobial, antiviral, anticancer properties [1] were also evidenced in the alcoholic extract. Fatty acids composition was as following: oleic, palmitic, 9-tetradecenoic, penta-decanoic, and docosatetranoic acids. An important natural antioxidant, vitamin E was evidenced in the analysed mistletoe young leaves and branches.

Table II

GC-MS analysis of aqueous extract of *Viscum album* young leaves and branches

Retention time, min	Compound name	Area, %
18.8	2,5,8-trimethyl-1,2-dihydronaphthalene	2.30
20.0	(3E, 5E)-7-isopropyl-8-methyl-3,5,7-nonatrien-2-one	1.17
20.2	pentadecanal	1.22
20.9	undecanoic acid	0.36
22.7	cedr-8-en-13-ol	1.10
23.0	L-celemene	0.78
24.2	4-(2,3,6-trimethlyphenyl_-2-butanone	1.54
24.8	1,2-naphthalene diol	1.59
25.0	2,2,3,3-tetramethyl-1-indanone	1.29
26.1	methyl-4-(3-hydroxy-3-methyl-1-butynyl) benzoate	3.65
26.5	azulol	0.95
26.7	1-(2,6,6-trimethyl-cyclohex-1-enyl_-butane-1,3-dione	1.33
26.9	1,3-dimethylpyrido[3,2-d]pyrimidine-2,4-(1H, 3H)-dione	2.26
27.4	5,7,8-trimethyl-2-chromanone	0.45
27.5	7,8-dehydro-8a-hydroxy isolongifolene	0.47
27.8	4,4,5,6,8-pentamethyl-3,4-2H-coumarin	0.71
28.9	myristic acid anhydride	2.01
29.9	E-2-methyl-3-tetradecen-1-ol acetate	1.77
30.8	2-cis-9-octadecenyloxyethanol	1.59
31.7	pentadecanoic acid, 14-methyl, methyl ester	0.99
32.5	i-propyl-9-hexadecenoate	2.97
33.1	Z-7-hexadecenoic acid	2.71
35.3	cis-10-heptadecenoic acid methyl ester	3.85
37.5	2,3-dihydroxypropyl elaidate	4.92
39.9	cis-10-nonadecenoic acid	1.38
41.9	2-tert-butyl-6-[(3-tert-butyl-2-hydroxy-5-methylphenyl)methyl]-4-methyl-phenol	1.23
44.0	3', 8, 8'-trimethoxy-3-piperidin-1-yl-2,2'-binaphthyl-1,1,4,4'-tetrone	1.04
47.1	17-pentatriacontene	1.13
52.4	betuline	1.11
54.1	5-(7a-isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-enal	1.23
56.0	lanosterol	1.68
56.6	γ-sitosterol	6.42
57.2	β-amyrin	8.79
57.4	δ7-chondrillastenol	3.27
58.0	lupeol	9.09
58.6	olean-12-en-3-yl acetate	11.12
59.5	lupeol acetate	10.55

The MS output for the derivatives obtained from the aqueous extract (Table II), were as following: 2,5,8-trimethyl-1,2-dihydronaphthalene, tetracyclic triterpenoids such as lanosterol which is involved in cholesterol synthesis [2]. γ-sitosterol, a steroid that has the property of reducing the lipid levels, was identified only in aqueous extract of mistletoe. Fatty acids such as pentadecanoic, hexadecenoic, heptadecenoic that exhibit antioxidant, antimicrobial and cancer preventive properties were also identified in *Viscum album* aqueous extract. B-amyrin, lupeol and lupeol acetate were found in both extracts. Tetralin derivatives were reported for the first time in this study. In both cell lines, MCF-7 and HepG2, a significant decrease in cell proliferation in the case of alcoholic extracts was observed and the inhibition of cell proliferation, dose depended, is significant (Figure 1, 2). Inhibition of cell proliferation was also observed in the case of aqueous extracts, significant at concentrations of 50 mg/mL and 75 mg/mL. The alcoholic extract was more efficient and proved a significant cytotoxic effect at the

concentration of 75 mg/mL, which may be due to the higher content of tetralin derivatives and also to the fatty acids content, compared to the aqueous extract.

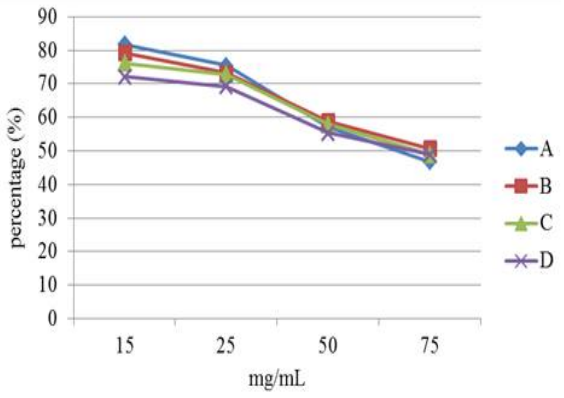

Figure 1.
MCF-7 cells proliferation: (A) aqueous extracts re-suspended in medium; (B) aqueous extracts resuspended in DMSO; (C) alcoholic extract resuspended in medium; (D) alcoholic extract resuspended in DMSO

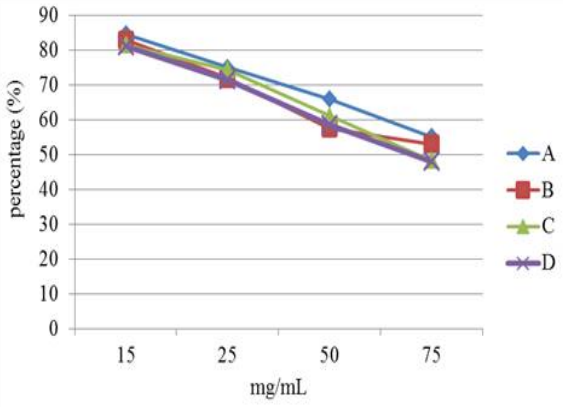

Figure 2.
The proliferation of HepG2 cells: (A) aqueous extracts resuspended in medium; (B) aqueous extracts resuspended in DMSO; (C) alcoholic extract resuspended in medium; (D) alcoholic extract resuspended in DMSO

Conclusions

The presence of some of the most important volatile compounds found in *Viscum album* extracts was reported. Components were successfully separated by gas chromatography and identified by ion trap mass spectrometry. Tetralin derivatives with important biological activity have been identified in both alcoholic and aqueous samples. Essential fatty acids such as palmitic, oleic, myristic and important phytosterols that possess biological activities were successfully separated and identified. The nature of active principles is extremely important for further studies regarding the correlation between structure and action mechanism and following that, GC-MS analysis represents the first step in understanding it. The biological potential of the identified compounds is worth being taken into consideration for detailed studies. The cytotoxic effect evaluated on MCF-7 and HepG2 cell lines revealed a significant inhibition of cell proliferation, which was dose dependent.

References

1. Ačimovič J., Rozman D., Steroidal Triterpenes of Cholesterol Synthesis. *Molecules*, 2013; 18: 4002-4017.
2. Balamurugan R., Stalin A., Aravinthan A., Kim J.-H., γ-sitosterol a potent hypolipidemic agent: *in silico* docking analysis. *Med. Chem. Res.*, 2015; 24: 124-130.
3. Bar-Sela G., White-Berry Mistletoe (*Viscum album* L.) as complementary treatment in cancer: Does it help? *E. J. of Int. Med.*, 2011; 3: e55-e62.
4. Choudhary S.P., Tran L.S, Phytosterols: Perspectives in Human Nutrition and Clinical Therapy. Current Medicinal Chemistry, 2011; 18(29): 4557-4567.
5. Loizou S., Lekakis I., Chrousos G.P., Moutsatsou P., Beta-sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. *Mol. Nutr. Food Res.*, 2010; 54(4): 551-558.
6. Mu L., Kou J., Zhu D., Yu B., Comparison of Neuroprotective Effects of flavonoids, Terpenoids, and Their Combinations from Ginkgo biloba on Ischemia-Reperfusion-Injured Mice. *Pharm. Biol.*, 2007; 45: 728-733.
7. Oluwatosin A., Massoud A., Mehran H.R., Parvin P., Moosavi-Movahedi A., Methanolic extract of African mistletoe (*Viscum album*) improves carbohydrate metabolism and hyperlipidemia in streptozotocin-induced diabetic rats. *A. P. J. of Trop. Med.*, 2012; 5(6): 427-433.
8. Orhan D.D., Orhan I., Fatty acids composition of *Viscum album* subspecies from Turkey. *Chem. of Nat. Comp.*, 2006; 42(6): 641-644.
9. Orhan D.D., Senol F.S., Hosbas S., Orhan I.E., Assessment of cholinesterase and tyrosinase inhibitory and antioxidant properties of *Viscum album* L. Samples collected from different host plants and its two principal substances. *Ind. Crops and Prod.*, 2014; 62: 341-349.
10. Rodino S., Butu A., Petrache P., Butu M., Dinu–Pîrvu C.E., Cornea C.P., Evaluation of the antimicrobial and antioxidant activity of *Sambucus ebulus* extract. *Farmacia*, 2015; 63(5): 751-754.
11. Sandeep A., Prakash N.B., Synthesis, characterization and antifungal activity of quinazoline thione derivatives of 3, 4-dihydro-1(2h)-naphthalenone. *Int. Res. J. Pharm.*, 2014; 5(6): 476-480.
12. Trifan A., Aprotosoaie A.C., Brebu M., Cioancă O., Gille E., Hăncianu M., Miron A., Chemical composition and antioxidant activity of Essential oil from romanian *Satureja montana* L. *Farmacia*, 2015; 63(3): 413-416.
13. Troger W., Galun D., Reif M., Schumann A., Stankovic N., Milicevic M., *Viscum album* [L.] extract therapy in patients with locally advanced or metastatic pancreatic cancer: A randomised clinical trial on overall survival. *E. J. of Cancer*, 2013, 49: 3788-3797.

14. Venkata R.B., Samuel L.A., Pardha S.M., Narashimha R.B., Naga V.K.A., Sudhakar M., Radhakrishnan T.M., Antibacterial, antioxidant activity and GC-MS analysis of *Eupatorium odoratum*. *Asian. J. Pharm. Clin. Res.*, 2012, 5(2), 99-106.
15. Weissenstein U.K., Kunz M., Urech K., Baumgartner S., Interaction of mistletoe preparations (*Viscum album*) with antitumor effects of several standard anticancer drugs *in vitro*. *E. J. of Integr. Med.*, 2015; 7(S1): 27.
16. Yang L., Yan-Li Z., Yong-Ping Y., Xiao-Li L., Chemical constituents of *Viscum album* var. *Meridianum*, *Biochem. System. and Ecology*, 2011; 39: 849-852.