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RESEARCH ARTICLE

Evaluation of Cytotoxic Effects on Ethereal Extracts of Some Selected Liverworts

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SUMMARY

The liverworts are interesting examples of the spore-forming plants with distinguishing chemical diversity of the secondary metabolites and a wide range of biological activities. In this study, Turkish liverworts have been investigated for their cytotoxic activities as a preliminary screening. Therefore, the ether extracts of some selected Turkish liverworts such as Riccia fluitans L., Porella cordaeana (Huebener) Moore, Porella platyphylla (L.) Pfeiff., Corsinia coriandrina (Spreng.) Lindb., Mannia androgyna (L.) A. Evans, Reboulia hemisphaerica (L.) Raddi, Plagiochasma rupestre (J.R. Forst et G. Forst) Steph. and Targionia hypophylla L. have been investigated by MTT assay using HeLa (Human cervical cancer cells), Sp2/0 (Mouse myeloma cells), YAC-1 (Mouse lymphoma cells) cell lines for the evaluation of cytotoxic activities. The most active extracts were determined, and the results were given as % inhibitions rates. The results compared with the phytochemical constituents of the liverworts that have been investigated previously on gas chromatography/mass spectroscopy (GC/MS).

Key Words: *MTT, liverworts, marchantiophyta, hepaticae, cytotoxicity, GC/MS*

Seçilmiş Bazı Ciğerotlarının Eterli Ekstrelerinin Sitotoksik Etkilerinin Değerlendirilmesi

ÖZ

Ciğerotları, sekonder metabolitlerinin oldukça farklı kimyasal çeşitliliği ve geniş bir biyolojik aktiviteye sahip olmaları ile sporlu bitkilerin ilginç örnekleridir. Bu çalışmada, Türkiye'de yetişen ciğerotlarının sitotoksik etkileri için bir ön tarama yapılmıştır. Bu nedenle, Riccia fluitans L., Porella cordaeana (Huebener) Moore, Porella platyphylla (L.) Pfeiff, Corsinia coriandrina (Spreng.) Lindb., Mannia androgyna (L.) A. Evans, Reboulia hemisphaerica (L.) Raddi, Plagiochasma rupestre (J. Forst ve G. Forst) Steph. ve Targionia hypophylla L. gibi bazı seçilmiş ciğerotu türlerinin eterli ekstrelerinin, sitotoksik aktiviteleri, HeLa (İnsan servikal kanser hücreleri), Sp2/0 (Fare miyeloma hücreleri), YAC-1 (Fare lenfoma hücreleri) hücre dizileri kullanılarak, MTT deneyi ile araştırılmıştır. En aktif ekstreler belirlenmiş ve sonuçlar % inhibisyon oranı olarak verilmiştir. Ayrıca bulunan sonuçlar, daha önce gaz kromatografisi / kütle spektroskopisi (GC/MS) analizleri ile fitokimyasal içerikleri aydınlatılmış olan, ciğerotlarının bileşenleriyle aktivite yönünden karşılaştırılmıştır.

Anahtar Kelimeler: *MTT, ciğerotları, marchantiophyta, hepaticae, sitotoksisite, GC/MS*

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INTRODUCTION

Nature has a large variety of bioactive compounds led to various biological effects. Plants and natural products have been used as a source of potential drug candidates (Verportee et al., 2006). Bryophytes are a small group of plants that cannot have real vascular systems. Morphologically very small, the difficulty to collect large quantities, the complications in defining and the insignificant use for people have led to neglect in scientific research (Dey & Mukherjee, 2011; Asakawa et al., 2013). The bryophytes are placed taxonomically between algae and pteridophytes and classified by quite separate evolutionary system into three coordinate classes such as Bryophyta (mosses/musci), Marchantiophyta (liverworts/Hepaticae), and Anthocerotophyta (hornworts); approximately represented more than 24.000 species grows almost everywhere in the world, especially in the moisture places (Asakawa, 2007). Although there is no strong scientific evidence in the literature, they are considered to be the oldest terrestrial plants (Asaksawa, 2008). Interestingly, bryophytes cannot be damaged by bacteria, fungi, and insects (Dey & Mukherjee, 2011; Asakawa et al., 2013). Moreover, they play an important role in the ecosystem by providing a buffer system for other plants (Chandra et al., 2016). There is no doubt that many of the bryophytes have been widely used as medicinal plants. The liverworts, the second largest group of bryophytes, are estimated with ~8000 species in 380 genera and 74 families in the world. (Crandall-Stotler et al., 2005; Asakawa, 2008; Asakawa et al., 2013) The liverworts have characteristic fragrant odors to emit volatile terpenoids or simple aromatic compounds (Asakawa, 2007), and intense pungent, sweet or bitter taste due to a wide variety of lipophilic terpenoids, and aromatic compounds. They have unique and quite interesting "cellular oil bodies," which are called membrane-bound cell organelles, which are easily extracted with organic solvents (Asakawa, 2007; 2008, Asakawa et al., 2013). According to the ancient philosophy known as "doctrine of signatures," the concept of Paracelsus, the liverworts used for the several disorders as their shape resemblance (Chandra et al., 2016). The most important point, the liverworts contain a number of bioactive molecules, and their biological effects have also been proven (Asakawa, 2008; Asakawa, 2011; Tosun et al., 2013; Ludwiczuk & Asakawa, 2015; Tosun et al., 2015; Dey & Mukherjee, 2015).

Turkey has very rich flora with bryophytes and totally 887 species recognized among which 163 are liverworts (Kurschner and Erdag, 2005; Asakawa et al., 2018a). Natural products are very important in the treatment of cancer. The determination of cytotoxic activity is considered the first step in the discovery of new anticancer molecules (Kutluay and Saraçoğlu, 2018). Cancer is still one of the most important cause of death in the world, and owing to many studies have been emphasizing anticancer, cytotoxic, antiproliferative effects, against cancer cell lines of the liverwort species (Asakawa et al., 2013; Dey & Mukherjee, 2015; Tan et al., 2017; Ozturk et al., 2018; Asakawa et al., 2018b), we aimed to investigated cytotoxic activity of ether extracts of some selected liverworts from Turkish flora such as *Riccia fluitans* L. (Ricciaceae), *Porella cordaeana* (Huebener) Moore (Porellaceae), *Porella platyphylla* (L.) Pfeiff. (Porellaceae), *Corsinia coriandrina* (Spreng.) Lindb. (Corsiniaceae), *Mannia androgyna* (L.) A. Evans (Aytoniaceae), *Reboulia hemisphaerica* (L.) Raddi (Aytoniaceae), *Plagiochasma rupestre* (J.R. Forst et G. Forst) Steph. (Aytoniaceae) and *Targionia hypophylla* L. (Targioniaceae) on HeLa, Sp2/0, YAC-1 cell lines. In addition, the activity results were discussed with respect to the major phytochemical components previously identified on GC/MS.

MATERIALS AND METHODS

Plant materials

All species were collected from Southwest of Turkey and determined by Dr. H. Özenoğlu from Adnan Menderes University, Department of Biology Education (Turkey). The detail information was mentioned below (Table 1). The voucher specimens were kept in Herbarium of Adnan Menderes University, Faculty of Education, Department of Biology Education, Aydın, Turkey.

Table 1. The liverworts collected from different localities

Species	Family	Herbarium No	Date	Location
***Corsinia coriandrina* (Spreng.) Lindb.**	Corsiniaceae	C11/228	10.01.2009	Muğla; Milas, Kapıkırı Village, Heraclea Archaic City Ruins, on the rocks and soil bank near the roadside, 30 m
***Mannia androgyna* (L.) A. Evans**	Aytoniaceae	C11/230	10.01.2009	Muğla; Milas, Kapıkırı Village, Heraclea Archaic City Ruins, on the rocks and soil bank near the roadside, 35 m
***Plagiochasma rupestre* (J.R. Forst et G. Forst) Steph.**	Aytoniaceae	C11/226	10.01.2009	Muğla; Milas, Kapıkırı Village, Heraclea Archaic City Ruins, theater area, on the rocks and soil ground, 70 m
***Porella cordaeana* (Huebener) Moore**	Porellaceae	C11/223	11.01.2009	Aydın; Koçarlı, Mersin Belen road 5. km, on the road in the stream bank, 696 m
***Porella platyphylla* (L.) Pfeiff.**	Porellaceae	C11/224	11.01.2009	Aydın; Koçarlı, Mersin Belen road 5. km, on the road in the stream bank, 696 m
***Reboulia hemisphaerica* (L.) Raddi**	Aytoniaceae	C11/227	11.01.2009	Aydın; Koçarlı, Mersin Belen road 5. km, on the road in the stream bank, 696 m
***Riccia fluitans* L.**	Ricciaceae	C11/225	10.01.2009	Izmir; Selçuk, Zeytinköy Village, Kazangöl Lake, 3 m
***Targionia hypophylla* L.**	Targioniaceae	C11/236	10.01.2009	Muğla; Milas, Kapıkırı Village, Heraclea Archaic City Ruins, on the rocks and soil bank near the roadside, 30 m

Extraction of plant materials

According to the literature, the extraction of oil bodies with *n*-hexane or ether, using ultrasonic apparatus is very easy for stem-leafy liverworts to give a large amount of crude extract. On the other hand, the thalloid liverworts are ground mechanically and then extracted with non-polar solvents (Asakawa et al., 2013; Tan et al., 2017).

In our experiments, half-dried liverworts were mechanically pulverized, then macerated with ether (500 mL) for 3 weeks in a cool and dark place. Erlenmeyer as occasionally agitating manually. The extracts were filtrated through celite filter (0.02–0.1mm particle size) (Celite 545, Merck 02693.0250, Darmstadt, Germany) embedded in a column and evaporated under 30°C to obtain crude green oil (extracts). Then, the crude extracts from each liverwort were weighed carefully and kept in a freezer until use. The amounts of starting materials and the extracts are given in Table 2.

Table 2. The amount of liverworts extracts

Species	Amount of Plants (g)	Amount of Extracts (mg)	Yield (mg/100g)
***Corsinia coriandrina* (Spreng.) Lindb.**	34.02	130	382.13
***Mannia androgyna* (L.) A. Evans**	110.00	320	290.91
***Plagiochasma rupestre* (J.R. Forst et G. Forst) Steph.**	56.00	1180	2107.14
***Porella cordaeana* (Huebener) Moore**	52.36	810	1546.98
***Porella platyphylla* (L.) Pfeiff.**	23.75	450	1894.74
***Reboulia hemisphaerica* (L.) Raddi**	36.41	410	1126.06
***Riccia fluitans* L.**	11.07	380	3432.70
***Targionia hypophylla* L.**	29.00	90	310.35

TLC analysis

The liverwort extracts were also examined by thin layer chromatography (TLC) (Figure 1) by using different ratios of *n*-hexane:ethyl acetate (4:1 and 1:1). The constituents on the TLC plate have been detected by the Godin reagent in daylight (mixing volume of a 1% vanillin solution in ethanol with one volume of 3% perchloric acid in water) after heating at a 100°C and later under UV light at 366 nm (just for checking).

Cell culture materials

The fetal bovine serum and RPMI-1640 medium were from PAA, and trypsin from Biochrom. MTT and all the other reagents were from Sigma or Fluca Chemical Co. Cell cultivating flasks were NUNC, and CO_2 incubator was from Sanyo MCO-15AC, Schoeller. In this research, HeLa cells were provided by Dr. Jan Kopecky (Czech Academy of Sciences). The other two mammalian cell lines were selected for the cytotoxicity assay, YAC-1 (mouse lymphoblast, induced by Moloney leukemia virus), and Sp2/0 (mouse myeloma cells). The cells were cultivated in RPMI 1640 medium (Sigma R-8005) with the addition of 5% fetal calf serum (PAA A15-04), 1% glutamine (Sigma G-5763), and 1% antibiotic-antimycotic solution (Sigma A-7292); all in plastic tissue culture flasks at 37°C. Prior to the experiments, cells were dyed with Trypan blue, in order to estimate viability, and counted in a Bürkers plate chamber in a light microscope. Only cell cultures with higher viability than 90% were used for the experiment. The cell suspension was centrifuged (1000 rpm, 10 min, 4°C), and an adequate amount of fresh RPMI medium was added, in order to obtain a concentration of 1.5×10^5 cells per well (200 µL RPMI).

Assessment of cell proliferation

Assessment of cell viability was determined by a colorimetric assay using [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide), MTT] (Mosmann, 1983). In order to detect the cytotoxicity, the cells were treated with 1 mg/mL of the extracts and incubated for 24-72 h, and the assay was performed in triplicate, and the culture plates were kept at 37°C with 5% CO_2 for desired incubation times. The concentration of the extracts was adjusted to 1 mg/mL. Subsequently, MTT has been added to the wells in final concentration 100 µg/mL, dissolved in phosphate-buffered saline and allowed to incubate for a further 4 h. After this incubation time, plates were centrifuged, supernatant removed, and crystals re-dissolved in DMSO (dimethylsulfoxide). The control group was treated with an equivalent amount of vehicle DMSO, the maximum of 1% in the assay mixture was used. The intensity of formazan, reduced product of MTT after reaction with active mitochondria of live cells, was determined by measuring the absorbance in 96-well UV/VIS microplate reader (Sunrise-TECAN) at a wavelength of 590 nm and 640 nm reference. The survival of cell lines was evaluated as the ratio of treated wells' absorbance to that of the control wells and expressed as a percent. The absorbance values of nontreated cells (control) were accepted as 100% viable and % inhibition of the extracts were calculated versus control.

RESULTS AND DISCUSSION

Discovery of new drugs has pumped to expand efforts to continue for the discovery of new and novel natural products/natural compounds with a higher level of activity and with therapeutic interest. So far, many new compounds have been isolated from the liverworts with interesting biological effects as a model for pioneer compounds. The liverwort flora is very rich in Turkey. It was indicated that the distribution profile of Turkish liverworts is closely related to that of Europe. In Turkish flora, *Porella platyphylla* is the most common species, *Reboulia hemisphaerica* and *Targionia hypophylla* are the second predominant species (Asakawa et al., 2018a).

In this study, some liverwort species growing in Turkey, at a specified concentration, is intended to scan and evaluate for their cytotoxic activity against various cancer cells. Previously, gas chromatography studies on these species have led to the evaluation of their phytochemical delete. (Tosun et al., 2013). In addition, a general distribution of the compounds has also been exhibited by the TLC analysis. The identification of their phytochemical content will help us in the evaluation and interpretation of their activities. GC/MS analyses of the selected liverworts were demonstrated that the volatile constituents are mostly composed of sesquiterpenoids. Consequently, the effect might be predominantly attributed to the sesquiterpene compounds. Further to that, several sesquiterpene lactones such as eudesmanolides, germacranolides and guaianolides isolated from liverworts showed cytotoxic activity against KB nasopharyngeal and P388 lymphocytic leukemia cells (Asakawa, 1995).

Investigating the bioactive constituents from the liverworts can lead to new pharmaceuticals. Indeed, the interest in liverworts is due to their structurally important and biologically active terpenoids. Many of liverworts exhibited cytotoxic activity such as *Marchantia, Riccardia, Radula, Frunella, Porella, Wiesnerella, Conocephalum* species (Asakawa, 2008; Asakawa et al., 2013; Chandra et al., 2016). There are many studies about on this subject, for instance, the ethereal extracts of *Porella cordaeana* growing in Turkey showed cytotoxic effects against MCF-7 (Human breast cancer cells) and HT-29 (Human colorectal cancer cells), but isolated new pinguisane derivatives could not exhibit a remarkable effect on the same cells (Tan et al., 2017). Activity-guided fractionation determined by the MTT assay on HL-60 (human promyelocytic leukaemia) and KB cells of the ethereal extract of *Dumortiera hirsute* led to the isolation of active bis-bibenzyl compound similar structure with marchantin A from the liverwort

Marchantia polymorpha and two bis-bibenzyl compounds known as isomarchantin C and isoriccardin C (Toyota et al., 2013). Marchantin A is a cyclic bisbibenzyl that has previously been isolated from *M. polymorpha,* and some of the other liverwort species has been shown to exert cytotoxic effects (Jensen et al., 2012). In the other study, the labdane-type diterpenoids (scapairrins A-Q) were isolated from the Chinese liverwort *Scapania irrigua* and tested against a small panel of human cancer cell lines, but the compounds G-J were exhibited inhibitory activities (Zang et al., 2015). The clerodane-type diterpenoids isolated from the MeOH extract of the Bornean liverwort *Gottschelia schizopleura* were also tested against HL-60, HT-29 (Human colon adenocarcinoma) and B16-F10 (Mus musculus skin melanoma); two of them showed active inhibition against HL-60 and B16-F10 cells (Ng et al., 2018). On the other hand, the pimarane-type diterpenoids, 2,3-secoaromadendrane-type sesquiterpenoids and fusicoccane-type diterpenoids from the Chinese liverwort *Plagiochila pulcherrima* have been tested for cytotoxic activity, some of the diterpenoids exhibited moderate inhibitory activity on the proliferation of human cancer cell lines HeLa, A172, and H460 (Wang et al., 2013). It is clear that many different types of terpenic compounds in the liverworts exhibit cytotoxic activity, and hundreds of studies on this subject can be found.

In the current study, TLC analysis demonstrated that the presence of aromatics, terpenoids, and hydrocarbons was observed in tested liverworts as usual. Moreover, the TLC analysis exhibited that the aromatic compounds were close to starting, terpenes in the subsequent area, and hydrocarbons in the region close to the frontal area (Figure 1). In the course of our screening for the cytotoxic activity of the selected liverworts was evaluated and the strong cytotoxicity was especially observed against Sp2/0 and YAC-1 cell lines. The most active species was the *Riccia fluitans* (93%) against YAC-1 cells following *Porella cordaeana* (86%) and *Targionia hypophylla* (85%) at 1 mg/mL concentrations. In addition, the most active species against to Sp2/0 cells can be considered as *T. hypophylla* and *P. cordaeana* in the same percentage (86%). Regarding the HeLa cells, the extracts have no significant effect on these cells (Figure 2).

This study was performed to elucidate the cytotoxic effects of the ether extracts from the eight liverworts species on several cancer cell lines. The current work demonstrated that the liverwort extracts exhibited remarkable inhibition on tested cancer cell lines. The scope of our ongoing studies on liverworts growing in Turkey is to be isolation of cytotoxic compounds, and further studies will be necessary for the isolation of active cytotoxic compounds, may yield promising agents. This study is important in screening of the cytotoxic activity for the first time on Turkish liverworts..

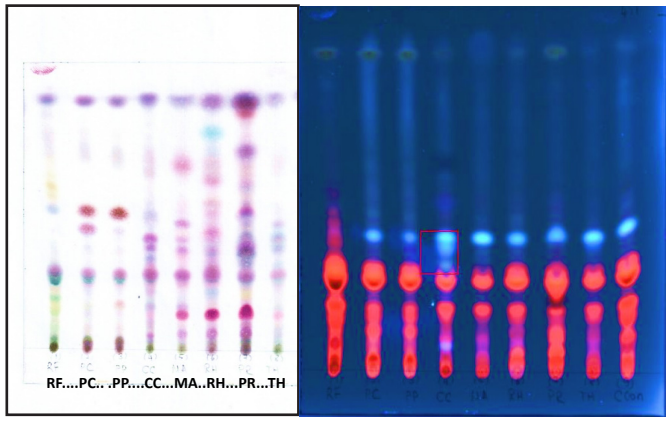

Figure 1. TLC profiles of the ether extract of selected liverworts from Turkish Flora

[Solvent system: Hexane:Ethyl acetate (4:1)]
(A) with Godin Reagent and (B) under UV light at 366 nm
CC: *Corsinia coriandrina* (Spreng.) Lindb., MA: *Mannia androgyna* (L.) A. Evans, PR: *Plagiochasma rupestre* (J.R. Forst et G. Forst) Steph., PC: *Porella cordaeana* (Huebener) Moore, PP: *Porella platyphylla* (L.) Pfeiff., RH: *Reboulia hemisphaerica* (L.) Raddi, RF: *Riccia fluitans* L., TH: *Targionia hypophylla* L.

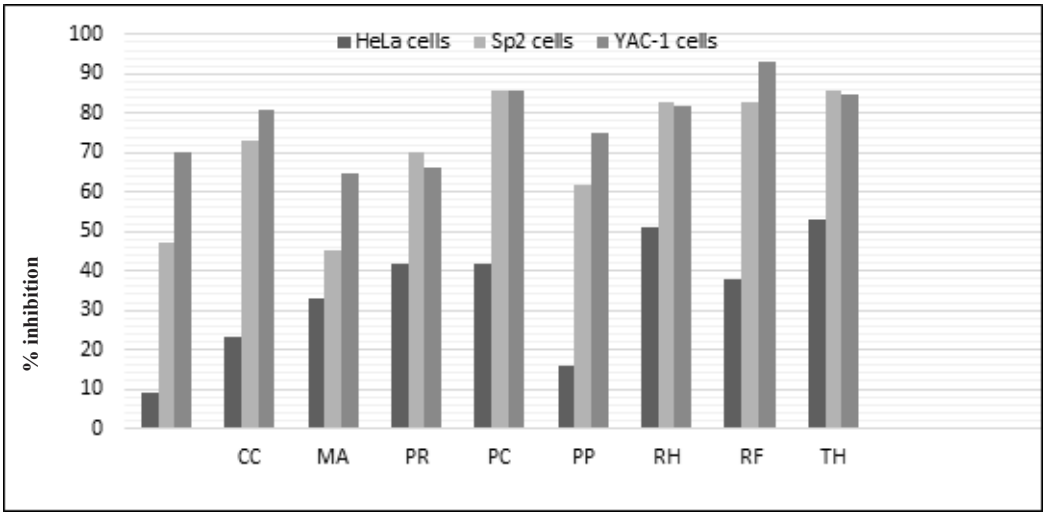

Figure 2. % Inhibition of the liverworts ether extracts

CC: *Corsinia coriandrina* (Spreng.) Lindb., MA: *Mannia androgyna* (L.) A. Evans, PR: *Plagiochasma rupestre* (J.R. Forst et G. Forst) Steph., PC: *Porella cordaeana* (Huebener) Moore, PP: *Porella platyphylla* (L.) Pfeiff., RH: *Reboulia hemisphaerica* (L.) Raddi, RF: *Riccia fluitans* L., TH: *Targionia hypophylla* L.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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