Kafkas Univ Vet Fak Derg
19 (3): 511-516, 2013
DOI: 10.9775/kvfd.2012.8196

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

In vitro Efficacy of *Quercus infectoria* Oliv. and *Achillea millefolium* L. Extracts Against *Blastocystis spp*. Isolates [1]

Ahmet ÖZBİLGİN [1] Cenk DURMUŞKAHYA [2] Ali Ahmet KİLİMCİOĞLU [1]
Hüsniye KAYALAR [3] Özgür KURT [4] Veciha Özge ERMİŞ [4] Tuba TABAK [1] İpek ÖSTAN [5]

[1] *The present study was financially supported by the Scientific Research Projects Department of Celal Bayar University (Project no: BAP, Tıp 2009-048)*

[1] Celal Bayar University, Faculty of Medicine, Department of Parasitology, TR-45030 Manisa - TURKEY
[2] Celal Bayar University, Faculty of Education, TR-45900 Manisa - TURKEY
[3] Ege University, Faculty of Pharmacy, Department of Pharmacognosy, TR-35100 İzmir - TURKEY
[4] Celal Bayar University, School of Science and Letters, Department of Biology, TR-45140 Manisa - TURKEY
[5] Celal Bayar University, Vocational School of Health Sciences, TR-45030 Manisa - TURKEY

Makale Kodu (Article Code): KVFD-2012-8196

Summary

Blastocystis is a common intestinal parasite that can inhabit the intestinal tract of humans and many animals. Despite it was firstly described almost 100 years ago; many subjects are still under debate about *Blastocystis*, including its life-cycle, pathogenic potential and treatment of infected individuals. Historically, local plant species have been used for therapeutic purposes by the local people of Anatolia. Here, hexane and methanol extracts of two local plants, *Quercus infectoria (Fagaceae)* and *Achillea millefolium*, which have been used against diarrhea in Anatolia, were examined for their *in vitro* efficacies against *Blastocystis*. LC_{50} and EC_{50} values of the plant extracts were determined by Brine Shrimp and Graphpad Prism 5® methods, respectively. The results showed that LC_{50} (500 µg/ml) and EC_{50} (198.8 µg/ml) concentrations of the methanol extract of *A. millefolium* were lowest compared to other extracts, its *anti-Blastocystis* activity was found to be comparable to metronidazole and it showed no cytotoxic activity. These initial results suggest that the methanol extract of *A. millefolium* may be a novel option for the treatment of *Blastocystis* infections in humans in future, if confirmed by further, larger-scale studies.

Keywords: *Blastocystis, treatment, Medicinal plants, Quercus infectoria, Achillea millefolium*

Quercus infectoria Oliv. ve *Achillea millefolium* L. Ekstrelerinin *Blastocystis spp*. İzolatlarına *in vitro* Etkileri

Özet

Blastocystis spp, insanların ve birçok hayvanın gastrointestinal sistemine yerleşen yaygın bir bağırsak parazitidir. Yaklaşık 100 yıl önce tanımlanmış olmasına rağmen, yaşam döngüsü, patojenitesi ve tedavisini içeren birçok konu halen gizemini korumaktadır. Geçmişten bugüne Anadolu'da çok sayıda bitki halk tarafından tedavi amacıyla kullanılmıştır. Bu projede ishale karşı kullanılan bitkilerden ülkemizde yetişen *Quercus infectoria (Fagaceae)* ve *Achillea millefolium*'un hekzan ve metanol ile hazırlanan ekstrelerinin *in vitro* ortamda Blastocystis spp.'lerin üremesi üzerine etkileri incelenmiştir. Bitki ekstrelerinin LC_{50} değeri "Brine Shrimp" yöntemi, EC_{50} değeri Graphpad Prism 5® istatistik yöntemi kullanılarak saptanmıştır. Sonuç olarak, *A. millefolium*'un metanol ekstresinin LC_{50} (500 µg/ml) ve EC_{50} (198.8 µg/ml) konsantrasyonları diğer ekstrelerle kıyaslandığında en düşük bulunmuş, *anti-Blastocystis* aktivitesinin ise metronidazol grubunun değerlerine en yakın olduğu ve sitotoksik aktivite göstermediği saptanmıştır. Bu sonuçlar *A. millefolium*'un metanol ekstresinin, ileride yapılacak geniş kapsamlı çalışmalarla doğrulandığında, *Blastocystis spp*. enfeksiyonlarının tedavisinde yeni bir seçenek olabileceğini göstermektedir.

Anahtar sözcükler: *Blastocystis, Tedavi, Tıbbi bitkiler, Quercus infectoria, Achillea millefolium*

INTRODUCTION

Blastocystis spp. was firstly described as yeast in 1911 by Alexieff; however, issues concerning its taxonomy, life cycle and pathogenic potential have long been debated. Its prevalence is 30-50% in developing countries, and it is

İletişim (Correspondence)
+90 236 2331466
a.ozbilgin@yahoo.com

probably the most common intestinal parasite in humans. However, there are contradictory reports about its clinical significance as it is isolated from both symptomatic and non-symptomatic patients. *Blastocystis spp.* has been reported as the only pathogenic agent in patients with gastrointestinal complaints that resolved after effective treatment [1]. Recent molecular studies have shown extensive genetic diversity among *Blastocystis* spp. isolates, which may explain why some patients showed symptoms, while others not [2]. Due to its varying clinical manifestations, many laboratories report *Blastocystis* spp. only when more than 5 parasites were identified under x400 magnification on the microscope using saline - Lugol direct examination method of the stool [3]. Direct Fluorescent Antibody Method, which is fast and practical, was also considered as a diagnostic method [4].

Treatment is suggested for symptomatic patients, if other gastrointestinal pathological agents can be excluded, infected with *Blastocystis* spp. and metronidazole is the drug of choice in the treatment. However, metronidazole may cause common side effects, which may refrain the patient from complying with treatment effectively [5-7].

In traditional medicine in Anatolia, leaves, bodies, fruits and seeds of many plant species have long been used as anti-diarrheic agents. As medical agents are relativey expensive and may cause significant side effects in patients, herbal compounds are used commonly by local people. Some of these herbal compounds may have significant potential therapeutic effects against different parasites, and may turn out to be registered drugs for these infections in the future.

Achillea millefolium is traditionally used against skin inflammations, hepatobiliary disorders and gastrointestinal complaints. It is mainly preferred for its spasmolytic, digestive, carminative, antiphlogistic and cholagoge effects. Its efficacy against dyspeptic complaints has been attributed to the presence of compounds in *A. millefolium* which could stimulate the digestive fluids in stomach, pancreas, and liver by increasing the tone of the vagal system [8]. The antioxidant and antimicrobial properties and the chemical profile of the essential oil obtained from *A. millefolium* have also been reported. Flavonoids, phenolic acids and sesquiterpene lactones are considered to be the most important groups of pharmacologically active compounds present in *Achillea* species [9-12]. The chemical composition of *Achillea* species has been analyzed in detail and extracts of this plant have been demonstrated to contain a number of pharmacological active ingredients, including alkaloids, such as choline, and flavonoids such as rutin and apigenin. Among these, choline was reported to be the active compound for the pharmacological effects of *A. millefolium* [9].

Quercus infectoria is a small tree widely distributed in Greece, Asia Minor and Iran. It has been evaluated in terms of its pharmacological effects and it was found that it had antiparkinsonian, antitremorine, antiinflammatory, antidiabetic and antioxidant effects. The constituents of the galls of *Q. infectoria* comprise a large amount of tannins, gallic acid, syringic acid, ellagic acid, beta sitosterol, amentoflavone hexamethyl ether, isocryptomerin, methyl betulate, methyl olenate, and hexagalloyl glucose [13]. Larvacidal activity of the gall extracts of *Quercus infectoria* was initially reported against *Anopheles stephensi* [14].

The aim of the present study was to assess the *in vitro* efficacies of the extracts of two local plants *Quercus infectoria* Oliv. belonging to *Fagaceae* family and *Achillea millefolium* L.(Yarrow) from *Asteraceae* family that have been used traditionally against diarrhea, on *Blastocystis* spp. isolates diagnosed by three different methods. In addition, genotyping was employed to identify any relationship between *Blastocystis* spp. subtypes and sensitivity to *A. millefolium* and *Q. infectoria*.

MATERIAL and METHODS

Blastocystis spp. Isolates

Cryopreserved stool samples of six patients found to be infected with *Blastocystis* spp. in Parasitology Laboratory of Celal Bayar University Medical School's Hospital were used in the study. These positive samples were read and cultured on the same day, and the remaining samples were kept for two weeks at +4°C before genotyping.

These stool samples were inoculated into Jones medium [15], which was commonly preferred for *Blastocystis* spp. culture. The cultures were kept at 37°C for 48-72 h, and one drop of culture fluid was then examined microscopically to detect whether *Blastocystis* spp. were reproduced.

Amplification and Genotyping of Blastocystis spp. Isolates

Both stool and culture samples were used for the molecular assessments. DNA isolation was conducted with QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), according to the instructions of the manufacturer. Two µls of DNA were taken for PCR analysis, using the primers F1 and BHCRseq that targeted the small subunits of ribosomal RNA, and standard conditions [16]. The amplicons were separated on 1.5% of agarose gel, and PCR products of 550-590 bp were considered positive for *Blastocystis* spp. PCR products were gel-purified using the UltraClean™ Gel Spin DNA Purification Sample kit (SANBIO, Uden, The Netherlands) and dideoxy sequenced in one direction using the BHCRseq3 primer as the sequencing primer. Sequence chromatograms were analyzed and aligned using the software program Bio Edit Sequence Alignment Editor [17]. Distance-based analysis was conducted with MEGA 3.1 [18], and trees were constructed using the UPGMA algorithm with the Kimura 2-parameter model;

Proteromonas lacertae (U37108) was used as the out-group. *Blastocystis* spp. subtype terminology as described [19]. Sequences were blasted against those in the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plant Materials

The samples of two plant species used in the present study were collected in Manisa province in western Turkey. The aerial parts of *Achillea millefolium* were collected from Spil Mountain (almost 1150 meters above sea level), and nut galls of *Quercus infectoria* were collected from Yagcilar village (almost 250 m asl), which was located 20 km away from the city centre. Collected plant materials were separated and identified technically [20], and voucher specimens for plant materials have been deposited in the Herbarium of Celal Bayar University, School of Science and Letters, Department of Biology.

Preparation of Plant Extracts and Determination of Cytotoxic Activity

The air dried and ground aerial parts of *Achillea millefolium* and nut galls of *Quercus infectoria* were extracted using n-hexane and methanol under stirring. The organic phases were filtered through 0.45 µm and distilled *in vacuo* to yield n-hexane and methanol extracts. Brine Shrimp Method was used to assess the biological activities of the plant extracts [21-23].

In vitro Sensitivity Tests and Determination of the Effective Concentration

Plant extracts were prepared at different concentrations ranging between 62.5 and 4000 µg/ml, while the control drug, metronidazole was between 0.6 and 40 µg/ml. Saline solution was used as control and 10^5 *Blastocystis* spp./ml were added to the tubes containing extract and saline solution. All tubes were cultivated for 48 h at 37°C and tube samples were suspended in 0.1% of eosine solution to count the living cells. Reproduction of *Blastocystis* spp. isolates as well as the presence of living cells were checked in all concentrations, and 1 ml of culture fluid was drawn from the tube just before the concentration presenting with no living cells or reproduction, and inoculated in a new culture tube for testing. Thus, lethal concentrations (LC) of each plant extracts *on Blastocystis* spp. isolates, if present, were determined. Effective concentrations (EC_{50}) were also assessed using Graphpad Prism 5® statistical method.

RESULTS

Blastocystis spp. isolates were thawed in water bath at 37°C after cryopreservation, and immediately inoculated into Jones medium. All six isolates reached 10^3 parasites/ml concentration within 48 h *(Fig. 1)*.

Genotypic assessments of the isolates revealed three subtypes; two Subtype 1, one Subtype 2 and three Subtype 3. No differences were noted in terms of the subtypes between stool and culture samples. No significant differences were identified between the isolates for reproduction efficacies of parasites (One-way variance analysis, P>0.05); each subtype showed similar reaction to each extract at the same concentrations. LC_{50} levels of the methanol extracts of *Q. infectoria* and *A. millefolium* were found to be 1000 µg/ml and 500 µg/ml, respectively. The methanol extract of *A. millefolium* was found to have the lowest EC_{50} value (198.8), compared to others *(Table 1, Fig. 2)*.

Cytotoxic activity assessments with Brine Shrimp Method revealed that the LC_{50} value of the methanol extract of *Q. infectoria* was 190.8605; no cytotoxicity was defined

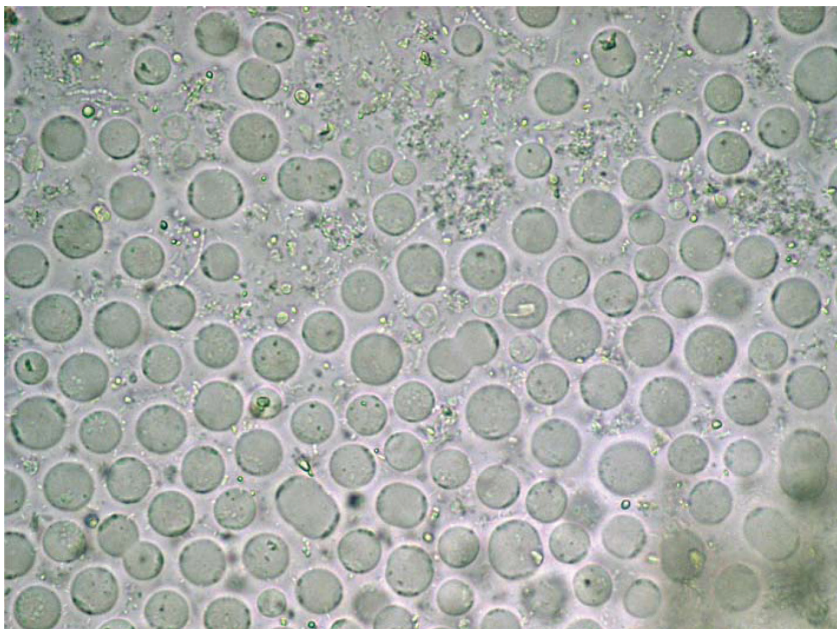

Fig 1. *Blastocystis* spp. isolates grown in Jones medium after cryopreservation

Şekil 1. Kriyoprezervasyondan sonra Jones besiyerinde üreyen *Blastocystis* spp. izolatları

Table 1. *Average numbers of Blastocystis spp. isolates 48 h after addition of plant extracts to study groups at different concentrations (Blastocystis spp. number x 10^7/ml)*

Table 1. *Çalışma gruplarına farklı konsantrasyonlarda bitki ekstresi eklendikten sonraki 48. saatte saptanan canlı Blastocystis sayısının ortalama değerleri (Blastocystis sayısı x 10^7/ml)*

Extract (µg/ml)	*Quercus infectoria* n-hexane	*Quercus infectoria* Methanol	*Achillea millefolium* n-hexane	*Achillea millefolium* Methanol	Extract (µg/ml)	Metronidazole	Control (Saline solution)
4000	0.98±0.08	0.00±0.00	0.00±0.00	0.00±0.00	**40.00**	0.00±0.00	5.02±0.08
2000	2.48±0.08	0.00±0.00	0.50±0.06	0.00±0.00	**20.00**	0.00±0.00	5.02±0.08
1000	2.98±0.08	0.00±0.00	1.03±0.05	0.00±0.00	**10.00**	0.00±0.00	5.05±0.14
500	4.03±0.10	1.00±0.06	2.05±0.12	0.00±0.00	**5.00**	0.00±0.00	5.07±0.14
250	4.98±0.12	2.02±0.08	2.48±0.08	1.03±0.05	**2.50**	0.48±0.08	5.00±0.11
125	5.05±0.08	2.50±0.06	4.05±0.05	2.52±0.08	**1.30**	1.05±0.05	4.98±0.08
62.5	5.08±0.08	3.03±0.08	5.03±0.10	3.07±0.08	**0.60**	2.48±0.12	5.00±0.11
EC_{50}*	3.458e+6	336.8	~ 546.5	198.8		0.1100	

EC_{50}: *Effective Concentration*

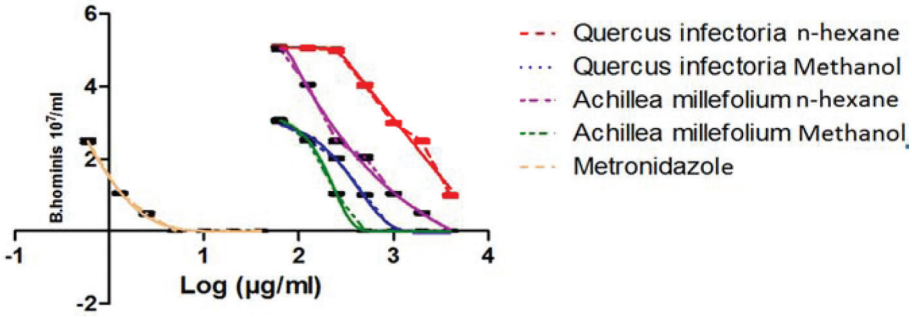

Fig 2. LC_{50} values of plant extracts

Şekil 2. Bitki ekstrelerinin LC_{50} değerleri

for n-hexane or methanol extracts of *A. millefolium* or n-hexane extract of *Q. infectoria*.

DISCUSSION

Metronidazole is a first line drug against intestinal protozoal infections, including blastocystosis. However, it has some drawbacks which are more severe in HIV-infected patients, such as nasty side effects, metallic taste, and headache [5-7,24,25]. Despite some other agents such as co-trimaxazole [26] was shown to be effective against *Blastocystis* spp., they are not commonly used and thus there is a need for new anti-protozoal agents which are safe and effective.

Medicinal plants have been used commonly in developing countries due to their availability, inexpensiveness and traditional use for centuries. In a study from Tailand, extracts of anti-diarrheic *Acacia catechu resin*, *Amaranthus spinosus* wholeplant, *Brucea javanica* seed (Bjs), *Piper longum* fruit (Plf) and *Quercus infectoria* nut gall (Qin) were assessed against *Blastocystis* spp. *in vitro* and dichloromethane and methanol extracts of Bjs were found to be effective [21]. Efficacy of the water extract of Bjs against an axenic strain of *Blastocystis* spp. was also reported [27]. In addition, anti-amebic [28] and anti-*Plasmodial* [29] activities were reported for Bjs.

Isolates of *Blastocystis* spp. have varying responses to plant extracts; this is probably due to different karyotypic features or isoenzyme patterns of the isolates [30-32]. *Quercus infectoria* nut gall (Qin) has been used as anti-diarrheic in traditional Taylandese medicine but there is no enough scientific data to support it. Its methanol extract showed anti-amebic activity in mice [33].

Sawangjaroen et al.[21] assessed *in vitro* anti-amebic activities of some plants. They reported that anti-amoebic activities of plants were dose-dependent and 1.000 mg/kg of Plf extract had the highest activity which was also achieved by 125 mg/kg of metronidazole. Lower doses of Plf could not kill the amoebas but limited their pathogenic effects in gut. Methanol extract of Qin showed efficacy against ceacum involvement of amebiasis in mice, but lower compared to Plf.

In another study, essential oils obtained from *Lavandula angustifolia* and *Lavandula intermedia* showed anti-parasitic activities against *Giardia lamblia*, *Trichomonas vaginalis* and a fish parasite, *Hexamita inflata* under 1% of concentrations [34]. Water, dichloromethane and methanol extracts of *Brucea javanica* and the methanol extracts of *Q. infectoria* had inhibitory effects against *Blastocystis* spp., which required further studies [21,35].

A. millefolium L. has been used traditionally in the treatment of inflammatory and spasmodic intestinal diseases, and hepatobiliary complaints [36]. It is used as a

deworming agent in animals; its anti-helminthic activity was shown in a study on sheep against gastrointestinal nematodes [35].

In vitro screening tests are essential for new drug assessments. In the present study, successful 24-month cryopreservation of *Blastocystis* spp. isolates followed by application of microscopy and culture for *in vitro* screening tests were demonstrated. Despite only six isolates were assessed in the study, it is noteworthy to report that no subtype differences were identified after the genotyping of stool samples and culture material.

No cytotoxic activity was demonstrated for n-hexane and methanol extracts of *A. millefolium* or n-hexane extract of *Q. infectoria*, which was significant for their reliabilities in biological investigations. Since the methanol extract of *Q. infectoria* had cytotoxic activity, and LC_{50} and EC_{50} concentrations of the methanol extract of *A. millefolium* had higher values, it was considered that the methanol extract of *A. millefolium* had higher anti-*Blastocystis* spp. activity, which warranted further assessments. In addition, the compounds that are responsible for the cytotoxic activity against *Blastocystis* spp. in the methanol extract of *Q. infectoria* should be identified.

This is the first study that involves the assessment of the efficacies of some plant extracts grown in Turkey and used as anti-diarrheic agents by local people, against cultured *Blastocystis* spp. isolates. Initial results, if confirmed by further assessments, demonstrate that the methanol extract of *A. millefolium* gave promising results and, could be used as an anti-protozoal agent in future.

Acknowledgement

We wish to thank to the technicians of Parasitology Department for their hard work during the study.

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