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

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**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. LC₅₀ and EC₅₀ values of the plant extracts were determined by Brine Shrimp and Graphpad Prism 5® methods, respectively. The results showed that LC₅₀ (500 µg/ml) and EC₅₀ (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

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**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. Geçişten 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, which have been used against diarrhea in Anatolia, were examined for their in vitro efficacies against Blastocystis. LC₅₀ ve EC₅₀ değerleri plant extracts were determined by Brine Shrimp ve Graphpad Prism 5® yöntemleri, sırasıyla. Sonuçlar, LC₅₀ (500 µg/ml) ve EC₅₀ (198.8 µg/ml) konsantrasyonlarca diğer ekstraktal konsantrasyonların altında, A. millefoliumun metanol ekstresinin anti-Blastocystis aktivitesi metronidazoel ile kıyaslandığında en düşük bulunmuş, anti-Blastocystis aktivitesinin ise metronidazoel grubunun değerlerine en yakın olduğu saptanmıştır. Bu sonuçlar A. millefoliumun metanol ekstresinin, ileride yapılacak geniş kapsamlı çalışmalara doğrudan önemli, Blastocystis spp. enfeksiyonlarının tedavisinde yeni bir seçenek olabileceğini göstermektedir.

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

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**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

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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. Recent molecular studies have shown extensive genetic diversity among Blastocystis spp. isolates, which may explain why some patients showed symptoms, while others not. 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. Direct Fluorescent Antibody Method, which is fast and practical, was also considered as a diagnostic method.

Treatment is suggested for symptomatic patients, if other gastrointestinal pathogenic 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.

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 relatively 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 spasmyloic, digestive, carminative, antiphlogistic and cholagogue 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. 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. 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.

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, amantoflavone hexamethyl ether, isocryptomerin, methyl betulate, methyl olenate, and hexagalloyl glucose. Larvical activity of the gall extracts of Quercus infectoria was initially reported against Anopheles stephensi.

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, 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 QIamp 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. 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. Distance-based analysis was conducted with MEGA 3.1 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 10⁵ Blastocystis spp./ml were added to the tubes containing extract and saline solution. All tubes were cultivated for 48 hours 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 (EC⁵₀) 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 10⁵ 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. LC⁵₀ 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 EC⁵₀ value (198.8), compared to others (Table 1, Fig. 2).

Cytotoxic activity assessments with Brine Shrimp Method revealed that the LC⁵₀ value of the methanol extract of Q. infectoria was 190.8605; no cytotoxicity was defined.
In vitro Efficacy of Quercus infectoria ...

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. Despite some other agents such as co-trimoxazole 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, *Brueca javanica* seed (Bjs), *Piper longum* fruit (Plf) and *Quercus infectoria* nut gall (Qin) were assessed against *Blastocystis* spp. dichloromethane and methanol extracts of Bjs were found to be effective. Efficacy of the water extract of Bjs against an axenic strain of *Blastocystis* spp. was also reported. In addition, anti-amebic and anti-*Plasmodial* 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. Metronidazole nut gall (Qin) has been used as anti-diarrheic in traditional Taylandese medicine but there is not enough scientific data to support it. Its methanol extract showed anti-amebic activity in mice.

Sawangjaroen et al. 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. Water, dichloromethane and methanol extracts of *Brueca javanica* and the methanol extracts of *Q. infectoria* had inhibitory effects against *Blastocystis* spp., which required further studies.

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

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**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)

| 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           | 0.14±0.08     | 5.05±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}        | 3.45±8e+6                   | 336.8                       | ~ 546.5                       | 198.8                         | 0.1100         |               |                         |

**EC_{50}: Effective Concentration**

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**Fig 2.** LC_{50} values of plant extracts

Şekil 2. Bitki ekstrelerinin LC_{50} değerleri
deworming agent in animals; its anti-helminthic activity was shown in a study on sheep against gastrointestinal nematodes 33.

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 is significant for their relevancies in biological investigations. Since the methanol extract of Q. infectoria had cytotoxic activity, and LC50 and EC50 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.

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