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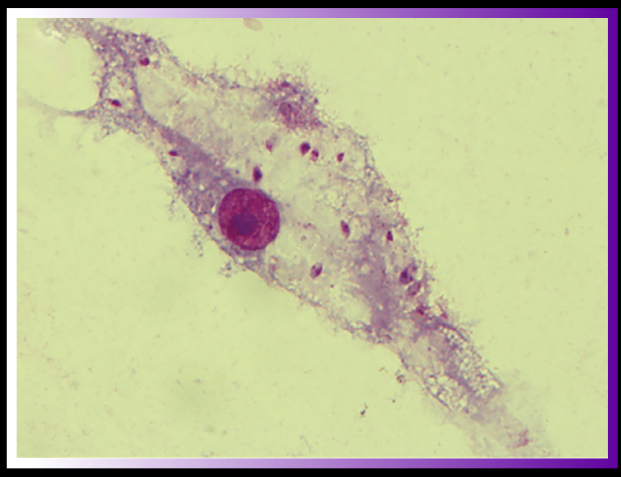

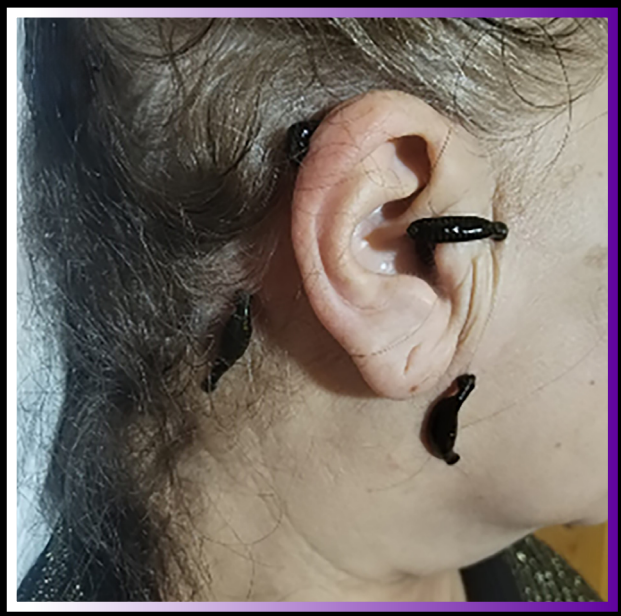

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EDİTÖRDEN

2025 yılının üçüncü sayısını ikisi yurt dışından olmak üzere 6 özgün araştırma makalesi ve 1 olgu sunumu ile çıkarmaktayız. Özgün araştırmalar arasında; *Leishmania* ile enfekte makrofajlarda sitokin sinyal yolağı ile ilişkili bir çalışma, *Acanthamoeba* kistlerinin mortalite oranını farklı yöntemlerle araştıran bir çalışma, Van ilimizde bir rehabilitasyon merkezinde tedavi alan hastalarda bağırsak parazit sıklığı bulgularını bildiren bir makale, Pakistan'da insanlarda kistik ekinokokkoz insidansını bildiren bir çalışma, adi ardıç bitkisi özütlerinin antiparazitik etkisini araştıran bir çalışma ile sülük tedavisine yer veren bir araştırma yer almaktadır. Olgu sunumunda da yine nadir görülen bir hidatik kist olgusuna yer verilmiştir.

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Expression of Cytokine Signaling Pathway Related Genes in *Leishmania*-infected Macrophages

Leishmania ile Enfekte Makrofajlarda Sitokin Sinyal Yolağı İlişkili Gen İfadesi

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ABSTRACT

Objective: Leishmaniasis, caused by protozoan parasites of the *Leishmania* spp., presents significant global health challenges, with visceral leishmaniasis (VL) and cutaneous leishmaniasis forms causing severe morbidity and mortality. Macrophages serve as primary host cells, where *Leishmania* spp. modulate immune 30 responses to ensure survival. Our study investigated gene expression changes in THP1-derived macrophages infected with *L. infantum* and *L. tropica* to elucidate host-pathogen interactions.
Methods: Macrophages were infected with stationary-phase promastigotes, and infection rates were confirmed via Giemsa staining. RNA was extracted, and real time-quantitative polymerase chain reaction was performed to analyze the expression of immune-related genes (*STAT1, STAT2, CCL4, IL23A, IL1R1, IL1RN*).
Results: Results demonstrated significant upregulation of STAT1 and STAT2, key mediators of the JAK-STAT pathway, in both infections, aligning with prior *in vivo* and *in vitro* studies. CCL4, a chemokine linked to macrophage recruitment, was also elevated, consistent with findings in VL and canine leishmaniasis. IL23A, associated with Th17 responses, showed increased expression, supporting its role in leishmanial immune modulation. Notably, IL1RN, an anti-inflammatory mediator, was upregulated, 40 suggesting a balancing mechanism to prevent excessive inflammation.
Conclusion: These findings highlight the complex interplay between pro- and anti-inflammatory responses during *Leishmania* infection and underscore potential targets for diagnostic and therapeutic strategies.
Keywords: *Leishmania*, macrophage, cytokine, JAK-STAT pathway

ÖZ

Amaç: *Leishmania* spp. protozoonlarının neden olduğu leishmaniasis, visseral leishmaniasis (VL) ve kutanöz leishmaniasis formlarıyla ciddi morbidite ve mortaliteye yol açarak küresel bir sağlık sorunu oluşturmaktadır. Makrofajlar, *Leishmania* spp.'nin hayatta kalmasını sağlamak için bağışıklık yanıtlarını modüle ettiği birincil konak hücrelerdir. Çalışmamızda, konak-patojen etkileşimlerini aydınlatmak amacıyla *L. infantum* ve *L. tropica* ile enfekte edilmiş THP-1 kaynaklı makrofajlardaki gen ekspresyon değişiklikleri incelenmiştir.
Yöntemler: Makrofajlar, durağan faz promastigotları ile enfekte edilmiş ve enfeksiyon 10 oranları Giemsa boyaması ile doğrulanmıştır. RNA izolasyonu sonrasında, bağışıklıkla ilişkili genlerin (*STAT1, STAT2, CCL4, IL23A, IL1R1, IL1RN*) ekspresyonunu analiz etmek için gerçek zamanlı-kantitatif polimeraz zincir reaksiyonu gerçekleştirilmiştir.
Bulgular: Sonuçlar, her iki enfeksiyonda da JAK-STAT yolunun önemli düzenleyicileri olan STAT1 ve STAT2'de belirgin bir artış olduğunu göstermiştir; bu bulgu, önceki *in vivo* ve *in vitro* çalışmalarla 15 uyumludur. Makrofaj birikmesiyle bağlantılı bir kemokin olan CCL4'ün de VL ve köpek leishmaniasisindeki bulgularla örtüşecek şekilde arttığı gözlemlenmiştir. Th17 yanıtlarıyla ilişkili IL23A'nın ekspresyonundaki artış, *Leishmania*'nın immün modülasyondaki rolünü desteklemektedir. Özellikle, anti-enflamatuvar bir mediatör olan IL1RN'deki yukarı regülasyon, aşırı enflamasyonu önlemeye yönelik bir denge mekanizmasına işaret etmektedir.
Sonuç: Bu bulgular, *Leishmania* enfeksiyonu sırasında pro- ve anti-enflamatuvar yanıtlar arasındaki karmaşık etkileşimi vurgulamakta ve tanısal/terapötik stratejiler için potansiyel hedefleri ortaya koymaktadır.
Anahtar Kelimeler: *Leishmania,* makrofaj, sitokin, JAK-STAT yolağı

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Leishmaniasis encompasses a spectrum of parasitic infections caused by different *Leishmania* spp., presenting diverse clinical challenges—from localized skin ulcers to fatal systemic infections affecting vital organs such as the liver, spleen, and bone marrow. The disease is endemic in over 90 tropical and subtropical countries across Asia, Africa, the Americas, and Europe (1). Classified as a neglected tropical disease, it affects more than 12 million people worldwide (2). Annually, 0.7-1.2 million new cases are reported, with approximately 350 million people at risk of infection (3).

Transmission occurs through the bite of infected female sand flies—Phlebotomus species in the Old World and Lutzomyia in the New World. To date, 21 *Leishmania* spp. and 30 sand fly vector species have been identified as capable of transmitting the disease to humans (4-5). The parasite exhibits a dimorphic life cycle, alternating between the motile promastigote stage in the vector and the non-motile amastigote stage in human hosts (2,6).

Clinically, leishmaniasis is categorized into four primary forms: Cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL), and post-kala-azar dermal leishmaniasis (PKDL). Among these, VL is the most severe, with the highest mortality rate due to its systemic nature (3). CL is primarily caused by *Leishmania aethiopica, L. infantum, L. tropica*, and *L. major* in the Old World, or *L. amazonensis, L. braziliensis, L. chagasi*, and *L. mexicana* in the New World (2). MCL is characterized by metastatic lesions that destroy mucous membranes and soft tissues in the mouth, nose, larynx, and pharynx. The main causative species—*L. braziliensis, L. panamensis, and L. guyanensis*—are predominantly found in Bolivia, Colombia, Ecuador, Peru, and Paraguay (7). In contrast, VL, also known as kala-azar, is a systemic infection caused by three *Leishmania* species: *L. chagasi, L. donovani,* and *L. infantum*.

Traditional diagnosis relies on detecting the pathogen via bone marrow aspirate smears or cultures (8). However, this method is invasive, often yields false negatives, and can lead to misdiagnosis. Moreover, VL lacks distinctive symptoms, making it difficult to distinguish from other illnesses, particularly in endemic regions (9). Consequently, there is a pressing need for improved diagnostic approaches.

Macrophages serve as the primary host cells for *Leishmania* spp. The parasites ensure their survival by altering macrophage metabolism (10). Studies using various host cell models have revealed gene expression changes during infection, shedding light on how *Leishmania* spp. modulates immune responses (11). Understanding gene expression patterns in *Leishmania* infection will improve our knowledge in disease biology which can positively impact both diagnosis and treatment. Therefore, in this study we investigated the changes in various gene expressions during *in vitro* macrophage infections of *L. infantum* and *L. tropica*.

METHODS

Monocyte Culture and Macrophage Differentiation

This study is designed as an *in vitro* experiment and does not require ethics committee approval. The human leukemia monocytic cell line THP-1 (kindly provided by Prof. Dr. Ayşe Nalbantsoy, Department of Bioengineering, Ege University) was maintained in 25 cm^2 sterile culture flasks. The cells were grown in RPMI-1640 medium (Biological Industries, USA) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, USA) and 1% penicillin/streptomycin (P/S, Gibco, USA). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere (Thermo Scientific, USA). To ensure optimal cell growth, the medium was refreshed every two days. THP-1 cells were seeded onto slides in 6-well plates at a density of 150,000 cells per well. To induce differentiation into macrophage-like cells and enhance their phagocytic activity, the cells were treated with 250 µg/mL PMA (Phorbol 12-myristate 13-acetate, Sigma, USA) and incubated for 24 hours prior to infection.

Promastigote Culture

Cryopreserved *L. infantum* (MCAN/TR/12/EP189) and *L. tropica* (MHOM/AZ/1974/SAF-K27) promastigotes were revived and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 27 °C. Initially, parasites were seeded at a density of 3 million per mL. Daily monitoring revealed logarithmic growth, with parasite counts reaching 8 million by day 3 and 15 million by day 5. Stationary-phase parasites were then harvested for macrophage infection.

Infecting Macrophages with *Leishmania*

Following differentiation of THP-1 cells into macrophages, the PMA-containing medium was removed and replaced with 1 mL of fresh medium containing *Leishmania* parasites at a concentration of 1,500,000 parasites/mL. The macrophage-parasite co-culture was then incubated for 24 hours at 37 °C. To determine the infection rate, Giemsa-stained slides were prepared, and the number of intracellular parasites per 100 macrophages was quantified via light microscopy.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from control and infected cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). RNA concentration and purity were assessed using a Nanophotometer N60 (Implen, Germany), and samples were stored at -80 °C. For cDNA synthesis, a commercial kit (Biolabs, New England) was employed. Briefly, random primers were added to RNA samples, followed by incubation at 65 °C for 5 minutes in a thermocycler. The sample mix was then combined with reverse transcriptase enzyme and incubated for 1.5 hours to complete cDNA synthesis. The resulting cDNA was stored at -20 °C pending further analysis.

Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

All quantitative RT-PCR experiments were conducted using the Light Cycler 480 system (Roche, USA). Primer sequences are provided in Table 1. β-actin served as the endogenous control for normalization, with the following primer sequences: forward 5´-ATGATGATATCGCCGCGCTC-3´ and reverse 5´-TCGTCGCCCACATAGGAATC-3´. Reactions were performed in 10 µL volumes containing: 2 µL cDNA template, 5 µL SYBR Green I master mix (1×, Roche, USA), 500 nM of each primer, and 1 µL nuclease-free water. The thermal cycling protocol consisted of: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation (95 °C, 10 s), annealing (60 °C, 10 s), and extension (72 °C, 10 s), with a final cooling step at 40 °C for 30 s. Amplification specificity was verified by melting curve analysis. Relative gene expression levels in *L. infantum*- and *L. tropica*-infected macrophages compared to uninfected controls were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Genes and primer sequences used in the study

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
STAT1	TGCTTGGATCAGCTGCAGAA	CCACCACAAACGAGCTCTGA
STAT2	GGGGCGCGAGGTTCTA	TGTCGAATGTCCACAGGCAG
CCL4	TGCTAGTAGCTGCCTTCTGC	CACTGGGATCAGCACAGACT
IL23A	GCTTCATGCCTCCCTACTGG	TGAGTGCCATCCTTGAGCTG
IL1R1	GAGCGGCAGGAATGTGACAA	CAAGGGGTCCAGCTTCTCAG
IL1RN	GACCTCCTGTCCTATGAGGC	GAGCATGAGGCTCAATGGGT

Statistical Analysis

All data were analyzed using GraphPad Prism version 9.2.0 for macOS (GraphPad Software, USA). Statistical significance was determined by One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons. A p-value <0.05 was considered statistically significant. All experiments included three replicates.

RESULTS

Infection of THP-1 derived macrophages with *L. infantum* and *L. tropica*

Infectiveness of promastigotes were ensured as described before (12). Promastigotes from the stationary phase of the culture were used to infect macrophages. After 24 hours, co-culture was washed and stained with Giemsa, and observed under microscope. Approximately 50% of the macrophages were infected with at least one or more amastigotes. Figure 1 shows representative infected macrophages under 100X magnification.

Gene Expression Levels

Expression levels of six genes (*STAT1*, *STAT2*, *CCL4*, *IL23A*, *IL1R1*, and *IL1RN*) were investigated for three experimental groups (Macrophage, *L. infantum*, and *L. tropica*) via RT-qPCR. Total RNA was isolated via TRizol method. cDNA was synthesized from total RNA and used in qPCR. All genes exhibited significant increase in infection with both species. No significant difference was found between *L. infantum* and *L. tropica* infections for STAT1, STAT2, CCL4, and IL1RN. *L. infantum* infected macrophages exhibited higher expression of IL23A and IL1R1 compared to *L. tropica* infected macrophages. Figure 2 shows relative expression levels.

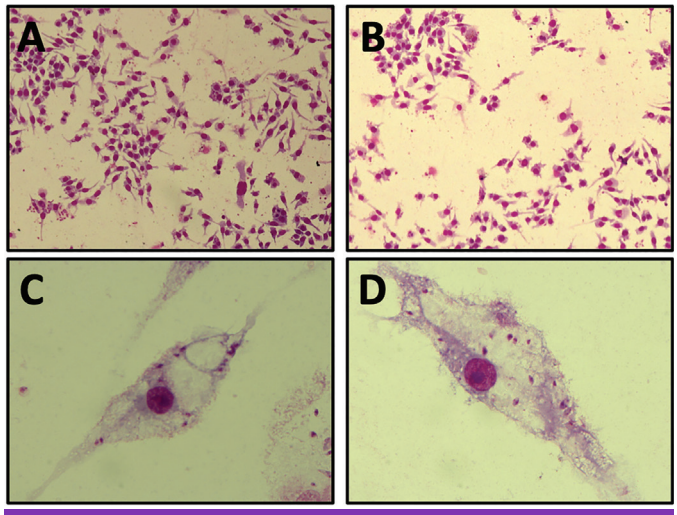

Figure 1. Infection of THP-1 derived macrophages. **A**) *L. infantum* amastigotes in macrophage (10X), **B**) *L. tropica* amastigotes in macrophage (10X), **C**) *L. infantum* amastigotes in macrophage (100X), **D**) *L. tropica* amastigotes in macrophage (100X)

DISCUSSION

In this study, THP-1 derived macrophages were infected with *L. infantum* and *L. tropica* species. Following the validation of infections under microscope, expression of several genes related to cytokine signaling were investigated via RT-qPCR. Namely, STAT1, STAT2, CCL4, IL23A, IL1R1, and IL1RN mRNA levels during *Leishmania* infections were measured.

Expression of STAT1 and STAT2, members of Signal Transducer and Activator of Transcription family were found to be significantly induced with the infection of both *L. infantum* and *L. tropica* species. The JAK-STAT pathway is a crucial signaling mechanism that mediates the effects of numerous cytokines. When this pathway malfunctions, it can contribute to the development and worsening of inflammatory and infectious diseases (13). In a study investigating cytokine pathway related gene expressions in skin lesions of *L. tropica* infected CL patients, authors found that five members of all STAT family, except STAT6 were significantly induced. STAT1 and STAT2 were among the most significantly increased members (14). In an *in vitro* study, Diotallevi et al. (15) infected U937-derived macrophages with *L. infantum, L. major,* and *L. tropica*. Following 24 and 48h, they performed RNA-Seq in order to reveal differentially expressed genes. They found and validated via qPCR that STAT1 expression is increased during infections by all three species (15). Our *in vitro* results correlate with both *in vivo* and *in vitro* data reported in the literature.

It was found that in VL induced by *L. donovani*, peripheral blood mononuclear cells (PBMC) expressed higher CCL4 compared to healthy controls (16). In canine VL caused by *L. infantum*, skin biopsy showed that the tissue is highly enriched with macrophages and CCL4 mRNA expression is significantly increased (17). It was also found upregulated in Diotallevi et al.'s (15) study mentioned above. Our results are consistent with the literature.

IL-23, a cytokine composed of two subunits, plays a key role in promoting Th17 cell differentiation (18). Microbial components—such as those from bacteria, viruses, fungi, and intracellular parasites—potently stimulate macrophages, monocytes, neutrophils, and dendritic cells to produce interleukin (IL)-23 (19). Hadifar et al. (14) found that intralesional expression of IL23A was significantly induced in *L. tropica* infected CL patients . Similarly, it was also found to be elevated in PBMC of CL patients (20). When cultured PBMC from PKDL patients were treated with total soluble *Leishmania* antigens, secreted IL23 was significantly

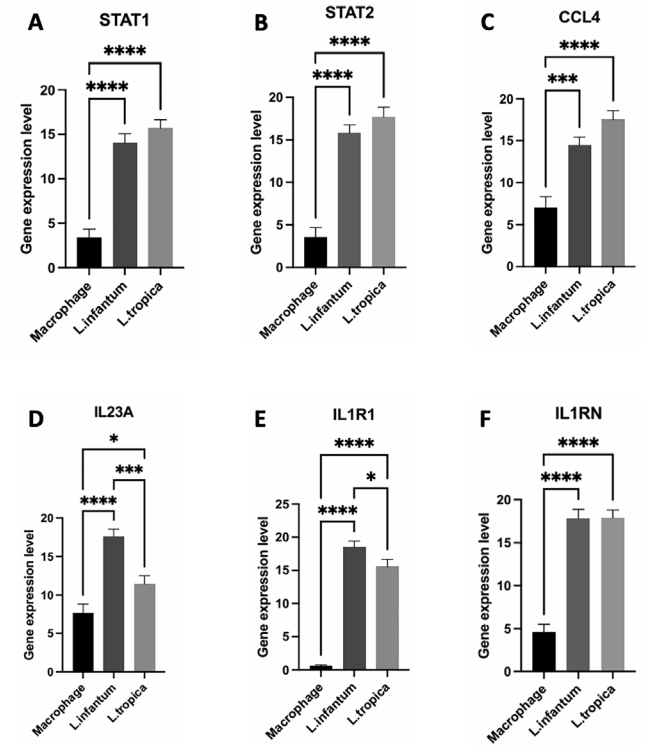

Figure 2. RT-qPCR results. Three experimental groups (uninfected Macrophage infected with *L. infantum*, and Macrophage infected with *L. tropica*) were investigated in terms of expression levels of six cytokine signaling-related genes. **A)** STAT1, **B)** STAT2, **C)** CCL4, **D)** IL23A, **E)** IL1R1, and **F)** IL1RN

**: p<0.05, ***: p<0.001, ****: p<0.0001, RT-qPCR: Real time-quantitative polymerase chain reaction*

increased compared to the PBMCs collected from healthy controls (21).

Fernandes et al. (11) infected THP-1 derived macrophages with three *Leishmania* spp. and compared the expression of long noncoding RNAs. They showed that IL1R1 was upregulated and coregulated with its antisense lncRNA in *L. infantum* infection.

Interestingly, along with pro-inflammatory cytokines, we found that anti-inflammatory IL1RN, antagonist of IL1 signaling, was also significantly upregulated in *L. infantum* and *L. tropica* infections. In an *in vivo* study, IL1RN KO mice manifested more severe disease progression (22), indicating that IL1R antagonist is important in restricting the immune response. Although parasite load was lower in IL1RN deficient mice, their survival rate was also decreased. Therefore, in the absence IL1R antagonist, the parasites are more efficiently cleared but at the same time the immune system is damaging the host. We found that IL1RN is upregulated in *Leishmania* infected macrophages *in vitro*.

Cytokines are pivotal in modulating the host's immune defense against *Leishmania* infection, influencing the balance between protective and pathological immune outcomes. Their biological effects are mediated through the activation of transcription factors, which translocate to the nucleus and regulate the expression of genes responsive to cytokine signaling. Understanding cytokine signaling and its regulation during leishmaniasis might result in better ways to both diagnose and treat the disease. Our *in vitro* results will contribute to literature in terms of revealing the role of cytokines in host defense against leishmaniasis.

CONCLUSION

This study demonstrates that *Leishmania* infection of macrophages triggers significant modulation of cytokine signaling pathways, characterized by upregulation of proinflammatory mediators alongside anti-inflammatory IL1RN. These findings underscore the dual role of host immune responses in controlling infection while preventing excessive inflammation. The consistent induction of JAK-STAT pathway genes across *L. infantum* and *L. tropica* infections highlights their potential as therapeutic targets. Further validation of these molecular signatures in clinical samples could advance diagnostic and immunomodulatory strategies for leishmaniasis.

*Ethics

Ethics Committee Approval: This study is designed as an *in vitro* experiment and does not require ethics committee approval.

Informed Consent: This study is designed as an *in vitro* experiment and does not require informed consent.

Footnotes

*Authorship Contributions

Concept: U.M., A.C., Design: U.M., H.A., A.C., Data Collection or Processing: U.M., H.A., C.M., A.C., Analysis or Interpretation: U.M., C.M., A.C., Literature Search: U.M., A.C., Writing: U.M., A.C.

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Comparison of the Synergistic Effects of Black Tea and Microwave with Gentamicin and Microwave on *Acanthamoeba* cyst Mortality *In vitro*

Acanthamoeba Kistinin İn vitro Mortalite Oranı Üzerinde Siyah Çay ve Mikrodalganın Sinerjistik Etkisinin Gentamisin ve Mikrodalganın Sinerjistik Etkisi ile Karşılaştırılması

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ABSTRACT

Objective: Drug resistance in *Acanthamoeba* poses a significant challenge, prompting the need for alternative treatments. This research aimed to explore the combined treatment of chemical or phytomedicines and microwaves radiation.
Methods: The *Acanthamoeba* strain was cultivated on non-nutrient agar. Black tea extracts were prepared using the maceration method. Final concentrations of 0.75 mg/mL and 0.375 mg/mL of gentamicin and tea, respectively, were used in this study. The samples were divided into 12 groups based on drug incubation time and repeated radiation exposure, either before or after incubation. The effects of combining gentamicin and black tea extracts with microwave exposure were then evaluated on the parasite.
Results: Our results showed that the growth inhibition of *Acanthamoeba* was significantly higher in the combined treatment groups compared to gentamicin, black tea, or microwave radiation alone ($p<0.0001$-$p<0.04$). It seems that the microwave radiation led to an increasing trend in growth inhibition within 72 hours.
Conclusion: Microwave radiation can play a significant complementary role in the treatment of *Acanthamoeba* cysts by gentamicin and black tea extracts. This effect was more significant on the irradiated cysts incubated with gentamicin and also depended on the increase in incubation time and the repetition of radiation.
Keywords: *Acanthamoeba*, black tea, combination treatment, drug resistance, gentamicin, radio frequency

ÖZ

Amaç: *Acanthamoeba*'nın ilaç direnci, alternatif yeni yöntem bulma konusunda önemli bir endişe kaynağıdır. Bu araştırmada kimyasal veya bitkisel ilaçlar ile mikrodalga radyasyonunun kombine tedavisinden faydalanılmaya çalışılmıştır.
Yöntemler: *Acanthamoeba*, besleyici olmayan agar üzerinde yetiştirildi. Siyah çay ekstraktları maserasyon yöntemiyle hazırlandı. Mevcut çalışmada kullanılan gentamisinin nihai konsantrasyonları 0,75 ve 0,375 mg/mL'dir. Örnekler ilaç inkübasyon süresi ve inkübasyon öncesi ve sonrası radyasyona tekrar maruz kalma (24, 48 ve 72 saat) açısından 12 gruba ayrıldı. Gentamisin bileşiği ve siyah çay ekstraktlarının mikrodalgaya maruz bırakılmasıyla parazit üzerindeki etkileri değerlendirildi.
Bulgular: Mevcut deneyler, gentamisin, siyah çay ve radyasyon tedavisi grubuyla karşılaştırıldığında bileşik tedavi gruplarında parazitin büyüme inhibisyonunun önemli ölçüde daha yüksek olduğunu gösterdi ($p<0,0001$-$p<0,04$). Mikrodalga radyasyonunun 72 saat içinde büyüme inhibisyonunun artan bir eğilime yol açtığı görülmektedir.
Sonuç: Mikrodalga radyasyonu, *Acanthamoeba* kistlerinin gentamisin ve siyah çay ekstraktları ile tedavisinde önemli bir tamamlayıcı rol oynayabilir. Bu etkinin gentamisin ile inkübe edilen ışınlanmış kistlerde daha belirgin olduğu ve inkübasyon süresinin artmasına ve radyasyon tekrarına da bağlı olduğu görüldü.
Anahtar Kelimeler: *Acanthamoeba*, siyah çay, kombinasyon tedavisi, ilaç direnci, gentamisin, radyo frekansı

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Acanthamoeba is a free-living amoeba and one of the most common protozoa found in nature. This opportunistic parasite has been isolated from diverse environments, including soil, dust, fresh water, seawater, swimming pools, dental units, air conditioners, and hospital spaces (1). There are two phases of in *Acanthamoeba* life cycle: trophozoite and cyst. The cysts of this parasite are resistant to lethal substances like chlorine and antibiotics and can withstand a wide range of temperatures. Notably, it causes eye infections known as *Acanthamoeba* keratitis (AK) (2). If AK is not treated, it can progress to stroma damage and perforation, leading to significant vision loss and possibly blindness (3). The rising use of contact lenses has led to a significant increase in AK, particularly affecting individuals aged 15 to 25 (4,5). Despite long-term treatment, AK may not always respond. The current therapeutic approach involves using various drugs, such as gentamicin, natamycin, neosporin, broline, propamidine isothionate, and miconazole (6,7). Gentamicin, frequently used for treating ocular keratitis, exerts its antibacterial effect by disrupting bacterial cell membranes and inhibiting protein synthesis. It is applied as eye drops to treat conditions like conjunctivitis, corneal ulcers, keratoconjunctivitis, and inflammation caused by microorganisms, including *Acanthamoeba* (8).

Scientific research continues to seek effective, low-risk treatments for infectious and non-infectious diseases (9,10). Herbal medicine is widely regarded as a potential alternative for treating parasitic diseases (9). Tea has long been used as an herbal remedy, prepared by brewing leaves, buds, or stems from various plants. In Iran, over 20 types of tea are made using different plant parts, each with unique properties in traditional medicine (11). In Iran, the most common teas are green, black, white, and sour tea. Green and black tea are derived from the *Camellia sinensis* plant (11). Black tea is richer, stronger, and more bitter than green tea due to the complete oxidation and drying of its leaves during processing (12). Black tea is rich in caffeine, theophylline, L-theanine, and antioxidants like catechins, while containing minimal fat, carbohydrates, and protein (13,14). Numerous studies have examined tea's effects and mechanisms in treating various diseases. One study found that tea can help prevent obesity, diabetes, metabolic syndrome, and cardiovascular diseases (15). Research has confirmed that both green and black tea inhibit oxidation (16). Studies also show that tea catechins can block toxins from bacteria like *Escherichia coli* and reduce its pathogenicity (17,18). Experimental studies suggest that high concentrations of tea may serve as an adjunct therapy alongside antibiotics (19,20).

The use of radio frequency electromagnetic waves, both *in vitro* and *in vivo*, is a novel method being explored for disease treatment. Radio waves, known for their high frequency, can penetrate tissue, generate heat, and act quickly. Modern life is continuously exposed to microwave radio waves from devices like mobile phones and microwave ovens (21,22). While many researchers study the harmful effects of waves, others have explored their potential to kill pathogens and treat diseases, making significant progress (23-25). Recent studies show that high-intensity microwaves from ovens, mobile phones, and other devices can increase the mortality of protoscolices, hydatid cyst walls, and *Acanthamoeba* cysts (26-29).

It is essential to create innovative approaches that utilize combination therapies to tackle the deficiencies of conventional methods, ultimately aiming to lessen the complications of infectious keratitis and boost clinical effectiveness. This study aims to examine the synergistic effects of microwave irradiation combined with tea extract or gentamicin on the mortality of *Acanthamoeba* cysts. While previous research has investigated the impact of microwaves from devices like cell phones and ovens on parasites (9,30), the combined effects of microwaves with tea extract or gentamicin on *Acanthamoeba* remain unexplored. This study builds on prior research that demonstrated the antimicrobial potential of microwaves and the therapeutic properties of tea extracts. It explores an innovative combination therapy aimed at overcoming the limitations of existing treatments, reducing complications associated with *Acanthamoeba* infections, and improving clinical outcomes. By examining the interactions between microwave irradiation and tea extract or gentamicin, this research seeks to provide insights into novel and more effective treatment strategies for AK.

METHODS

Parasite Preparation

Acanthamoeba strain MG066681 (T4 genotype), kept in Parasitology Laboratory of Arak University of Medical Sciences was used for this study. This *Acanthamoeba* strain used in the study was an environmental (soil) (31). The parasite was cultured on non-nutrient agar plates coated with killed *Escherichia coli* at laboratory temperature (28 °C) (32). After 1-3 weeks, the surface of the plate was washed with sterile Page's saline, and the cysts were collected and concentrated by centrifugation at 1500 × g for 5 minutes (33). The cysts washed 3 times with phosphate-buffered saline (PBS). The viable and non-viable cysts were counted using trypan blue staining (unstained viable and stained non-viable cyst) and by hemocytometer. The parasitic suspension in which ≥90% of the cysts were alive was used for the study. Finally, a parasitic suspension containing 2×10^5 cysts per mL was prepared for this experimental study. The amount of parasite was 4×10^4 in 200 µL of parasite suspension.

Tea Extract Preparation

Black tea (*Camellia sinensis*) was obtained from the Medical Plants Research Center, SKUMS, and transferred to the Infectious Diseases Research Center, Faculty of Medicine University of Medical Sciences. The extracts of the black tea were prepared via maceration as follows: 100 g black tea was transferred into an Erlenmeyer flask; 1 L of 70% ethanol was added and the solution was placed at the laboratory temperature for 24 h. The extract was filtered through filter paper, dried via rotary evaporation, and stored at 4 °C (34). The final concentration of tea extract was used in experiment stage was 30 mg/mL.

Drug Preparation

Gentamicin was prepared (Sina Darou, Tehran, Iran). The results of some studies have shown that a concentration of 3 mg/mL of this drug leads to complete mortality of *Acanthamoeba* cysts (35). So, in the current study, lower concentrations of this drug were used. Final concentrations of 0.75 and 0.375 mg/mL of drug used in the current study.

Microwave Generator

The device used for irradiation of microwaves was Thermatur m250, Uniphy Elektromedizin Company, made in Germany. The radiant power of the device was 250 W (antenna of the device) and a frequency of 2450 MHz with an intensity of 2150 W/m (Figure 1).

Microwave Radiation Intensity Measurement

The measurements were performed by a TES 92 device with the capability to measure the EMF frequency range of 3.5 MHz-50GHz.

Experiments

200 µL of parasitic suspension were exposed with PBS, gentamicin (final concentration 0.75 mg/mL), tea extract (final concentration 30 mg/mL) and microwave radiation (25 minutes) as control groups 1 to 4, respectively. Parasite mortality was recorded up to 72 hours at 24-hour intervals in these groups. The treatment experiments were performed by four different protocols (I to IIII). Each protocol included two treatment groups (I_1 and I_2), resulting in a total of eight treatment groups (Groups 5 to 12).

Group I: Incubation before irradiation

• **I_1:** Incubation with gentamicin, followed by repeated cycles of microwave radiation and incubation (A-F).

• **I_2:** Incubation with tea extract, followed by repeated cycles of microwave radiation and incubation (A-F).

Group II: Irradiation before incubation

• **II_1:** Initial microwave irradiation, then incubation with gentamicin, followed by alternating cycles of irradiation and incubation (G-L).

• **II_2:** Initial microwave irradiation, then incubation with tea extract, followed by alternating cycles of irradiation and incubation (G-L).

Group III: Simultaneous incubation and irradiation

• **III_1:** Microwave irradiation performed simultaneously with incubation using gentamicin, followed by alternating cycles of irradiation and incubation (M-R).

• **III_2:** Microwave irradiation performed simultaneously with incubation using tea extract, followed by alternating cycles of irradiation and incubation (M-R).

Figure 1. Microwave irradiation generator

Group IV: Double irradiation before incubation

• **IV_1:** Two microwave irradiation cycles first, followed by incubation with gentamicin and subsequent incubations (S-U).

• **IV_2:** Two microwave irradiation cycles first, followed by incubation with tea extract and subsequent incubations (S-U).

At the end of each exposure time, the cysts were washed three times by PBS to remove residual drugs. The washed cysts resuspended in 100 µL PBS and used for investigated of viable and non-viable cysts and calculating percent of eliminated cysts (or % growth inhibition) by Trypan blue exclusion assay (36,37). All experiments were repeated 3 times. After 25 min of microwave exposure, the temperatures were increased from 22.6±23 to 23.2±23.9 °C. The average temperature was 0.9 °C.

Calculation of growth inhibition percent (or % eliminated cysts)

Summary, the cyst suspension and trypan blue were mixed at 1:1 ratio. The mixture was loaded in hemocytometer and number of viable and non-viable cysts counted under light microscope. The calculation was performed as follows (36).

% viable cysts = Total number of viable cysts per mL/total number of cysts per mL × 100

% growth inhibition (or % eliminated cyst) =100-% viable cysts.

Statistical Analysis

The analysis of data was done by SPSS (version 23, SPSS/PC Inc., Chicago, IL, USA) and Excel (2016). The results were presented as mean ± standard deviation and percent of eliminated cyst (or % growth inhibition). Differences between the growth inhibitions of groups were analyzed by One-Way ANOVA test. Statistical significance was defined as $p<0.05$.

Ethical Approval

All experiments were approved by Ethical Committee of the Arak University of Medical Sciences in this research (number: REC.1400.198, date: 2021-10-31).

RESULTS

The assessment of growth suppression, along with the survival and viability rates of *Acanthamoeba* in the control groups, was conducted, and the results for each group are presented in Table 1.

The treatment groups were shown in the Table 2a. The incubation time was 24 h and the irradiation time was 25 minutes in all treatments.

The levels of growth inhibition, along with the viability and survival rates of *Acanthamoeba* in the treatment groups (5-12), were evaluated. The findings for each treatment phase (A-U) are detailed in Table 2b. It should be noted that the parasite survival number is expressed as $\times 10^{-4}$.

An analysis of the 12 groups based on the duration of exposure revealed that all treated groups (groups 5 to 12) exhibited significantly greater growth inhibition compared to the control groups (groups 1 to 4) ($p<0.05$), except for group 2 (gentamicin control) and group 9 (Table 2b).

The growth inhibition rates of the treatment groups (gentamicin and tea) under protocols I to IV are presented in Figure 2.

Table 1. Growth inhibition and viable cyst percentages of *Acanthamoeba* in control groups

	Group 1			**Group 2**		
Exposure time (h)	**□ Survival cyst/mL mean ± SD**	**% viability mean ±SD**	**% growth inhibition**	**□ Survival cyst/mL mean ± SD**	**% viability mean ± SD**	**% growth inhibition**
24	20±0	100±0	0	14.6±0.06	73±0.30	27
48	20±0	100±0	0	14.2±0.07	71±0.35	29
72	20±0	100±0	0	13.8±0.08	69±0.39	31
	Group 3			**Group 4**		
Exposure time (h)	**□ Survival cyst/mL mean ± SD**	**% viability mean ±SD**	**% growth inhibition**	**□ Survival cyst/mL mean ± SD**	**% viability mean ± SD**	**% growth inhibition**
24	20±0	100±0	0	19.2±0.07	96±0.2	4
48	19.8±0.03	99±0.17	1	18.8±0.05	94±0.25	6
72	19.8±0.04	99±0.2	1	18.2±0.05	91±0.24	9

Group 1: PBS, Group 2: Gentamicin, Group 3: Tea extract, Group 4: Microwave, SD: Standard deviation, □: $\times 10^{-4}$

Table 2a. The treatment groups and the stages of treatment

Treatment groups	**Treatment subgroups**	**Protocols**
5	**$I_{1:}$** Parasite suspension + gentamicin (final concentration: 0.75 mg/mL)	**I** First, the *Acanthamoeba* cyst suspension was incubated with gentamicin or tea for 24 hours (**A**), followed by microwave irradiation (25 min) according to the protocol (*).
6	**$I_{2:}$** Parasite suspension + tea extract (final concentration: 30 mg/mL)	
7	**$II_{1:}$** Parasite suspension + exposure then adding gentamicin (final concentration: 0.75 mg/mL)	**II** First, the *Acanthamoeba* cyst suspension was exposed to microwave irradiation (25 min) (**G**), followed by a 24-hour incubation with gentamicin or tea. The samples were then irradiated again following protocol (**).
8	**$II_{2:}$** Parasite suspension + exposure then adding tea extract (final concentration: 30 mg/mL)	
9	**III_{1}:** Parasite suspension + gentamicin (final concentration: 0.75 mg/mL) + microwave irradiation (25 min)	**III** *Acanthamoeba* cyst suspension was exposed to simultaneous microwave irradiation (25 min) and 24-hour incubation with either gentamicin or tea (**M).** The samples were then irradiated again following protocol (***)
10	**III_{2}:** Parasite suspension + tea extract (final concentration 30 mg/mL) + microwave irradiation (25 min)	
11	**IV_{A}:** Parasite suspension + double microwave irradiation followed by the addition of gentamicin (final concentration: 0.75 mg/mL)	IV Parasites undergo two consecutive rounds of irradiation instead of one (25 min), followed by incubation with gentamicin or tea for 24 hours (**S**), and then the incubation protocol (****) is applied.
12	**IV_{B}:** Parasite suspension + double microwave irradiation followed by the addition of tea extract (final concentration: 30 mg/mL)	

* **B:** 25 minutes microwave radiation**, C:** 24-hour incubation, **D:** 25 minutes microwave radiation**, E:** 24-hour incubation**, F:** 25 minutes microwave radiation
** **H:** 24-hour incubation with tea or gentamicin, **I:** 25 minutes microwave radiation, **J:** 24-hour incubation, **K:** 25 minutes microwave radiation, **L:** 24-hour incubation
*** **N**: 25 minutes microwave radiation, **O:** 24-hour incubation, **P:** 25 minutes microwave radiation, **Q:** 24-hour incubation, **R:** 25 minutes microwave radiation
**** **T:** 24-hour incubation, **U:** 24-hour incubation

Table 2b. Inhibition effects and percent of viable *Acanthamoeba* cysts treated at stages of protocols I-IV

Process	Protocol I						Process	Protocol II					
	Group 5*			Group 6*				Group 7**			Group 8**		
	Survival cyst/mL mean ± SD	% viability mean ± SD	% growth inhibition	Survival cyst/mL mean ± SD	% viability mean ± SD	% growth inhibition		Survival cyst/mL mean ± SD	% viability mean ± SD	% growth inhibition	Survival cyst/mL mean ± SD	% viability mean ±SD	% growth inhibition
A	14±0.03	70±0.17	30	17.8±0.02	89±0.1	11	G	11.8±0.08	59±0.4	41	17.6±0.03	88±0.15	12
B	12±0.034	60±0.17	40	17.2±0.08	86±0.4	14	H	10±0.06	50±0.3	50	17.4±0.03	87±0.15	13
C	12±0.03	51±0.3	49	16±0.03	80±0.15	20	I	7.8±0.03	39±0.15	61	17±0.04	85±0.2	15
D	8.6±0.03	43±0.15	57	15.6±0.01	78±0.05	22	J	6.2±0.03	31±0.15	69	16.6±0.02	83±0.1	17
E	7.6±0.03	38±0.15	62	15.2±0.02	76±0.1	24	K	5.2±0.02	26±0.1	74	16.4±0.04	82±0.2	18
F	7.2±0.08	36±0.4	64	15±0.03	75±0.15	25	L	4.8±0.04	24±0.2	76	16±0.03	80±0.15	20
			Protocol III							Protocol IV			
		Group 9***			Group 10***				Group 11****			Group 12****	
Process	□ Survival cyst/mL mean ± SD	% viability mean ± SD	% growth inhibition	Survival cyst/mL mean ± SD	% viability mean ± SD	% growth inhibition	Process	Survival cyst/mL mean ± SD	% viability mean ± SD	% growth inhibition	□ Survival cyst/mL nean ± SD	% viability mean ± SD	% growth inhibition
M	17.4±0.04	87±0.2	13	16.2±0.01	81±0.05	19	S	13±0.02	65±0.1	35	16.6±0.03	83±0.15	17
N	16.4±0.02	82±0.1	18	16.2±0.03	81±0.15	19	T	10.8±0.04	54±0.2	46	16±0.02	80±0.1	20
O	16.2±0.02	81±0.1	19	15.8±0.01	79±0.05	21	U	7.6±0.04	38±0.2	62	15.6±0.03	78±0.15	22
P	14±0.03	70±0.15	30	15.6±0.01	78±0.05	22							
Q	13.6±0.03	68±0.15	32	15.6±0.04	78±0.2	22							
R	13.6±0.01	68±0.05	32	15.4±0.02	77±0.1	23							

*: Parasite was incubated with control drug or tea prior to microwave exposure
**: Parasite exposure to microwaves prior to incubation with control drug or tea
***: Simultaneous exposure of parasites to microwaves and control drug or tea treatment and followed by incubation
****: parasite exposure to double irradiation microwave before incubation with control drug or tea
A: 24-h incubation with control drug or tea, **B:** 25 minutes microwave radiation, **C:** 24-hour incubation, **D:** 25 minutes microwave radiation, **E:** 24-hour incubation, **F:** 25 minutes microwave radiation,** **G:** 25 minutes microwave radiation, **H:** 24-h incubation with control drug or tea, **I:** 25 minutes microwave radiation, **J:** 24-hour incubation, **K:** 25 minutes microwave radiation, **L:** 24-hour incubation
*** **M:** Simultaneous exposure microwaves (25 min) and control drug or tea treatment followed by 24-h incubation, **N:** 25 minutes microwave radiation, **O:** 24-hour incubation, **P:** 25 minutes microwave radiation, **Q:** 24-hour incubation, **R:** 25 minutes microwave radiation
****S.** Double irradiation microwave followed by, 24-h incubation with control drug or tea, **T:** 24-hour incubation, **U:** 24-hour incubation
□: $\times 10^{-4}$, SD: Standard deviation

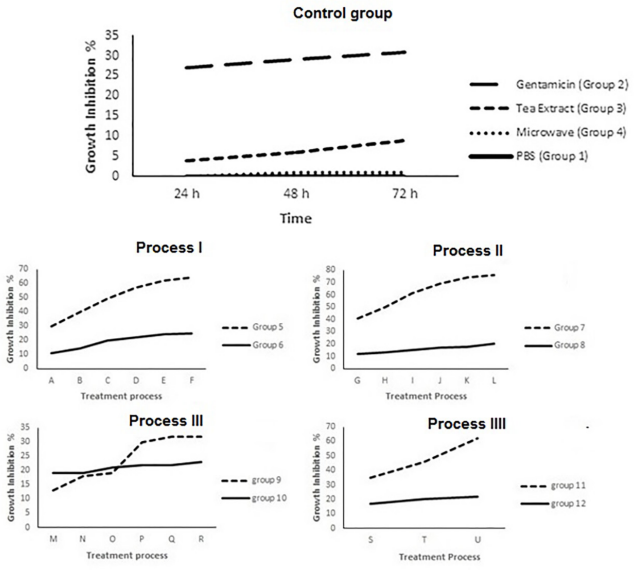

Figure 2. Comparison of *Acanthamoeba* growth inhibition across groups

DISCUSSION

This study revealed that microwave radiation (250 W, 25 minutes) alone was ineffective in achieving significant mortality in *Acanthamoeba* cysts. The lack of effectiveness can be attributed to insufficient moisture within the parasites to induce intracellular oscillations and a negligible increase in temperature during treatment. Studies have shown that the thermal and non-thermal effects of microwaves are heavily dependent on the water content of the target organism. Zhang et al. (23) reported that moist environments significantly enhance the efficacy of microwave radiation against microbial cysts by facilitating intracellular heating. Previous findings emphasize that effective microwave treatments require higher moisture content and elevated temperatures to deactivate *Acanthamoeba* cysts, as demonstrated by studies reporting complete cyst mortality at higher power settings and prolonged exposure times (22,30).

Among the treatment protocols tested, the conventional drug Gentamicin in protocol II demonstrated the highest growth inhibition rate, particularly in Group 7. Gentamicin's efficacy can be attributed to its established bactericidal mechanism, targeting the bacterial-like ribosomes of *Acanthamoeba* (15). In contrast, the tea extract in protocol III initially exhibited a greater inhibitory effect on parasite growth than gentamicin. This effect is likely due to the bioactive compounds in tea, such as catechins and tannins, which disrupt cellular membranes and metabolic pathways (9). By the end of the experiment, however, the tea extract's effectiveness became comparable to other protocols, suggesting a transient potency that diminishes over time.

A comparative analysis of Protocols II and III indicates that increasing the frequency of radiation exposure significantly enhanced growth inhibition, particularly in Group II. This aligns with Shaw et al. (38), who demonstrated that the non-thermal effects of pulsed microwave radiation effectively inactivated *Escherichia coli* and *Staphylococcus aureus*, potentially boosting the efficacy of concurrent treatments. A study investigated the use of gold nanoparticles combined with microwave radiation as an antiparasitic treatment. The results showed that increased microwave exposure time in the presence of gold nanoparticles led to a significant decline in parasite survival rates, indicating a synergistic effect that enhances growth inhibition (39). A research demonstrated that microwave can significantly enhance the sensitivity of cancer cells to anticancer drugs (40). This suggests that microwave exposure can improve the efficacy of co-administered therapeutic agents, potentially applicable to antiparasitic treatments.

However, the results indicate that while microwave exposure aids growth inhibition, gentamicin remains more effective overall than the tea extract. This observation is consistent with earlier findings that highlight the superior efficacy of conventional drugs over botanical alternatives in some contexts (41,42).

The combination of microwave radiation with gentamicin or tea extract yielded mixed results. In groups 9 and 10, the initial 48-hour period showed that tea extract combined with microwave radiation exhibited a stronger inhibitory effect on *Acanthamoeba* than gentamicin. This finding suggests a potential synergistic effect between microwave radiation and tea extract during the early stages of treatment. Synergistic effects may arise from microwave-induced structural weakening of the cyst wall, enhancing the penetration of bioactive compounds (43).

Comparison of protocols III with II shows that microwaves reduced drug efficiency to less than half that of protocols II. However, the overall inhibition rates in groups 11 and 12 were similar to those in groups 5-8, suggesting an inconsistent synergistic effect over time. This variability may be influenced by factors such as treatment sequence, exposure duration, and parasite heterogeneity.

Exposure timing also appeared to have limited impact on tea treatment group. Early or later exposure of the parasites to microwave radiation relative to the tea extract did not significantly enhance growth inhibition. This finding contrasts with studies emphasizing the importance of treatment timing in maximizing synergistic effects. Cheng et al. (44) demonstrated that pre-treatment with microwaves followed by drug exposure increased efficacy in bacterial biofilms by disrupting extracellular matrices.

Previous studies have shown that tea extracts and electromagnetic waves individually have anti-*Acanthamoeba* effects. Hajihossein et al. (9) found that green and black tea extracts were more effective than the anti-keratitis drug Natamycin, with efficacy depending on incubation time and extract concentration. Similarly, the study on high-intensity microwave radiation (e.g., 1550 W, 2450 MHz) reported complete cyst mortality within minutes due to substantial temperature increases (29). However, this study found lower efficacy for both microwave treatments and tea extracts compared to gentamicin. This discrepancy may result from differences in microwave intensity, exposure duration, or tea extract concentrations. The reduced mortality rate with tea extract compared to gentamicin may also reflect variations in the type of drug or extract concentrations used.

Recommendation:

- Evaluate the effects of higher power settings, longer exposure times, and varying frequencies to determine the most effective microwave treatment conditions.
- Investigate other plant-derived extracts with potential anti-*Acanthamoeba* properties, focusing on concentration, incubation time, and their interaction with microwave radiation.

- Test combination therapies on diverse *Acanthamoeba* strains and under different environmental conditions to enhance generalizability.
- Assess the feasibility, safety, and cost-effectiveness of integrating microwave radiation with conventional or botanical therapies in clinical and field settings.

CONCLUSION

The integration of microwave radiation with gentamicin or tea extract shows promise as a combination therapy against *Acanthamoeba* cysts. However, the inconsistent results observed in this study highlight the complexity of synergistic interactions and the need for further comprehensive studies to confirm and refine these findings.

*Ethics

Ethics Committee Approval: All experiments was approved by Ethical Committee of the Arak University of Medical Sciences in this research (number: REC.1400.198, date: 2021-10-31).

Informed Consent: This study was conducted exclusively on *Acanthamoeba* cysts *in vitro*. No human participants or animal subjects were involved; therefore, ethical approval and informed consent were not required.

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Footnotes

*Authorship Contributions

Surgical and Medical Practices: H.S., R.H., Y.F., Concept: Z.E., H.S., Design: Z.E., H.S., Data Collection or Processing: Z.E., H.S., Analysis or Interpretation: Z.E., H.S., Literature Search: Z.E., H.S., Writing: Z.E., H.S.

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Frequency of Intestinal Protozoa in Patients Receiving Treatment at Van Special Physioactive Special Education and Rehabilitation Center

Van Özel Fizyoaktif Özel Eğitim ve Rehabilitasyon Merkezinde Tedavi Alan Hastalarda İntestinal Protozoonların Görülme Sıklığı

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ABSTRACT

Objective: The aim of this study is to determine the frequency of intestinal protozoa in disabled patients attending a rehabilitation center, thereby highlighting the significance of intestinal protozoa in individuals with disabilities.
Methods: The study included a total of 300 individuals, comprising 200 disabled patients and 100 non-disabled individuals. Stool samples were collected from all participants and examined using the native-Lugol and modified acid-fast methods.
Results: In the study, intestinal protozoa were detected in 41% of disabled individuals and in 9% of individuals in the control group (p=0.001). *Blastocystis* was detected in 18% of patients, *Cryptosporidium* spp. in 15%, *Giardia intestinalis* in 9%, *Cyclospora cayetanensis* in 5%, and *Entamoeba coli* in 4%. Among the subgroups, spina bifida patients had a protozoan infection rate of 83.3%, while the rates in other groups were lower. A significant relationship was detected between the presence of parasites and diarrhea, constipation, and loss of appetite (p<0.05).
Conclusion: Disabled individuals, especially those with spina bifida, are at risk for intestinal protozoa, and regular screening for opportunistic protozoa is essential for these patients.
Keywords: Down syndrome, disability, intestinal protozoa, rehabilitation, spina bifida

ÖZ

Amaç: Bu çalışmanın amacı, bir rehabilitasyon merkezine devam eden engelli hastalarda intestinal protozoonların sıklığını belirleyerek intestinal protozoonların engelli bireylerdeki önemini ortaya koymaktır.
Yöntemler: Çalışmaya, 200 engelli hasta ve 100 engelsiz birey olmak üzere toplam 300 kişi dahil edildi. Çalışmaya dahil edilen tüm bireylerden dışkı örnekleri alınarak, nativ-Lugol ve modifiye asit fast yöntemleriyle incelendi.
Bulgular: Çalışmada, engelli bireylerin %41'inde ve kontrol grubundaki bireylerin %9'unda bir ya da daha fazla intestinal protozoon saptandı (p=0,001). Hastaların %18'inde *Blastocystis*, %15'inde *Cryptosporidium* spp., %9'unda *Giardia intestinalis*, %5'inde *Cyclospora cayetanensis* ve %4'ünde *Entamoeba coli* saptandı. Hasta alt gruplarından spina bifida hastalarının %83,3'ünde intestinal protozoon bulunurken diğer gruplardaki oranlar daha düşük bulundu. Ayrıca engelli bireylerde protozoon varlığı ile ishal, kabızlık ve iştahsızlık arasında anlamlı bir ilişki olduğu belirlendi (p<0,05).
Sonuç: Engelli bireylerin, özellikle de spina bifida hastalarının intestinal protozoonlar açısından risk altında olduğu ve bu hastaların özellikle fırsatçı protozoonlar yönünden düzenli olarak taranması gerektiği kanaatine varıldı.
Anahtar Kelimeler: Down sendromu, engelli, intestinal protozoonlar, rehabilitasyon, spina bifida

INTRODUCTION

Intestinal parasitic infections constitute a significant global health issue, with widespread prevalence. Particularly common in developing nations, these infections can lead to high rates of morbidity and mortality. They are more prevalent in tropical and subtropical regions worldwide, affecting an estimated two billion individuals. The widespread occurrence of these infections underscores the need for increased efforts in their prevention and healthcare improvement on a global scale (1-3).

Intestinal protozoa, common intestinal parasites, are phylogenetically diverse and are widely distributed

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across human and animal populations (4). Geographical disparities, socio-economic conditions, education levels, climate, and environmental factors play pivotal roles in the transmission of intestinal protozoan infections. Factors such as inadequate infrastructure, poor sanitation, and malnutrition particularly facilitate the spread of these infections (5-7). The prevalence of intestinal protozoan infections is notably high among individuals with gastrointestinal complaints. Intestinal parasites are associated with various symptoms, including diarrhea, abdominal pain, nausea, vomiting, weight loss, indigestion, bloating, and constipation (8).

Intestinal protozoa pose a significant risk to specific groups of people, including children, immunocompromised patients, and individuals with disabilities. However, due to the limited inclusion of disabled individuals in research related to intestinal protozoa, there remains insufficient information and awareness in this regard. Motor and/or cognitive developmental disorders observed in disabled individuals can adversely affect the neuromotor function of the digestive system, leading to insufficient digestion and malabsorption. Additionally, learning and comprehension difficulties, weaknesses in personal care skills, and challenges related to personal hygiene increase the susceptibility of disabled individuals to intestinal protozoan infections. Therefore, it is essential for disabled individuals to receive education on healthcare and personal hygiene and undergo regular screening tests (9,10).

The aim of this study is to determine the frequency of intestinal protozoa in disabled patients attending a rehabilitation center, thereby highlighting the significance of intestinal protozoa in individuals with disabilities.

METHODS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Ethical approval for this study was obtained from the Non-Invasive Clinical Research Ethics Committee of Van Yüzüncü Yıl University (date: 14.10.2022, number: 2022/10-09).

The study was conducted between November 10, 2022, and April 25, 2023. A total of 300 participants were included in the study: 200 patients receiving treatment at the Van Special Physioactive Special Education and Rehabilitation Center, and 100 individuals without intellectual or physical disabilities who visited the Parasitology Research Laboratory of Van Yüzüncü Yıl University Medical Faculty. Informed consent forms were obtained from all participants. For each collected sample, gender, age, and address information of the patient were recorded. Furthermore, the patient's gastrointestinal complaints, physical development status, and disability information were documented.

The collected stool samples were first examined macroscopically. Subsequently, a rice grain-sized portion of stool was taken from each sample using a stick, and these samples were microscopically examined for intestinal protozoa using the native-Lugol method. Preparations were examined under a 40X objective to identify protozoan cysts and trophozoites. Following this, the samples were stained using a modified acid-fast staining method to detect *Cryptosporidium* spp. and *Cyclospora cayetanensis*, and they were then examined under a light microscope using a 100X objective.

Statistical Analysis

The comparison of proportions for categorical variables was conducted using the Z (t) test. Additionally, the chi-square test was employed to determine the relationship between categorical variables. A statistical significance level of 5% was utilized for calculations, and the SPSS (version 21) and MINITAB (version 14) statistical software packages were employed for these calculations.

RESULTS

Demographic Findings

Out of the 200 participants included in the study, 50 had cerebral palsy (25%), 40 had hemiplegia (20%), 24 had spina bifida (12%), four had multiple sclerosis (2%), four had Parkinson's disease (2%), 20 had autism (10%), 20 had Down syndrome (10%), 28 had other physical disabilities (14%) including amputations, congenital hip dislocation, scoliosis, burns, and fractures, and 10 had other intellectual disabilities (5%) including learning disabilities, mental retardation, and attention deficit disorders.

The participants included in the study ranged in age from 2 to 65 years, with a mean age of 20.1±21.3. Among the patients, 136 (68%) were under 18 years of age, while 64 (32%) were 18 years or older. Among the patients aged 18 and above, 59.3% were found to have hemiplegia. Regarding the living locations of the patients, four lived in rural areas, eight lived in urban centers, and the remaining 188 lived in suburban neighborhoods. It was determined that the patients generally had low to moderate income levels.

Parasitic Findings

Out of the 200 patients included in the study, intestinal protozoa were detected in 82 individuals (41%), while among the 100 individuals in the control group, 9 (9%) had one or more intestinal protozoa. A statistically significant difference was observed in the statistical evaluation of parasite frequency between the patient group and the control group (p=0.001). The frequency of intestinal parasites was highest among spina bifida patients (83.3%), and the positivity rates for other patient groups are provided in Table 1.

In the patient group, one intestinal protozoa was detected in 62 patients (31%), while two intestinal protozoa were found in 20 patients (10%). Among the patient group, *Blastocystis* was identified in 36 patients (18%), *Cryptosporidium* spp. in 30 patients (15%), *Giardia intestinalis* in 18 patients (9%), *C. cayetanensis* in 10 patients (5%), and *Entamoeba coli* in 8 patients (4%). The distribution of parasites detected in patient subgroups is presented in Table 2.

In the control group, one intestinal protozoa was detected in seven (7%) individuals, while two intestinal protozoa were found in two (2%) individuals. Among the control group, *Blastocystis* was detected in six (6%) individuals, *E. coli* in three (3%) individuals, *G. intestinalis* in one (1%) individual and *Cryptosporidium* spp. in one (1%) individuals.

When examining protozoa frequency according to age groups, intestinal parasites were detected in 60 out of 136 patients (44.1%) who were under 18 years old and in 22 out of 64 patients (34.4%) who were 18 years and older. There was no statistically significant difference in protozoa frequency between the two

age groups (p=0.182) (Table 3). Additionally, there were no significant differences in the frequency of *Blastocystis* (p=0.537), *Cryptosporidium* spp. (p=0.480), *G. intestinalis* (p=0.311), and *E. coli* (p=0.324) between the two age groups based on statistical evaluation. However, since *C. cayetanensis* was only detected in patients under 18, a significant difference was observed in the frequency of this parasite among age groups (p=0.001).

When analyzing parasite frequency by gender, intestinal protozoa were identified in 38 out of 106 male patients (35.8%) and in 44 out of 94 female patients (46.8%). Statistical evaluation did not reveal a significant difference between gender and parasite frequency (p=0.114) (Table 3).

When examining the distribution of protozoa presence in patients according to clinical symptoms, statistical evaluation revealed significant relationships between parasite frequency and diarrhea (p=0.046), constipation (p=0.035), and loss of appetite (p=0.034) for each respective symptom. However, no significant associations were found between protozoa presence and symptoms such as weakness, abdominal pain, fever, nausea, vomiting, growth retardation, and weight loss based on the statistical analysis (Table 4).

DISCUSSION

Parasitic diseases can adversely impact the health of billions of people worldwide, leading to serious health problems. Intestinal parasites continue to pose a threat to public health in underdeveloped and developing countries. Factors such as low socio-economic status, inadequate or unbalanced dietary habits, crowded living environments, lack of clean water and safe food sources, poor environmental hygiene, and inadequate infrastructure contribute to the spread of parasites. Environmental factors such as temperature, precipitation, humidity, and soil type also influence the prevalence of parasites (11,12).

The immune system plays a significant role in the frequency and clinical course of intestinal parasitic infections. While some

Table 1. Prevalence of intestinal protozoa in patient and control groups

Group	Positive patients (%)	Negative patients (%)	p
Control group (n=100)	9 (9.0)	91 (91.0)	0.001
Patient group (n=200)	82 (41.0)	118 (59.0)	
Cerebral palsy (n=50)	14 (28.0)	36 (72.0)	0.001
Hemiplegia (n=40)	16 (40.0)	24 (60.0)	
Spina bifida (n=24)	20 (83.3)	4 (16.7)	
Multiple sklerosis (n=4)	0 (0)	4 (100)	
Parkinson (n=4)	2 (50.0)	2 (50.0)	
Other physical disabilities (n=28)	14 (50.0)	14 (50.0)	
Autism (n=20)	4 (20.0)	16 (80.0)	
Down syndrome (n=20)	8 (40.0)	12 (60.0)	
Other intellectual disabilities (n=10)	4 (40.0)	6 (60.0)	

Table 2. Distribution of detected parasites among patient subgroups

Subgroup	*Blastocystis* (%)	*Cryptosporidium* spp. (%)	*C. cayetanensis* (%)	*E. coli* (%)	*G. intestinalis* (%)
Cerebral palsy (50)	6 (12.0)	6 (12.0)	2 (4.0)	-	2 (4.0)
Hemiplegia (40)	8 (20.0)	4 (10.0)	-	2 (5.0)	4 (10.0)
Spina bifida (24)	6 (25.0)	6 (25.0)	8 (33.3)	4 (16.7)	2 (8.3)
Multiple sklerosis (4)	-	-	-	-	-
Parkinson's (4)	2 (50.0)	-	-	-	2 (50.0)
Other physical disabilities (28)	4 (14.3)	8 (28.6)	-	-	4 (14.3)
Autism (20)	4 (20.0)	2 (10.0)	-	-	2 (10.0)
Down syndrome (20)	4 (20.0)	4 (20.0)	-	-	2 (10.0)
Other intellectual disabilities (10)	2 (20.0)	-	-	2 (20.0)	-

Table 3. Distribution of parasite frequency by age and gender

Group		Positive case count (%)	Negative case count (%)	p
Age	≤18 (n=136)	60 (44.1)	76 (55.9)	0.182
	>18 (n=64)	22 (34.4)	42 (65.6)	
Gender	Male (n=106)	38 (35.8)	68 (64.2)	0.114
	Female (n=94)	44 (46.8)	50 (53.2)	

Table 4. Distribution of parasite presence in patients according to symptoms

Group		Positive case count (%)	Negative case count (%)	p
Weakness	Absent (n=146)	62 (42.5)	84 (57.5)	0.488
	Present (n=54)	20 (37.0)	34 (63.0)	
Diarrhea	Absent (n=192)	76 (39.6)	116 (60.4)	0.046
	Present (n=8)	6 (75.0)	2 (25.0)	
Abdominal pain	Absent (n=162)	66 (40.7)	96 (59.3)	0.878
	Present (n=38)	16 (42.1)	22 (57.9)	
Fever	Absent (n=150)	58 (38.7)	92 (61.3)	0.245
	Present (n=50)	24 (48.0)	26 (52.0)	
Nausea	Absent (n=192)	78 (40.6)	114 (59.4)	0.597
	Present (n=8)	4 (50.0)	4 (50.0)	
Vomiting	Absent (n=186)	76 (40.9)	110 (59.1)	0.884
	Present (14)	6 (42.9)	8 (57.1)	
Growth retardation	Absent (n=126)	52 (41.3)	74 (58.7)	0.919
	Present (n=74)	30 (40.5)	44 (59.5)	
Weight loss	Absent (n=108)	48 (44.4)	60 (55.6)	0.283
	Present (n=92)	34 (37)	58 (63)	
Constipation	Absent (n=120)	42 (35)	78 (65)	0.035
	Present (n=80)	40 (50.0)	40 (50.0)	
Loss of appetite	Absent (n=104)	50 (48.1)	54 (51.9)	0.034
	Present (n=96)	32 (33.3)	64 (66.7)	

parasites may not cause any issues in individuals with intact immune systems, they can lead to various symptoms and life-threatening severe infections in individuals with compromised or suppressed immune systems (13).

Numerous studies have been conducted to investigate parasite prevalence in immunocompromised patients. In Indonesia, a study found that 76% of 318 HIV/AIDS patients with chronic diarrhea were infected with parasites (14). In Iran, among 265 patients including hemodialysis patients, kidney transplant recipients, cancer patients, and HIV/AIDS patients, the prevalence of parasites was 11.7% (15). In Brazil, 61.6% of 73 cancer patients undergoing chemotherapy were found to be infected with parasites (16). In Egypt, 30% of 100 patients with diagnoses of malignancy, diabetes mellitus, or chronic kidney failure were infected with parasites (17). In Türkiye, among 80 children diagnosed with leukemia and with an absolute neutrophil count below 1000/mm^3, 41.2% had one or more intestinal parasites (15). Studies involving immunocompromised or immunosuppressed patients consistently reveal that intestinal parasites remain a significant health concern. It is believed that intestinal parasites could also pose a substantial health problem for intellectually and physically disabled individuals whose immune systems are compromised for various reasons, and who may not adhere to hygiene practices. It has been suggested that intellectual disability could directly affect hygiene habits, potentially facilitating the transmission of intestinal parasitic infections (18). However, there is a limited number of studies on the prevalence of intestinal parasites in intellectually and physically disabled individuals, and no studies related to this topic were found in Türkiye.

In Iran, three separate studies conducted in institutions with mentally disabled patients yielded the following results: In the first study, intestinal parasites were detected in 20.4% of 225 patients (19). In the second study, 26.1% of 119 mentally disabled patients were found to have intestinal parasites (20). In the third study, 54.7% of 126 mentally disabled patients were found to have one or more intestinal parasites (21). Similar findings were reported in other countries: In Egypt, 43.5% of 200 mentally disabled individuals had intestinal parasites (22), while in Thailand, 57.6% of 1086 mentally disabled individuals were affected (23). In Korea, the prevalence was 35.7% among 112 mentally disabled individuals (24), and in Italy, it was 23% among 550 mentally disabled individuals (25). In Ethiopia, 56.7% of 104 mentally disabled children had one or more intestinal parasites (26).

In a study conducted in Iran involving both physically and mentally disabled individuals, intestinal protozoa were detected in 12.3% of 196 participants. The prevalence of parasites was 9.2% among mentally disabled individuals and 2.5% among physically disabled individuals (27). Similar trends were observed in other regions: In Thailand, 38.46% of 52 physically disabled individuals were affected (28), while in Brazil, 8.3% of 156 physically disabled children had intestinal parasites (29).

This study investigated 200 patients with either mental and/or physical disabilities, with 41% showing the presence of intestinal protozoa. This rate aligns with previous studies (19-22) highlighting the significant health concern posed by intestinal parasites in disabled individuals. The findings suggest a higher frequency of intestinal parasites among mentally disabled individuals (20,21) compared to physically disabled individuals (27,29). Specifically, this study found a higher occurrence of intestinal parasites in physically disabled individuals who faced challenges in maintaining proper hygiene due to conditions like

spina bifida and hemiplegia, which hindered their ability to use lavatories comfortably. Moreover, conditions such as urinary incontinence, bowel fullness, and absorption disorders resulting from bowel dysfunction in spina bifida patients could potentially weaken the immune system. This study, for the first time, identified an 83.3% frequency of intestinal protozoa among spina bifida patients, underscoring the need to investigate intestinal protozoa in disabled individuals, especially those with spina bifida.

According to studies conducted in Türkiye, *G. intestinalis* is one of the most common pathogenic parasites. This parasite exhibits a wide distribution from temperate regions to tropical zones. While it is generally observed at rates of 2-5% in industrialized countries, this rate can range from 9% to 20-30% in developing countries (30). The prevalence of *G. intestinalis* in communities influences the positivity rates in disabled individuals. In studies conducted on disabled individuals in Iran, the prevalence ranged from 1.6% to 6.2% (19-21,27), 8% in Thailand (23), 8.5% in Egypt (22), and 0.9% in Korea (24). In this study, *G. intestinalis* was detected in 9% of patients, which is higher than in other studies. Considering factors such as the low to moderate income levels of the included patients, their predominantly residing in peripheral neighborhoods, and the widespread presence of *G. intestinalis* in the Van Region, we believe that the prevalence of *G. intestinalis* is higher in this study compared to other studies.

Blastocystis is a widely prevalent parasite worldwide, particularly observed more frequently in developing countries (31). In developed countries, the prevalence of *Blastocystis* is generally between 5% and 20%. In developing countries, however, the prevalence is over 60% (32). The prevalence of *Blastocystis* among disabled individuals varies between 4% and 25.4% in previous studies (19-22,27). In this study, *Blastocystis* was detected in 18% of disabled individuals. While this rate might be considered normal for the general population, it should not be overlooked in immunocompromised disabled individuals.

Cryptosporidium spp. species are among the leading causes of diarrhea in both immunocompromised and immunocompetent individuals. The prevalence of cryptosporidiosis varies between developed countries, where it ranges from 1% to 2%, and developing countries, where it can be between 3% and 20% (33). Individuals living in areas with poor nutrition, inadequate nutrition, suppressed immune systems, and low sanitation are at greater risk for cryptosporidiosis (34). Similarly, such conditions are often observed among individuals with intellectual and physical disabilities. Therefore, determining the prevalence of *Cryptosporidium* spp. among disabled individuals is crucial. Studies conducted on disabled populations have reported a prevalence of *Cryptosporidium* spp. of 23.5% in Egypt (22) and 1.7% in Iran (20). In this study, *Cryptosporidium* spp. was detected in 15% of cases. This finding underscores the significance of *Cryptosporidium* spp., an opportunistic protozoan, as a serious health concern for disabled individuals, emphasizing the necessity to consider this pathogen within this specific patient group.

Another opportunistic protozoan, *C. cayetanensis*, causes severe gastroenteritis in both immunocompetent and immunosuppressed individuals (35). Although cases of cyclosporidiosis are sporadic, they are reported worldwide and are particularly prevalent in tropical and subtropical countries (36). Given that it can lead to severe symptoms in individuals with weakened immune systems, determining the prevalence of *C. cayetanensis* among disabled individuals is of importance. In a study conducted on disabled patients, *C. cayetanensis* was detected at a rate of 7.5% in Egypt (22). In this study, a rate of 5% for *C. cayetanensis* was found, with 80% of the positive cases being spina bifida patients. This identified rate demonstrates that disabled individuals, especially those with spina bifida, are at risk for opportunistic protozoan infections.

Entamoeba coli has a global distribution and is commonly found in regions with inadequate sanitation conditions, particularly in rural areas (37). Various studies have investigated the prevalence of *E. coli* in disabled patients in different countries, and reports indicate varying rates of *E. coli* within this patient group. In a study conducted in Thailand, *E. coli* infection was detected at a rate of 23.1% (27), while in Iran, rates of 9.7%, 5.6%, and 10.1% were reported (19-21). In Egypt, the rate was 2.5% (22), and in Korea, it was 25% (24). In this study, a rate of 4% for *E. coli* was observed. Although *E. coli* is considered non-pathogenic, some studies suggest that it can disrupt the intestinal microbiota. Therefore, we believe that *E. coli* should not be overlooked, especially in disabled individuals who often have disrupted gut flora.

The studies conducted on disabled individuals have not found a statistically significant difference between parasite frequency and gender (19-23,28). Similarly, in this study, no statistically significant difference was observed between gender and parasite frequency.

Limited research conducted on disabled children indicates a higher risk of intestinal parasitic infection in this group. Particularly in cases of intellectual disability, these children may struggle to maintain proper personal hygiene practices, making them more susceptible to intestinal parasitic infections (29). In some studies involving disabled individuals, it has been reported that the frequency of intestinal parasites decreases with age (19,21,25). However, in a study conducted by Fentahun et al. (26), no significant difference was observed between age and intestinal parasite prevalence. Although the frequency of intestinal protozoa in patients under 18 years old was higher than in those over 18 years old in this study, the difference was not statistically significant. The lack of statistical difference between these two groups may be attributed to the fact that 59.3% of patients over 18 years old were individuals with hemiplegia who may not pay sufficient attention to personal hygiene.

Among the symptoms caused by intestinal parasites are nausea, vomiting, diarrhea, abdominal pain, growth retardation, loss of appetite, weight loss, and anemia. The long-term effects of these parasites can lead to serious complications. Especially in children, they can result in disturbances in nutrient absorption, inadequate nutrition, mental retardation, and irritability. These conditions can negatively impact the healthy growth and developmental processes of children, particularly causing deficiencies in both intellectual and physical development (38-40). However, it's worth noting that these symptoms are commonly observed in disabled individuals for various reasons. Consequently, attributing the symptoms in disabled individuals solely to intestinal parasites becomes challenging. In this study, a significant relationship was found between the presence of intestinal protozoa and symptoms such as diarrhea ($p=0.046$), constipation ($p=0.035$), and loss of appetite ($p=0.034$). On the other hand, there was no significant relationship observed between the presence of protozoa and symptoms such as weakness, abdominal pain, fever, nausea, vomiting, growth retardation, and weight loss.

Study Limitations

This study has several limitations. The first is that only native-Lugol and modified acid-fast staining method of stool samples were performed. Another limitation is that only disabled individuals registered in a single centre were included in the study.

CONCLUSION

In conclusion, this study reveals a high prevalence of intestinal protozoa, reaching up to 41%, among individuals with intellectual and/or physical disabilities. This high frequency underscores that intestinal protozoa pose a serious health concern for this specific patient population. Furthermore, it emphasizes the necessity of considering intestinal protozoa such as *Blastocystis*, *Cryptosporidium* spp., *G. intestinalis*, and *C. cayetanensis*, especially in individuals with intellectual and/or physical disabilities. The elevated risk of transmission of *Cryptosporidium* spp. may contribute to its high prevalence in these populations. The findings of our study underscore the significance of cryptosporidiosis once again. Taking into account the risk factors that predispose to infection, it is imperative to educate disabled individuals, caregivers, and parents about intestinal parasites. Additionally, increased attention to hygiene measures is essential to prevent the transmission of parasitic diseases, especially among individuals with conditions like spina bifida and hemiplegia.

***Information:** The results of the current study were summarized from a Master thesis of MS.

*Ethics

Ethics Committee Approval: Ethical approval for this study was obtained from the Non-Invasive Clinical Research Ethics Committee of Van Yüzüncü Yıl University (date: 14.10.2022, number: 2022/10-09).

Informed Consent: Informed consent was obtained from all participants involved in the study. Participation in this study was completely voluntary and anonymous.

Footnotes

*Authorship Contributions

Concept: H.Y., M.S., Design: H.Y., M.S., S.A., Data Collection or Processing: M.S., S.A., A.E., Analysis or Interpretation: M.S., S.A., A.E., Literature Search: M.S., S.A., Writing: M.S., S.A., H.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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Retrospective Evaluation of the Incidence of Cystic Echinococcosis in Humans Between 2012-2023 in Pakistan

Pakistan'da 2012-2023 Yılları Arasında İnsanlarda Kistik Ekinokokkoz Görülme Sıklığının Retrospektif Olarak Değerlendirilmesi

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ABSTRACT

Objective: Present study aimed to determine the demographic, epidemiological and pathological features of human cystic echinococcosis (CE) cases using patients' hospital based clinical history from 2012-2023.
Methods: The current retrospective study was conducted from June-December and aimed to investigate the incidence of human CE in Pakistan. A total of 74 surgically confirmed patients' data was retrieved from the hospital records. All epidemiological, radiological, histopathological and treatment characteristics of the patients were recorded.
Results: The results showed that the highest number of CE cases were recorded in 2019 (15/74, 20.2%), followed by 2018 (11/74, 14.8%). In age categories, the highest frequency of CE cases was recorded in the age group 11-20 (19/74, 25.7%), followed by 31-40 (18/74, 24.3%), 41-50, and 51-60 (9/74, 12.1%), respectively. Gender-wise findings showed that females were more infected (46/74, 62.2%) as compared to males (28/74, 37.83%). Among reported cases, most infected organs were liver (24/74, 32.4%) and the lungs (14/74, 18.9%), followed by the bone, bladder, and abdominal cavity (3/74, 4.1%), kidney, chest (2/74, 2.7%), while and others. Surgery was performed after echinococcal cyst detection by diagnostic imaging methods such as computed tomography, magnetic resonance imaging, or ultrasound. All patients received albendazole anthelminthic medication after their surgical procedures.
Conclusion: The present research reveals that CE is persistently endemic in Pakistan. Lack of knowledge and dedicated work on behalf of public healthcare and veterinarians to control CE pose a challenge in Pakistan. A lot of research and strong management programs are required to combat the disease.
Keywords: Cystic echinococcosis, hydatid cyst, retrospective, surgical, Pakistan

ÖZ

Amaç: Bu çalışma, 2012-2023 yılları arasında hastane kayıtları kullanılarak insan kistik ekinokokkoz (KE) olgularının demografik, epidemiyolojik ve patolojik özelliklerini belirlemeyi amaçlamıştır.
Yöntemler: Bu retrospektif çalışma Haziran-Aralık ayları arasında yürütülmüş olup, Pakistan'da insan KE insidansını araştırmayı hedeflemiştir. Cerrahi olarak doğrulanmış toplam 74 hastaya ait veriler hastane kayıtlarından elde edilmiştir. Tüm epidemiyolojik, radyolojik, histopatolojik ve tedaviye ilişkin özellikler kayıt altına alınmıştır.
Bulgular: Sonuçlara göre en fazla KE olgusu 2019 yılında kaydedilmiştir (15/74, %20,2), bunu 2018 yılı izlemiştir (11/74, %14,8). Yaş gruplarına göre en fazla olgu 11-20 yaş grubunda görülmüş (19/74, %25,7), bunu sırasıyla 31-40 yaş (18/74, %24,3), 41-50 ve 51-60 yaş grupları (her biri 9/74, %12,1) takip etmiştir. Cinsiyete göre değerlendirildiğinde, KE olgularının kadınlarda (46/74,

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Türkiye PARAZİTOLOJİ Dergisi

%62,2) erkeklere (28/74, %37,83) kıyasla daha yüksek olduğu saptanmıştır. En sık etkilenen organlar karaciğer (24/74, %32,4) ve akciğerler (14/74, %18,9) olmuş, bunu kemik, mesane ve karın boşluğu (3/74, %4,1), böbrek, göğüs (2/74, %2,7), ve diğerleri. takip etmiştir. Ekinokok kistleri, bilgisayarlı tomografi, manyetik rezonans veya ultrason gibi görüntüleme yöntemleri ile tespit edildikten sonra cerrahi müdahale uygulanmıştır. Tüm hastalara cerrahiyi takiben antihelmintik olarak albendazol verilmiştir.

Sonuç: Bu araştırma, KE'nin Pakistan'da sürekli endemik olduğunu ortaya koymaktadır. Halk sağlığı uzmanları ve veteriner hekimlerin KE'yi kontrol altına alma konusundaki bilgi eksikliği ve yetersiz çalışmaları, ülkede hastalığın kontrolünü zorlaştırmaktadır. Bu hastalıkla mücadele için daha fazla araştırma ve etkili kontrol programlarına ihtiyaç vardır.

Anahtar Kelimeler: Kistik ekinokokkoz, hidatik kist, retrospektif, cerrahi, Pakistan

INTRODUCTION

Cystic echinococcosis (CE) is a global zoonotic disease in humans and livestock caused by *Echinococcus granulosus* tapeworms. Globally, CE has been detected in populations on every continent excluding Antarctica, while alvelolar echinococcosis (AE) is confined to the northern hemisphere (1). An estimated 1.2 million people worldwide are affected by human CE, and 1 to 3 million disability-adjusted life years are lost worldwide, although these numbers are probably underestimated (1-3). *E. granulosus* ranked second among the top 8 food-borne parasites of worldwide public health significance in 2012, according to a joint FAO/World Health Organization (WHO) expert committee (4).

The life cycle of parasites involves two mammalian hosts: A definitive canine host in which parasite adult form develops and an intermediate host (domestic and wild ungulates) harboring the larval stage of parasite (5), while humans are accidental and dead-end host of parasite (6). Humans are infected by accidental ingestion of parasite eggs via contaminated food and water (7,8).

Human echinococcosis is a worldwide common disease (9). Among all the species of *Echinococcus*, two are of public health concern: CE caused by *Echinococcus granulosus* sensu lato and *Echinococcus multilocularis*, the causative agent of alveolar echinococcosis, due to their wide distribution and their medical and economic impact (10). *Echinococcus granulosus* s.l. is a cosmopolitan species, as it is widely distributed (1) causing CE echinococcosis in humans, and has a broad range of intermediate hosts (5).

The primary stages of CE are always asymptomatic until complications occur, which depend on the size of the cyst, numbers, localization, and CE stages. Cyst rupture by chance and subsequently cyst contents spilling could be the outcome of secondary infection (11). Disruption of the cysts can be lethal due to anaphylactic shock. *Echinococcus* can infect any organ, but the most common infected organs are the liver and lungs. Other organs like the spleen, kidney, muscles, and bones are less frequently infected by CE (12).

Numerous imaging techniques such as ultrasound (US), radiography, computed tomography (CT), and magnetic resonance imaging (MRI) as well as laboratory testing techniques like antibody, antigen, and cytokine detection are used for CE diagnosis (13). Early detection of CE is very difficult because of the complicated life cycle of *Echinococcus granulosus* and the fact that the disease develops very slowly after infection (14), but there are several factors on which the detection of CE depends. These factors include cyst size, multiple or single cyst, organ that is infected, cyst localization, integrity of the cyst, and immunity of the infected individual (15). Recently, researchers have introduced various techniques for the detection of *Echinococcus* spp. These molecular techniques include nested polymerase chain reaction (PCR), real-time quantitative PCR, multiplex PCR, and nucleic acid isothermal amplification technology (14).

For treatment of CE, percutaneous therapy, surgery, and anti-infective medication are viable alternatives; however, they are image-based, stage-specific methods. In some cases, a wait-and-watch strategy is recommended, based on the cyst's stage (16). In 2003 a standardized ultrasound-based classification for CE was created by WHO Informal Working Group on Echinococcosis, providing visibility of all cyst stages (17). This system further classifies cysts according to clinical groups to aid in treatment options according to stage of disease (e.g., operation, medication, percutaneous, observation) (18). It enables individualized, economical treatment depending on the character of the cyst and the parents features and local conditions. Nevertheless, such a strategy is not used in many endemic countries resulting in non-optimized care and unnecessary health costs (19).

Research assessing CE epidemiology in Central Asia confirmed that *E. granulosus* is a public health concern. Pakistan's economy is primarily based on the animal husbandry, and because of poor hygienic conditions, CE continues to be a health concern for humans (20). Even though echinococcosis is endemic in neighboring countries, there is paucity of information regarding the circulating genotypes of *E. granulosus* in Pakistan (21). Keeping facts about CE in mind, the present study was designed to determine the demographic, epidemiological and pathologic features of human CE cases using patients' hospital-based history from 2012-2023.

METHODS

Study Area

The present retrospective study was conducted in Islamabad, Pakistan. The capital city of Pakistan, Islamabad, is located at 33.43°N 73.04°E, near the base of the Margalla Hills and on the northern edge of the Pothohar Plateau. The entire area of Islamabad city is 906.50 km^2, of which 220.15 km^2 are urban and 466.20 km^2 are rural. It is situated in Pakistan's northern area, which has a subtropical humid climate with cold winters, hot summers, and a monsoon season (22).

Study Duration and Sampling Procedure

A total of 74 clinical reports on human CE were collected from the Pakistan Institute of Medical Sciences (PIMS) hospital located in Islamabad from June 2021 to December 2023. The histopathology department of the hospital postoperatively confirmed the patients' clinical data from 2012 to 2023, which was also included in this investigation. Samples were collected from the hospital with the written informed consent of patients and hospital administration for sample characterization.

The epidemiological (age and gender), histopathological (size, cyst location, number of cysts, macroscopic and microscopic) and treatment data of each patient were retrieved from hospital records. According to the medical records, during CT scans and biopsies examination, only infected organs with cysts were removed from the patient body and preserved in block form (formalin-fixed paraffin-embedded tissue) so that they could be studied further.

Statistical Analysis

All collected data were compiled in a Microsoft 365 Excel database to calculate the frequency of human CE cases. Statistical analysis was performed using SPSS (Version 26.0). Chi-square test (X^2) was used to evaluate the association between different groups and variables. The p-value <0.05 was considered statistically significant.

RESULTS

A total of 74 patients with CE were diagnosed and surgically treated in PIMS hospital, Islamabad during 2012-2023 and their age and gender wise distribution is shown (Figure 1). The clinical data of the CE patients provided the framework for the current findings. The findings showed that a highest number of CE patients were reported in the year 2019 (15/74, 20.2%), followed by 2018 (11/74, 14.9%), 2016 (8/74, 10.8%), 2012 and 2022 (7/74, 9.5%), 2013 and 2015 (5/74, 6.8%), 2017 and 2021 (4/74, 5.4%), 2014 and 2020 (4/74, 5.4%), and 2023 2/74 (2.7%). The statistical analysis showed that CE cases were statistically significant across different study years (Table 1). The highest number of positive cases was recorded in the age group 11-20 (19/74, 25.7%), followed by 31-40 (18/74, 24.3%), 41-50 (9/74, 12.1%), 51-60 (9/74, 12.1%), and 21-30 (7/74, 9.5%) (Figure 2).

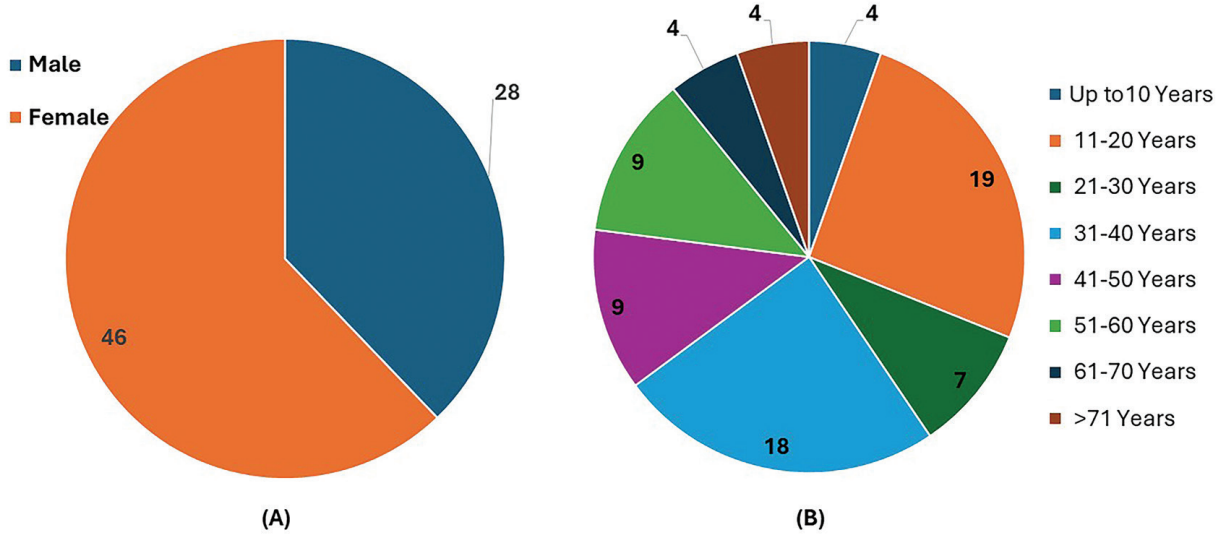

Figure 1 (A, B). Gender and age wise distribution of CE patients
CE: Cystic echinococcosis

Table 1. Year-wise frequency and chi-square analysis of human CE cases in PIMS Hospital, Islamabad, Pakistan

S. no	Year	Total	Frequency (%)	Statistical analysis (X^2)
1	2012	7	9.5	X^2=25.24 df=11 p<0.008
2	2013	5	6.8	
3	2014	3	4.0	
4	2015	5	6.8	
5	2016	8	10.8	
6	2017	4	5.4	
7	2018	11	14.9	
8	2019	15	20.2	
9	2020	3	4.0	
10	2021	4	5.4	
11	2022	7	9.5	
12	2023	2	2.7	

CE: Cystic echinococcosis, PIMS: Pakistan Institute of Medical Sciences

The frequency of human hydatid cyst disease was 37.8% in males and 62.2% in females, respectively. The results showed that more CE cases were observed in females (46/74, 62.2%) compared to males (28/74, 37.83%) (Figure 3). Statistical analysis showed there is a significant association between the frequencies of both the genders (Table 2).

In the present study, hydatid cyst disease was observed in different organs. The study showed that in most CE patients, the liver was the most infected organ (24/74, 32.4%), followed by the lungs (14/74, 18.9%), bone, bladder, and abdominal cavity (3/74, 4.1%), kidney, chest (2/74, 2.7%), while others (Table 3, Figure 4). Statistical analysis ($p<0.000$) showed that there is a significant association across different organs (Table 3). With respect to organ involvement, multiple organ involvement was less (19/74, 25.6%), as compared to single organ involvement (55/74, 74.3%). Table 4 shows that most cysts (9/74, 11.0%) had a diameter of less than 4 cm, followed by 7-8 cm (8/74, 11.0%).

Surgery was performed after echinococcal cyst detection by diagnostic imaging methods such as CT, MRI, or US. All patients received albendazole anthelminthic medication after their surgery. Albendazole is usually recommended for CE patients who underwent surgery in Pakistan.

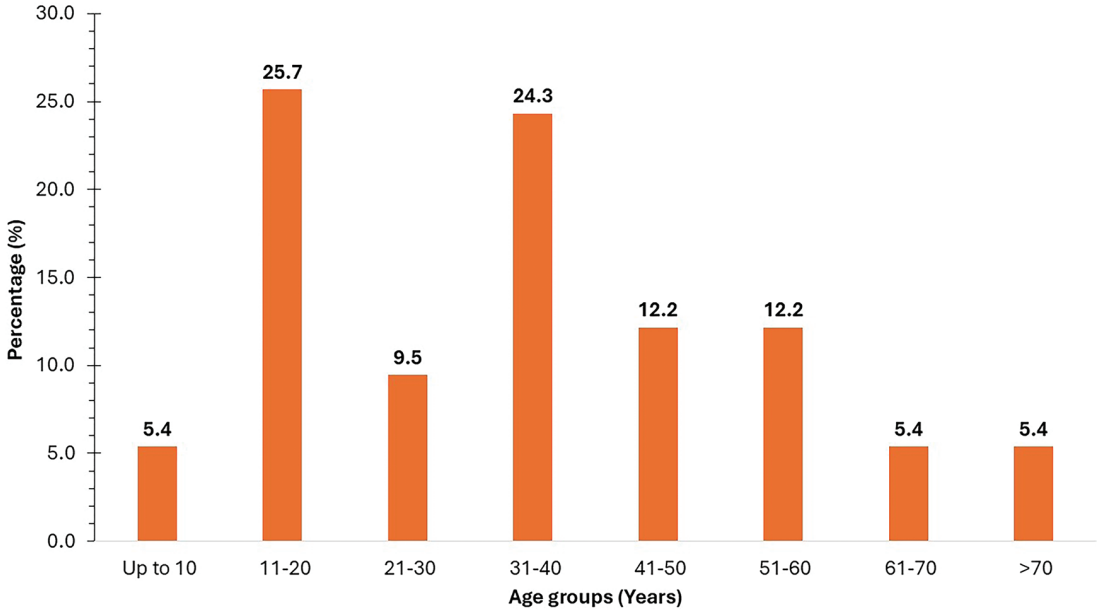

Figure 2. Age wise frequency (%) of human hydatid cyst disease

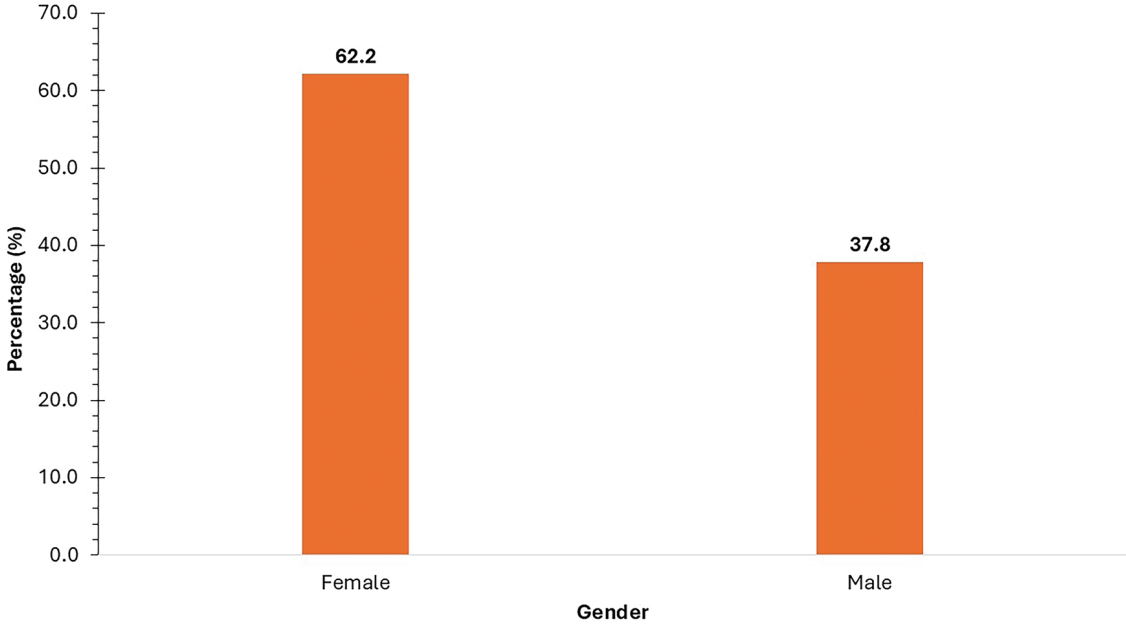

Figure 3. Gender wise frequency (%) of human hydatid cyst disease

Table 2. Gender-wise frequency of human CE cases

S. no	Gender	Total	Frequency (%)	Statistical analysis (x^2)
1	Female	46	62.2	X^2=4.38; df=1; p<0.03
2	Male	28	37.8	

CE: Cystic echinococcosis

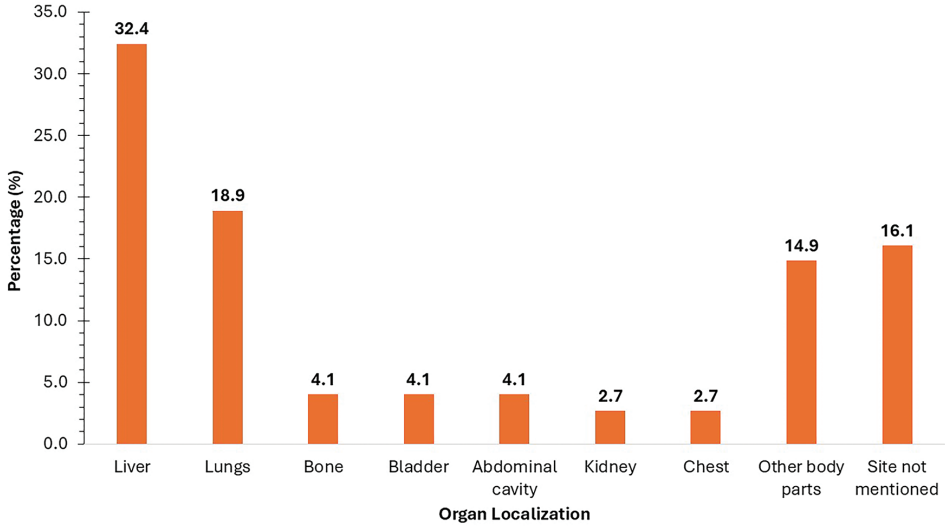

Figure 4. Organ wise localization of human CE cases
CE: Cystic echinococcosis

Table 3. Site of involvement of the cyst

S. no	Organs	Total	Frequency (%)	Statistical analysis (X)
1	Liver	24	32.4	X^2=58.56 df=8 p<0.000
2	Lung	14	18.9	
3	Kidney	2	2.7	
4	Bone	3	4.1	
5	Chest	2	2.7	
6	Bladder	3	4.1	
7	Abdominal cavity	3	4.1	
8	Not mentioned	12	16.1	
9	Others	11	14.9	

Table 4. Size-wise distribution of single and multiple cysts in human hydatid cyst cases

S. no	Size (cm)	Number of cysts		
		Multiple	Single	Total
1	≤4	3 (16.0%)	6. (11.0%)	9 (12.0%)
2	5-6	0 (0%)	5 (9.0%)	5 (7.0%)
3	7-8	1 (5.0%)	7 (13.0%)	8 (11.0%)
4	9-10	1 (5.0%)	3 (5.0%)	4 (5.0%)
5	11-12	1 (5.0%)	2 (4.0%)	3 (4.0%)
6	13-14	0 (0%)	1 (2.0%)	1 (1.0%)
7	15-16	1 (5.0%)	1 (2.0%)	2 (3.0%)
8	17-18	0 (0%)	0 (0%)	0 (0%)
9	19-20	0 (0%)	0 (0%)	0 (0%)
10	≥21	0 (0%)	1 (2.0%)	1 (1.0%)
11	Not mentioned	12 (63.0%)	29 (52.0%)	41 (55.0%)

DISCUSSION

Echinococcosis is a global neglected tropical zoonotic disease that affects both human and animals (23). CE is a rapidly growing global health threat, present in many parts of the world, including Pakistan (11). Better human clinical management, CE control programs, by-product management at slaughterhouses especially disposal of contaminated organs may reduce the infection of *Echinococcus* spp. Even though Pakistan's health care system has experienced major changes and advancements recently, there are still many issues, such as inadequate management, a scarcity of trained staff, and a lack of scrutiny of health policy. Main cause of the CE in animals in Pakistan maybe due to socio-cultural practices like home slaughtering, animals' organs feed to the dogs, not deworming of pets and huge number of stray dogs that may support the transmission of the parasite (24). Such ideal conditions increase the probability of contact and may lead to parasitic infection. Humans can become infected with the disease through the accidental ingestion of parasitic eggs excreted by the faeces of definitive hosts such as dogs, foxes, and other canids (15).

The findings of our study revealed variations in the frequency of human hydatid cysts. The highest number of patients were diagnosed in 2019 (20.2%), followed by 2018 (14.8%). The results in accordance with precious published data reports the similar type of observations with higher CE cases in 2019 followed by 2018 (25). The study showed that the most common age group for CE was 11-20 followed by 31-40 and then 41-50 age group. These findings are consistent with past retrospective research that showed increased rates of CE infections in similar age groups (25-28). This study identified a 5.4% infection rate in children under 10 years old. Variations in the distribution of age groups can be due to the asymptomatic nature of the disease, making diagnosis difficult and challenging.

The findings of the current study showed that the incidence of echinococcosis was higher in the females as compared to males, and these results are consistent with study reported in 2022 in the Khyber Pakhtunkhwa region (25). Same results were observed in previous studies (8,26,27) indicating that male were less affected than females. While our findings are contrary with (24), where CE frequency in females were less than males. Gender disparities and social constraints in the country like Pakistan may have influenced the current study's findings towards females.

In the current study, we examined the frequency of human hydatid cyst disease across several organs. The results revealed that the highest frequency rate was observed in the liver followed by the lungs. These results are concord with many previous studies (25,28). Additionally, rare anatomical sites such as kidneys, chest, bones, bladder, abdominal cavity, and ovaries were reported to be infected in the current investigation. This investigation regarding rare infection sites with an extremely low occurrence rate also correlates with published report (29).

CONCLUSION

CE is a zoonotic and neglected tropical disease that has a high prevalence in Pakistan and poses a serious threat to public health. Although there is a paucity of data on the prevalence of CE in Pakistan, the current retrospective research attracts the consideration to CE as a public health concern. Additionally, medical histories remain usually inadequate, as records are unstructured and lack all CE treated cases in the hospitals. Therefore, the current research serves as an initial step in reducing the information gap regarding CE cases in the capital city, Islamabad, Pakistan. Present study will serve as a baseline for evaluating human CE infection and might help in developing preventative strategies in Pakistan.

*Ethics

Ethics Committee Approval: The Departmental Ethics Review Board (ERB) at the COMSATS University Islamabad (CUI), Pakistan has approved the research under no. CUI/Bio/ERB/2021/43.

Informed Consent: Samples were collected from the hospital with the written informed consent of patients and hospital administration for sample characterization.

Footnotes

*Authorship Contributions

Concept: H.S., H.K., H.A., Design: H.S., H.K., H.A., Data Collection or Processing: H.S., N.K., Analysis or Interpretation: A.K., As.K, G-J.Y., R.M.K.S., Literature Search: H.S., H.K., Writing: H.S., H.K., A.K., As.K, G-J.Y., R.M.K.S., N.K., H.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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Investigation of Antiparasitic Effect of *Juniperus communis* L. Fruits Extracts

Juniperus communis Meyve Ekstrelerinin Antiparaziter Etkisinin Araştırılması

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ABSTRACT

Objective: *Juniperus communis* (*J. communis*) (common juniper) is a plant that has been used for medicinal purposes for centuries. This study aims to evaluate the antiparasitic effects of ethanol, methanol, chloroform, and water extracts of *J. communis* fruits against *Plasmodium falciparum*, *Leishmania tropica*, *Trichomonas vaginalis*, and *Blastocystis*.

Methods: The antiparasitic activities of fruit extracts prepared at room temperature using the shaking maceration method were tested against *Plasmodium falciparum* using the ring stage survival test, and against *Leishmania tropica*, *Trichomonas vaginalis*, and *Blastocystis* using the broth microdilution method.

Results: The chloroform extract of *J. communis* fruits was found to be effective on *Plasmodium falciparum*, *Leishmania tropica*, *Trichomonas vaginalis*, and *Blastocystis* parasites at concentrations of 15, 10, 30 and 30 µg/mL, respectively.

Conclusion: The chloroform extract of *J. communis* fruits has shown strong antiparasitic activity against the investigated parasite species. These findings support the plant's antiparasitic potential and hold promise for future medical applications. Especially its effectiveness against metronidazole-resistant *Trichomonas vaginalis* strains is important for the development of alternative treatment options. This study highlights the potential use of *J. communis* as a medicinal plant and will contribute to the literature on research related to the isolation and structural determination of its active compounds

Keywords: Antiparasitic, *Juniperus communis*, *Blastocystis*, *Juniperus communis*, *Leishmania*, *Plasmodium*, *Trichomonas*

ÖZ

Amaç: *Juniperus communis* (*J. communis*) (adi ardıç), tıbbi amaçlarla uzun süredir kullanılan bir bitkidir. Bu çalışma, *J. communis* meyvelerinden hazırlanan etanol, metanol, kloroform ve su ekstrelerinin *Plasmodium falciparum*, *Leishmania tropica*, *Trichomonas vaginalis* ve *Blastocystis* üzerindeki antiparaziter etkilerini değerlendirmeyi amaçlamaktadır.

Yöntemler: Çalkalamalı maserasyon yöntemi oda sıcaklığında hazırlanan meyve ekstrelerinin antiparazitik aktiviteleri, *Plasmodium falciparum*'a karşı ring stage survival testi ile *Leishmania tropica*, *Trichomonas vaginalis* ve *Blastocystis*'e karşı aktiviteleri ise sıvı mikrodilüsyon yöntemi ile araştırılmıştır. Her bir parazit türü üzerinde ekstrelerin farklı konsantrasyonlarda etkinlikleri test edilmiştir.

Bulgular: *J. communis* meyvelerinin kloroform ekstresinin, *Plasmodium falciparum*'a karşı 15 µg/mL, *Leishmania tropica*'ya karşı 10 µg/mL, *Trichomonas vaginalis*'e karşı 30 µg/mL ve *Blastocystis*'e karşı ise 30 µg/mL konsantrasyonda etkili olduğu gözlenmiştir.

Sonuç: *J. communis* meyvelerinin kloroform ekstresi, araştırılan parazit türlerine karşı güçlü antiparaziter etkinlik göstermiştir. Bu bulgular, bitkinin antiparaziter potansiyelini desteklemekle birlikte gelecekteki tıbbi uygulamalar için umut vadetmektedir. Özellikle metronidazol dirençli *Trichomonas vaginalis* suşları üzerinde etkili olması, alternatif tedavi seçeneklerinin geliştirilmesi açısından önemlidir. Bu çalışma, *J. communis*'un tıbbi bitki olarak kullanım potansiyelini ortaya koymakta olup, aktivite eşliğinde izolasyon ve yapı tayini ile ilgili araştırmalar için literatüre katkı sağlayacaktır.

Anahtar Kelimeler: Antiparazitik, *Juniperus communis*, *Blastocystis*, *Juniperus communis*, *Leishmania*, *Plasmodium*, *Trichomonas*

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Malaria remains one of the greatest threats to human health. Among the *Plasmodium* species that cause this disease, *Plasmodium falciparum* (*P. falciparum*) and *P. vivax* are responsible for high mortality rates globally (1). The development of resistance to existing drugs necessitates the discovery of cost-effective and fast-acting new therapeutics (2-4). Similarly, leishmaniasis, a disease caused by *Leishmania* species, affects millions of people worldwide. The emergence of drug resistance in leishmaniasis treatment highlights the need for new drug candidates (5,6).

Trichomonas vaginalis (*T. vaginalis*) is a protozoan that is sexually transmitted and widely prevalent worldwide. The increasing prevalence of metronidazole-resistant strains underscores the necessity for investigating alternative treatment approaches (7).

The prevalence of *Blastocystis* ranges from 30% to 60% in developing countries. Individuals with immunosuppression and those in close contact with animals have been identified as being at particularly high risk of infection. *In vitro* and *in vivo* studies have reported its potential association with various gastrointestinal disorders and its significant role in irritable bowel syndrome (8).

Antimicrobial resistance is classified as one of the top 10 global health threats, requiring global collaboration to combat its life-threatening consequences. This situation prioritizes the discovery of new and effective antimicrobial agents. Accordingly, plant metabolites, such as terpenes, phenolic compounds, and alkaloids, have been widely studied for their antibacterial, antiviral, antifungal, and antiparasitic properties (9).

Juniperus communis (*J. communis*) (common juniper) is a plant historically used for medicinal and therapeutic purposes. In recent years, studies have investigated the antimicrobial, anticancer, and antiparasitic effects of different extracts of this plant (10).

This study aims to evaluate the antiparasitic activities of ethanol, methanol, chloroform, and water extracts of *J. communis* against various parasites, thereby exploring the potential of natural products in medical applications. The findings obtained will enhance our understanding of this plant's antiparasitic properties and guide future research.

METHODS

Ethical Approval

This research was approved by the Ethics Committee of the Faculty of Medicine, Manisa Celal Bayar University (approval date: 29/03/2023; approval number: 20.478.486/1773).

Preparation of *J. communis* Extracts

Ethanol, methanol, chloroform, and water extracts of *J. communis* fruits were obtained by the maceration technique. 5 g of ground fruits were stirred with 100 mL of solvent and macerated at room temperature for 24 hours. Each extract solution was filtered, and the solvent was evaporated under reduced pressure in a rotary evaporator until dryness. The extracts were stored at -20 °C until use.

Parasite Isolates

The parasite strains used in this study, *P. falciparum* (3D7), *Leishmania tropica* (MHOM/TR/2012/CBCL-LT), *T. vaginalis* (ATCC-50143), and *Blastocystis*, were obtained from the Parasite Bank of the Faculty of Medicine, Manisa Celal Bayar University.

In vitro Cultivation of *P. falciparum* and Screening of Extracts

The cultivation of the *P. falciparum* 3D7 strain was performed using a specialized medium prepared with 10.43 g of RPMI 1640, 25 mL of 1 M HEPES solution, 2 g of $NaHCO_3$, 0.5 mL of gentamicin solution, 0.272 g of hypoxanthine, and 5 g of albumax II.

A suspension containing 1% parasitemia was distributed into 96-well microplates. The microplates were placed in a chamber (microaerophilic incubation environment) with a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. The chamber was incubated at 37 °C for 2.5 hours.

Following incubation, plant extracts were added to the parasite suspension in the microplates at final concentrations ranging from 250 µg/mL to 2.5 µg/mL, and the plates were incubated for 6 hours. After incubation, the microplates were washed, fresh medium was added, and the plates were incubated for 66 hours.

At the end of the incubation, thin smears were prepared from the microplate wells. The smears were stained with Giemsa stain and examined under a light microscope (11-14).

In vitro Cultivation of *Leishmania tropica* and Screening of Extracts

The *Leishmania tropica* isolate MHOM/TR/2012/CBCL-LT, which was isolated in Türkiye and stored in liquid nitrogen, was thawed under appropriate conditions and cultured in Novy-MacNeal-Nicolle (NNN) medium. Promastigotes grown in the NNN medium were subsequently inoculated into RPMI-1640 (Roswell Park Memorial Institute medium 1640) medium containing 10% fetal bovine serum. The growth status of the parasites was monitored every other day after inoculation. For the experiments, *L. tropica* promastigotes in the logarithmic phase, reaching a density of 10^6 promastigotes/mL, were used.

The efficacy of *J. communis* extracts against *L. tropica* promastigotes was evaluated using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) kit (15-17).

In vitro Cultivation of *T. vaginalis* and Screening of Extracts

The *T. vaginalis* strain ATCC-50143 (metronidazole-resistant), stored in liquid nitrogen, was thawed under appropriate conditions and cultured in a Trypticase-Yeast Extract-Maltose medium. The growth intensity of the parasites was assessed by checking the media on consecutive days. For the experiments, *T. vaginalis* trophozoites in the logarithmic phase, reaching a density of 10^4 trophozoites/mL, were used.

The efficacy of *J. communis* extracts against *T. vaginalis* was evaluated using the microdilution method (7).

In vitro Cultivation of *Blastocystis* and Screening of Extracts

The *Blastocystis* strain, stored in liquid nitrogen, was thawed under appropriate conditions and transferred into tubes containing modified Iscove's Dulbecco's Medium supplemented with 10% inactivated horse serum. The tubes were incubated in an anaerobic environment at 35 °C. The growth status of the parasites was monitored every 2-3 days. For the experiments, *Blastocystis* parasites in the logarithmic phase, reaching a density of 10^5 parasites/mL, were used.

The efficacy of *J. communis* extracts against *Blastocystis* was evaluated using the microdilution method (18-20).

Statistical Analysis

During all *in vitro* experiments, statistical analyses were conducted to compare the effects of different *J. communis* fruit extracts (ethanol, methanol, chloroform, and water) on *P. falciparum*, *L. tropica*, *T. vaginalis*, and *Blastocystis*. The efficacy of the extracts was evaluated based on IC_{50} and LD values. Data analysis was performed using the chi-square test. All statistical analyses were done using SPSS (Statistical Package for the Social Sciences) software (version 21).

RESULTS

In vitro Screening of *P. falciparum* for Active Extracts

Examination of Giemsa-stained preparations revealed that the chloroform extract of *J. communis* fruits eliminated parasites at a concentration of 15 µg/mL. In contrast, parasites were observed in all dilutions of the other extracts.

In vitro Cultivation of *Leishmania tropica* and Screening of Active Extracts

The antileishmanial activities of *J. communis* extracts were evaluated at dilutions ranging from 250 µg/mL to 2.5 µg/mL. The IC_{50} value of amphotericin B was determined to be 0.06 µM. The IC_{50} values for the ethanol, methanol, and water extracts of *J. communis* were >250 µg/mL, while the IC_{50} value for the chloroform extract was determined to be 10 µg/mL.

In vitro Cultivation of *T. vaginalis* and Screening of Active Extracts

The IC_{50} values representing the antitrichomonal activities of the extracts were evaluated. The IC_{50} values for the ethanol, methanol, and water extracts of *J. communis* fruits were >250 µg/mL, whereas the IC_{50} value for the chloroform extract was determined to be 30 µg/mL.

In vitro Cultivation of *Blastocystis* and Screening of Active Extracts

The activities of *J. communis* extracts against *Blastocystis* were assessed at dilutions ranging from 250 µg/mL to 2.5 µg/mL. The lethal concentration (LD) values for the ethanol, methanol, and water extracts were found to be >250 µg/mL, whereas the LD value for the chloroform extract was determined to be 30 µg/mL.

Statistical Analysis

According to the chi-square test, a significant difference was observed between the chloroform extract and the other extracts in the *in vitro* efficacy against *P. falciparum*, *L. tropica*, *T. vaginalis*, and *Blastocystis* ($p<0.05$).

DISCUSSION

Juniperus species have long been used in various cultures for their antiparasitic properties (21). Similarly, the leaves and fruits of *Juniperus oxycedrus* (prickly juniper) have been applied topically to treat parasitic diseases (22). In this study, the ethanol, methanol, chloroform, and water extracts of *J. communis* were comprehensively evaluated for their *in vitro* effects on *P. falciparum*, *L. tropica*, *T. vaginalis*, and *Blastocystis*. The results indicate that this plant exhibits antiparasitic properties.

The antimalarial potential of *Juniperus* species has been particularly investigated for *J. communis*. Essential oils derived from *J. communis* have been reported to be tested against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. Analyses using Gas Chromatography and Gas Chromatography-Mass Spectrometry have shown that the essential oils contain common components such as α-pinene, although the specific components vary between oils. However, it has been reported that the essential oil of *J. communis* exhibits limited antimalarial activity, with IC_{50} values exceeding 1000 µg/mL, indicating low efficacy in inhibiting parasite growth (23).

In our study, the chloroform extract of *J. communis* fruits demonstrated significant activity against *P. falciparum*. The chloroform extract exhibited a completely parasiticidal effect at a concentration of 15 µg/mL. However, the low efficacy of the ethanol, methanol, and water extracts suggests that these compounds have limited antimalarial activity.

In addition, *Juniperus procera*, a close relative of *Juniperus communis*, has been reported to exhibit notable antimalarial and antileishmanial properties. The n-hexane fraction of the ethanol extract of *Juniperus procera* was found to exhibit the most prominent activity among the tested fractions. Various diterpenes, such as abieta-7,13-diene, were isolated from this fraction, and this compound demonstrated moderate antimalarial activity against *P. falciparum* D6 and W2 clones, with IC_{50} values of 1.9 and 2.0 µg/mL, respectively. The IC_{50} values of the crude n-hexane fraction were 5.8 and 4.4 µg/mL, suggesting potential synergistic effects among the components of the extract or the presence of additional potent antimalarial compounds (24).

More comprehensive studies on *Juniperus excelsa* have revealed its significant antileishmanial effects. A study evaluating the efficacy of Greek juniper leaf and fruit extracts against leishmaniasis reported high antileishmanial activity. The petroleum ether and chloroform fractions demonstrated particularly strong activity, showing high efficacy even at low concentrations. These findings highlight the potential of Greek juniper extracts as effective antileishmanial agents (25). In our study, the high IC_{50} values of ethanol, methanol, and water extracts against *Leishmania tropica* promastigotes indicate weak antileishmanial activity for these extracts. However, the chloroform extract exhibited strong activity at a concentration of 10 µg/mL.

A comprehensive study conducted in 2013 at Shiraz University of Medical Sciences evaluated the antileishmanial activities of *Juniperus excelsa* leaf and fruit extracts, as well as leaf fractions, against *Leishmania major* in both *in vitro* and *in vivo* models. The leaf extract was reported to exhibit the highest efficacy (IC_{50}: 0.97±3.53 mg/mL), and the ethyl acetate fraction showed significant activity (IC_{50}: 1.95±5.30 mg/mL). In the *in vivo* study, a significant reduction in lesion size was observed in the test group compared to the control group ($p<0.05$), suggesting that *Juniperus excelsa* could contribute to antileishmanial therapy (26).

Additionally, a placebo-controlled clinical trial evaluated the efficacy of a topical formulation of *Juniperus excelsa* leaf extract for the treatment of cutaneous leishmaniasis. Seventy-two patients were treated with the extract in a placebo-controlled manner for three months, with cryotherapy administered as standard treatment. Weekly assessments showed that 82% of patients in the extract group achieved complete recovery, compared to 34%

in the placebo group, demonstrating a significant difference ($p<0.001$). The recovery time was also shorter in the extract group ($p=0.04$), with no major side effects reported other than mild local irritation in some patients. The researchers concluded that *Juniperus excelsa* extract is an effective adjunctive treatment for cutaneous leishmaniasis when combined with cryotherapy, increasing recovery rates and shortening recovery time (27).

In this study, the chloroform extract of *J. communis* demonstrated activity against metronidazole-resistant *T. vaginalis* at a concentration of 30 µg/mL, suggesting its potential as an alternative treatment for resistant strains. In contrast, the ethanol, methanol, and water extracts exhibited lower efficacy. Further investigation into the antitrichomonal properties of these extracts may help to elucidate the observed differences in activity.

The chloroform extract demonstrated lethal activity against *Blastocystis* at a concentration of 30 µg/mL. In contrast, the ethanol, methanol, and water extracts showed limited efficacy, even at higher concentrations, consistent with previous findings for *P. falciparum*, *L. tropica*, and *T. vaginalis* samples.

These findings highlight the diverse antiparasitic properties of *Juniperus* species, demonstrating their broad potential for medical applications. However, the effectiveness of different *Juniperus* species and their extracts varies against different parasites. While *J. communis* showed limited activity against malaria, *Juniperus procera* and *Juniperus excelsa* have shown more promising results against leishmaniasis and malaria. Further investigation of the active components in these species and the optimization of potential therapeutic applications are needed.

Future studies should focus on understanding the full spectrum of components contained within these plants and exploring the synergistic effects of these components. This will enable the development of more effective and safer antiparasitic therapies using *Juniperus* species.

Although our study comprehensively evaluated the antiparasitic activities of various *J. communis* extracts, there are some important limitations. The cytotoxic activities of the extracts have not yet been tested, which poses a limitation for their potential therapeutic applications in humans. Additionally, the efficacy of the extracts has not been tested in *in vivo* models, creating a gap in confirming their biological activity in complex systems and assessing their clinical potential.

CONCLUSION

This study demonstrates that the chloroform extract of *J. communis* exhibits strong antiparasitic activity against specific parasite species. The findings support the medicinal potential of this plant and offer promising prospects for future therapeutic applications. Notably, its effectiveness against metronidazole-resistant *T. vaginalis* strains is significant for the development of alternative treatment options. However, the low efficacy of ethanol, methanol, and water extracts highlights the need for more detailed investigations into their components and application methods. This study underscores the potential of *J. communis* as a medicinal plant and contributes to the literature for further research.

*Ethics

Ethics Committee Approval: This research was approved by the Ethics Committee of the Faculty of Medicine, Manisa Celal Bayar University (approval date: 29/03/2023; approval number: 20.478.486/1773).

Informed Consent: Since this study was conducted solely using archived parasite isolates in laboratory settings, informed consent was not required.

Footnotes

*Authorship Contributions

Concept: İ.Ç., Y.Ö., V.T., H.K., K.Y., A.Ö., Design: V.T., K.Y., A.Ö., Data Collection or Processing: Y.Ö., V.T., Analysis or Interpretation: H.K., K.Y., A.Ö., Literature Search: İ.Ç., Y.Ö., Writing: İ.Ç., A.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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Efficacy of Medicinal Leech Therapy in Diverse Clinical Applications: A Comprehensive Study from Azerbaijan

Çeşitli Klinik Uygulamalarda Tıbbi Sülük Tedavisinin Etkinliği: Azerbaycan'dan Kapsamlı Bir Çalışma

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ABSTRACT

Objective: Hirudotherapy (HT), the therapeutic use of medicinal leeches, has been practised for centuries, and the interest in modern medicine has recently been renewed. This study evaluates the clinical outcomes of HT at Herba Medical Center in Azerbaijan between 2020 and 2024, focusing on its efficacy across 11 medical conditions.

Methods: A total of 181 patients were treated using disposable medicinal leeches (*Hirudo orientalis*) sourced from hygienic farms approved by Azerbaijan's Ministry of Ecology and Natural Resources. Treatment protocols were tailored to disease severity, with sessions scheduled daily, every 3 days, or weekly, depending on the condition. Success rates were calculated based on post-treatment examinations, patient feedback, and physician evaluations. Statistical analyses, including Pearson correlation analysis and paired t-test, were used to compare treatment success rates between conditions.

Results: The overall success rate of HT was found to be 82.68±29.25%. 100% success was achieved in the treatment of osteoarthritis pain (n=50), lipoma (n=8), Raynaud disease (n=3) and scleroderma (n=2). High success rates were also observed in thyroiditis (94.44%, n=18), Baker's cyst (80%, n=25), ear diseases (80%, n=10) and diabetic foot ulcers (80%, n=5). Moderate success was achieved in eye diseases (75%, n=20), and the lowest efficacy was observed in the treatment of varicose veins (33.33%, n=30). HT effectively relieved pain and improved symptoms. However, it was limited in reversing structural deformities (e.g., hallux valgus) or tissue loss (e.g., diabetic foot ulcers).

Conclusion: These findings suggest that HT may have broader indications. We propose that HT can effectively relieve pain, regulate blood circulation, and treat some chronic diseases with fewer side effects. Further and more detailed research is needed to understand the mechanism of this treatment method better.

Keywords: Hirudotherapy, leech therapy, traditional medicine, complementary treatment, pain management, chronic diseases

ÖZ

Amaç: Tıbbi sülüklerin terapötik kullanımı olan hirudoterapi (HT), yüzyıllardır uygulanmaktadır ve modern tıbba olan ilgi son zamanlarda yenilenmiştir. Bu çalışma, 2020 ile 2024 yılları arasında Azerbaycan'daki Herba Tıp Merkezi'nde HT'nin klinik sonuçlarını değerlendirerek 11 tıbbi durumdaki etkinliğine odaklanmaktadır.

Yöntemler: Toplam 181 hasta, Azerbaycan Ekoloji ve Doğal Kaynaklar Bakanlığı tarafından onaylanan hijyenik çiftliklerden elde edilen tek kullanımlık tıbbi sülükler (*Hirudo orientalis*) kullanılarak tedavi edildi. Tedavi protokolleri, duruma bağlı olarak günlük, 3 günde bir veya haftalık olarak planlanan seanslarla hastalığın şiddetine göre uyarlandı. Başarı oranları, tedavi sonrası muayenelere, hasta geri bildirimlerine ve doktor değerlendirmelerine göre hesaplandı. Koşullar arasındaki tedavi başarı oranlarını karşılaştırmak için Pearson korelasyon analizi ve eşleştirilmiş t-testi de dahil olmak üzere istatistiksel analizler kullanıldı.

Bulgular: HT'nin genel başarı oranı %82,68±29,25 olarak bulundu. Osteoartrit ağrısı (n=50), lipom (n=8), Raynaud hastalığı (n=3) ve skleroderma (n=2) tedavisinde %100 başarı sağlandı. Tiroidit (%94,44, n=18), Baker kisti (%80, n=25), kulak hastalıkları (%80, n=10) ve diyabetik ayak ülserlerinde (%80, n=5) de yüksek başarı oranları gözlendi. Göz hastalıklarında orta düzeyde başarı (%75, n=20) elde edilirken, en düşük etkinlik varis tedavisinde (%33,33, n=30) görüldü. HT ağrıyı etkili bir şekilde hafifletti ve semptomları iyileştirdi. Ancak yapısal deformiteleri (örneğin, halluks valgus) veya doku kaybını (örneğin, diyabetik ayak ülserleri) geri döndürmede sınırlı kaldı.

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Sonuç: Bu bulgular HT'nin daha geniş endikasyonlara sahip olabileceğini düşündürmektedir. HT'nin ağrıyı etkili bir şekilde hafifletebileceğini, kan dolaşımını düzenleyebileceğini ve bazı kronik hastalıkları daha az yan etkiyle tedavi edebileceğini ileri sürüyoruz. Bu tedavi yönteminin mekanizmasını daha iyi anlamak için daha fazla ve daha detaylı araştırmalara ihtiyaç vardır.
Anahtar Kelimeler: Hirudoterapi, sülük tedavisi, geleneksel tıp, tamamlayıcı tedavi, ağrı yönetimi, kronik hastalıklar

INTRODUCTION

The therapeutic properties of medical leeches have been recognised for centuries, and the treatment method involving these creatures is known as hirudotherapy (HT). Historical evidence of this treatment dates back to ancient Egyptian hieroglyphs from 3500 years ago, where leeches were utilised for bloodletting. The practice continued through the Roman Period, with Galen (AD 130-201) using leeches for medical purposes. In the Middle Ages, leech therapy was popularised by Avicenna, who detailed the method in his work "The Law of Medicine" in the 10th century (AD 980-1037). The peak of leech therapy's usage was in the early 19th century (1825-1850) (1-6). In France, more than five million leeches were used annually for treatment in hospitals in the 19th century. While the practice declined in the 20th century, it has experienced a resurgence in popularity over the last 40 years (7).

HT techniques have a rich historical background and have recently garnered increased attention due to their potential mechanisms of action. The importance of medicinal leech species, the main actors in this treatment method, has also increased. Among the Hirudinea family, the European medicinal leech, *H. medicinalis*, stands out as a well-known member, having been utilised for therapeutic benefits in various medical cultures such as Arab, Chinese, Greek, and Roman since ancient times (8). Six medicinal leech species under the *Hirudo* genus are employed in the Eurasian region for therapeutic purposes (9). These extraordinary creatures can even be found in European pharmacies, and more than 300 HT clinics offer medicinal leech treatment in Germany alone (10). In particular 2004, the US Food and Drug Administration authorised medicinal leeches in plastic and reconstructive surgery (11). As a result, their adoption of HT has gained global momentum and led many countries to establish legal frameworks to regulate their use (12-15).

HT is a commonly utilised treatment for various conditions, including pain, circulatory system disorders, diabetic complications, and arthritis. Leeches secrete vasodilators like hirudin, calin, hyaluronidase, and histamine while feeding, preventing blood clotting, offering pain relief, inducing muscle relaxation, and modulating the immune response. Plastic surgeons favour using medical leeches to enhance venous blood circulation, particularly in replantation surgeries. Furthermore, surgeons acknowledge leeches as an efficacious approach to managing venous disorders, hematomas, and persistent wounds (16). The discovery of hirudin, a potent anticoagulant from leech saliva, and the identification of leech saliva's components have increased scientific interest in leeches (17).

Recent studies have provided robust evidence supporting the efficacy of HT in various medical conditions. For example, a meta-analysis by Lauche et al. (18) demonstrated that HT significantly reduces pain in knee osteoarthritis compared to placebo (19). Similarly, Flecken and Michalsen (20) reported that HT alleviates symptoms of chronic venous insufficiency by reducing venous congestion. In the field of plastic surgery, HT has been shown to prevent venous congestion in flap and replantation procedures, highlighting its versatility (13). However, the long-term effects and mechanisms of HT warrant further investigation, particularly through well-designed randomized controlled trials (21).

There are still some concerns about the use of medicinal leeches in HT applications. One of the most important concerns is whether the leeches used in HT are taken from hygienic medical leech farms. There are 11 medical leech farms in Azerbaijan for this purpose. In these farms, *Hirudo orientalis*, known as a Caucasian medicinal leech, is grown, and this leech species is widely used in leech treatment (22). Historically, medicinal leeches were employed in Azerbaijani traditional medicine within palaces and among the populace through HT, either solely with leeches or with cupping. This practice persists in specific clinics today (23). This study represents one of the first studies on leech therapy in Azerbaijan, providing baseline data for prospective studies. It aims to contribute to a deeper understanding of the mechanisms and applications of HT by analysing patient outcomes and success rates in 11 different medical conditions.

METHODS

This study was conducted at Herba Medical Center, which practices traditional and complementary medicine (TCM) in Baku, Azerbaijan. The study aims to analyse the health status data of patients who applied to the health centre and received medical leech therapy before and after treatment. The research covers data recorded between 2020-2024 and includes data on 181 patients treated.

No regulation is prepared within the framework of TCM practices in Azerbaijan. Hospitals and clinics that want to apply leech therapy can use it with the hospital's approval or if the physician decides on leech therapy. In this context, the results of this study include the clinical results of patients who agreed on HT by Herba Medical Center and the physicians of this clinic. Although Azerbaijan lacks a formal ethics committee system for TCM applications, efforts are being made to align with international ethical standards to enhance such studies' global acceptance and credibility.

Patient Selection and Data Collection

The patients evaluated in the study represent 11 different cases and diseases admitted to Herba Medical Center and treated with medical leech therapy. Each patient's treatment process was determined after a detailed anamnesis and physical examination. In this process, treatment parameters such as the area where the leeches will be applied, the number of sessions and the number of leeches to be used are planned according to the patient's specific needs. A treatment protocol specific to each disease and patient was created and recorded. In addition to standard treatment protocols, patient feedback regarding pain relief, symptom improvement, and overall satisfaction was recorded to provide a holistic understanding of HT outcomes. The success rate of the applied leech therapy on the disease was calculated proportionally by rating it according to the feedback of the patients, their healthy participation in daily life and the evaluations of our physicians.

Supply and Use of Medicinal Leeches

The leeches used in the treatment were obtained from medical leech farms approved by Azerbaijan's Ministry of Ecology and Natural Resources for cultivation under hygienic conditions. The leeches used were disposable for each patient and were not used in other patients. The used leeches were first anaesthetised in 10% alcohol for 30 minutes and then euthanised in 70% alcohol and disposed of as medical waste.

Treatment Protocols

Treatment sessions were planned according to the severity and progression of each condition. Sessions were organized at intervals of daily, every 3 days, every 7 days, or every 10 days, depending on the disease. For chronic conditions, treatment was repeated every 3, 4, or 6 months. The number of leeches applied per session ranged from 1 to 12, depending on the condition's type, size, and severity. Detailed protocols for each condition are outlined below:

Osteoarthritis: In this study, 50 patients with osteoarthritis of the neck, waist, and shoulders were treated using leech therapy. Each patient received 14 sessions, divided into two periods of 7 sessions each, with a 4-month interval between periods. For neck and lumbar osteoarthritis, 12 leeches were applied per session, distributed evenly across the neck, waist, and back (Figure 1A). Similarly, 12 leeches were administered around the shoulder area in each session for osteoarthritis. Thus, each session involved 12 leeches, resulting in 84 leeches per treatment period (Table 1).

Varicose veins: Thirty patients with varicose veins sought treatment at the health centre. After confirming the absence of anaemia and low blood pressure in all patients, 16 sessions of leech therapy were administered, divided into two periods of 8 sessions each, with a 6-month interval between periods (Figure 1B). In both periods, sessions were conducted weekly. During each session, 9 to 12 leeches were applied, with the total number of leeches used in each treatment period ranging from 72 to 96, depending on disease severity (Table 1).

Baker's cyst: A total of 25 patients with Baker's cyst were treated with leech therapy for 6-8 sessions, depending on the severity of the disease. For treatment, an average of 5 leeches (4-8 leeches) were applied around the cyst formed behind the kneecap at 7-day intervals (Figure 1C). Thirty-fourty leeches were used for Baker's cyst treatment (Table 1).

Eye diseases: Leech treatment was applied to patients who applied to the health centre with complaints of glaucoma, macular disease, eye allergy, diabetic retinopathy and eye trauma. A total of 20 patients were admitted to the clinic with complaints of the above-mentioned diseases, at least two patients each. All patients were treated with leech therapy for nine sessions at 7-day intervals. In each session, 2-6 leeches were applied to the patients (Figure 1D, E). A total of 18-54 leeches were used to treat eye diseases (Table 1).

Inflammation of the thyroid gland: A total of 18 patients with thyroid gland inflammation underwent six sessions of leech therapy. Each session involved the application of 10 leeches, five placed around the salivary gland and five around the thyroid gland (Figure 1F). Over the course of the treatment, 60 leeches were used (Table 1).

Figure 1. Application points of medicinal leeches according to some diseases. **A)** Osteoarthritis, **B)** Varicose veins, **C)** Baker's cyst, **D)** Eye diseases (right), **E)** Eye diseases (left), **F)** Inflammation of the thyroid gland, **G)** Ear diseases, **H)** Hallux valgus, **I)** Lipoma, **J)** Diabetic foot ulcer, **K)** Raynaud's disease, **L)** Scleroderma

Table 1. Diseases for which hirudotherapy is applied in Azerbaijan include the number of patients, treatment protocols, and treatment success rates

Cases	Number of patients	Number of treatment sessions	Session frequency	Number of treatment repetitions	The time between the two treatments	The mean number of leeches in each session (± SD)	Treatment site	Positive result	Treatment success rate (%)
Osteoarthritis	50	7	7 days apart	2	4 months	12	Neck, Waist, Shoulder	50	100
Varicose veins	30	8	7 days apart	2	6 months	10±2 (9-12)	Varicose veins area	10	33.33
Baker's cyst	25	6-8	7 days apart	1	-	6±2 (4-8)	Behind the knee, the Baker cyst area	20	80
Eye diseases	20	9	7 days apart	1	-	6	Around the eyes	15	75
Thyroid gland inflammation	18	6	10 days apart	1	-	10	Salivary gland and thyroid gland surroundings	17	94.44
Ear diseases	10	7-8	7 days apart	1	-	7	Around the ear and neck	8	80
Hallux valgus	10	8	7 days apart	1	-	7	Toes and top of foot	-	0
Lipoma	8	6-8	7 days apart	1	-	5±2 (3-7)	Lipoma circumference	8	100
Diabetic foot ulcer	5	8-10	Every day	2	3 months	4.75±2.36 (3-8)	Junction area of necrotic area and healthy tissue	4	80
Raynaud's disease	3	6-8	3 days apart	1	-	3±2 (1-4)	Toes where the disease develops	3	100
Scleroderma	2	7	7 days apart	2	3 months	5	Damaged sub-district	2	100
General results	**181**	**6-10**	**1-10 days apart**	**1-2**	**3-6 months**	**6.11±3.44**		**159**	**82.68±29.25**

SD: Standard deviation

Ear diseases: Ten patients sought treatment at the health centre for various ear conditions, including external otitis, middle ear inflammation, and tinnitus. These patients underwent 7-8 sessions of leech therapy at weekly intervals. Seven leeches were utilised during each session, totalling 49-56 for treating ear diseases. Specifically, four leeches were applied around the ear, while three leeches were placed in the neck area, 2 cm below the ear-head junction (Table 1, Figure 1G).

Hallux valgus: In the research, ten patients diagnosed with hallux valgus underwent leech therapy, which consisted of eight sessions. The treatment involved the application of seven leeches on the toes at seven-day intervals (Figure 1H). A total of 56 leeches were utilised to treat hallux valgus (Table 1).

Lipoma: The eight patients diagnosed with lipoma underwent 6-8 sessions of leech therapy. The treatment involved applying 3-7 leeches around the affected area, depending on the severity of the condition, at weekly intervals (Table 1, Figure 1I).

Diabetic foot ulcer: A total of five patients presented at the health centre with complaints of diabetic foot ulcers. Each patient underwent 8-10 sessions of leech therapy, divided into two periods spaced three months apart. During each treatment period, 3-10 leeches were applied to the patients daily (Figure 1J). The total number of leeches used per treatment period ranged from 30 to 80, depending on the severity of the disease. Leech application targeted the junction points between necrotic and intact tissues. The number of treatment sessions was escalated until the colour transition between necrotic and intact tissues lightened (Table 1).

Raynaud's disease: In the study, three patients diagnosed with Raynaud's disease underwent 6 to 8 sessions of leech therapy. The treatment involved the application of 1-4 leeches to the affected areas, based on the disease's severity, with a 3-day interval between sessions (Figure 1K). This treatment regimen was repeated every three months until an improvement in blood flow

and the normalisation of skin colour in the affected area were observed (Table 1).

Scleroderma: Two patients with a diagnosis of scleroderma received seven sessions of leech therapy. The treatment involved applying five leeches around the hardened scar tissue at 7-day intervals (Figure 1L). Thirty-five leeches, given in sets of five per session, were used for treating scleroderma (Table 1).

Statistical Analysis

Statistical analyses were conducted to evaluate the efficacy of HT across different diseases. Descriptive statistics, including mean, standard deviation, and percentages, were used to summarize treatment sessions, success rates, and the number of leeches per session. The Pearson correlation analysis was used to explore the relationship between the number of treatment sessions and treatment success rates, identifying potential trends in treatment outcomes. A paired t-test was applied to evaluate the effectiveness of HT on pain management in different diseases. Pre-treatment and post-treatment pain scores were compared for each patient. Statistical significance was set at $p<0.05$-0.001.

RESULTS

In the study, 181 patients were treated using medicinal leeches for 11 cases and diseases. While a positive response was obtained in 159 patients, no result was obtained in 22. The average success rate of HT applied in 11 different diseases in the clinic was 82.68±29.25 (Table 1). Treatment results have revealed the need for personalised treatment protocols according to different diseases and the complexity of each case.

It is observed that the most common patients applying to the treatment centre have pain caused by osteoarthritis. As a result of the treatment used on 50 patients to relieve neck, waist and shoulder osteoarthritis pain, it was observed that the patients' pain started to decrease significantly from the first session. It was determined that all patients' pain disappeared entirely with the completion of the treatment sessions. It was determined that leech therapy was 100% successfully relieved osteoarthritis pain (Figure 2A).

Leech therapy applied to 20 patients with large varicose veins did not improve the dilation of the veins. It was determined that the vascular structure returned to normal in 10 patients with dark-coloured vascular structures in the capillaries. It was defined that HT was effective, especially in varicose capillaries and non-chronic cases (Figure 2A).

Baker's cyst, characterised by the accumulation of excess joint fluid in the bursa at the back of the knee, often leads to severe pain and circulatory issues over time. As a result of leech application within the protocol framework for this disease, it was determined that 20 out of 25 patients recovered completely, and the treatment success rate was 80% (Figure 2A).

Fifteen out of 20 patients with glaucoma, macular disease, eye allergy, diabetic retinopathy, blood leakage into the fundus of the eye and eye trauma recovered, and the success rate of leech therapy was 75% (Figure 2A). Three patients with macular disease and two patients with diabetic retinopathy did not get positive results from leech therapy in the clinic. Although the overall success rate was high, it showed potential difficulties in treating advanced or chronic vascular conditions in the eye with leeches therapy.

Successful results were obtained in 17 out of 18 patients who received leech therapy due to thyroid gland inflammation (thyroiditis), making the success rate 94.44% (Figure 2A). It was determined that the patient, without successful results, had chronic thyroiditis. The application of HT in different protocol combinations in chronic cases may give more positive results.

Eight out of 10 patients were cured with leech treatment for complaints of otitis media, otitis externa, and tinnitus. Positive results were obtained in three out of five patients treated for otitis media. While the success rate in the treatment of otitis media is 60%, 100% success has been achieved in tinnitus and otitis externa (Figure 2A). The general success rate of leech treatment in ear diseases was 80%.

Hallux valgus was determined that pain decreased in 8 out of 10 patients who received leech therapy for the disease, but no deformity improvement occurred (Figure 2A). Although an improvement in pain reduction was observed in these patients, leech therapy had no positive effect on deformation.

Improvement was observed in all eight patients who were treated for lipoma complaints. The success rate of leech treatment in lipoma is 100% (Figure 2A).

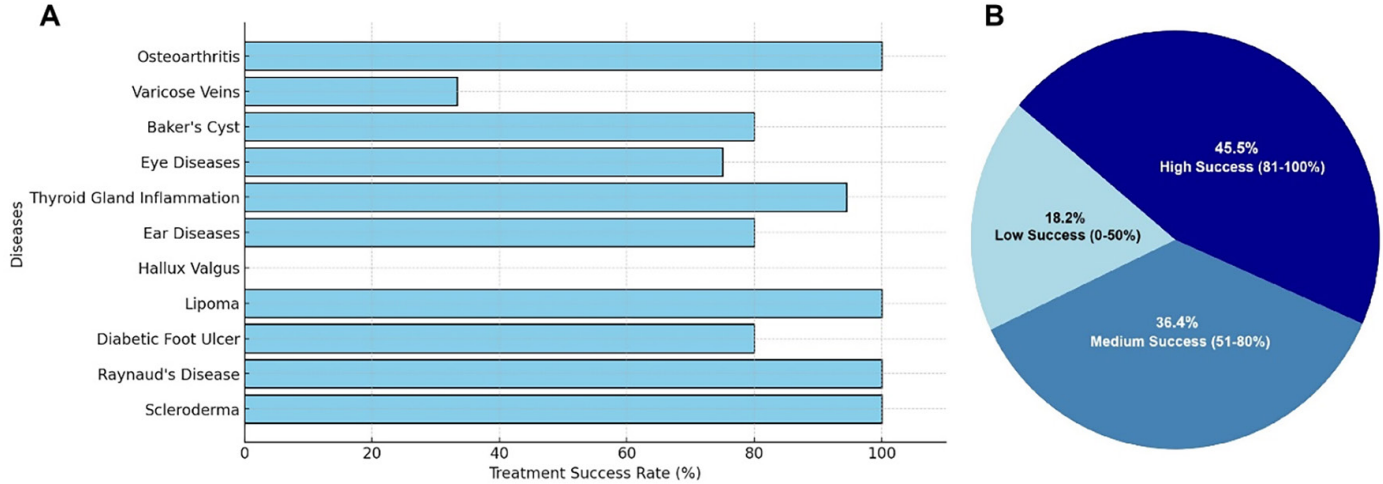

Figure 2. Success rates of hirudotherapy on a disease-by-disease and grouped basis. **A)** Hirudotherapy success rates by disease in Azerbaijan, **B)** Proportion of diseases by treatment success rate

Four out of five patients who applied with diabetic foot ulcer complaints had positive results from treatment with leeches. The success rate of leech treatment in diabetic foot ulcers was 80% (Figure 2A). While leech treatment was successful, especially in the initial stages of wounds, it was observed that leeches were insufficient in the treatment of chronic and enlarged ulcers. It was observed that although the abscess and necrotic structure healed, the tissue loss did not reverse.

Raynaud's disease is characterised by the formation of colour changes or bruising due to insufficient blood flow in the veins, especially at the tips of the pointed organs such as the toes, fingers and ear tips. In three patients treated with Raynaud's disease, 100% success (Figure 2A) was achieved by getting positive results from treatment with medicinal leeches.

In two patients, 100% success (Figure 2A) was obtained from leech therapy against scleroderma manifested by skin thickening. In both patients, it was determined that the skin thickening returned to normal after HT.

Leech therapy showed low success rate (18.2%) in two diseases (varicose veins and hallux valgus), moderate success rate (36.4%) in four diseases (Baker's cyst, eye diseases, ear diseases, diabetic foot ulcer) and high success rate (45.5%) in five diseases (osteoarthritis, thyroid gland inflammation, lipoma, Raynaud's disease, scleroderma) (Figure 2B).

Pearson correlation analysis revealed a weak and negative correlation between the number of leeches (Figure 3) used per session and treatment success ($r=-0.179$, $p=0.599$). This value shows that the increase in leeches does not significantly increase the treatment success and may even show a slight inverse relationship. Still, this relationship is not statistically significant ($p>0.05$). This result shows that the treatment success is not directly related only to the number of leeches; other factors, such as the type of disease, the patient's health status, and the applied treatment protocol, are also effective.

When the relationship between the total number of sessions applied for leech treatment and the success rate is taken into consideration, the success rate was observed at levels of 75% and above in diseases with an average of 7-8 sessions of HT. However, increasing the number of sessions did not always increase the success rate, for example, in the treatment of varicose veins, the success rate remained low despite 8 sessions (33.33%). This suggests that treatment success may be related not only to the number of sessions, but also to the protocol applied and the nature of the disease (Figure 4).

According to the paired t-test analysis results, a statistically significant decrease in pain levels was observed after HT treatment in osteoarthritis ($t=26.85$, $p<0.001$), varicose disease ($t=23.71$, $p<0.001$), Baker's cyst ($t=21.26$, $p<0.001$), thyroid gland inflammation ($t=18.39$, $p<0.001$) and ear diseases ($t=11.77$, $p<0.001$). These findings reveal that HT has a significant effect in reducing pain and can be considered as a potential complementary treatment method.

However, since sufficient patient numbers were not reached for other diseases, the change was not statistically significant. Therefore, supporting studies with larger sample groups are required. It reveals that HT is effective in pain management in some diseases, but it may not be equally effective for every disease.

DISCUSSION

Both clinical studies and case reports confirm the effectiveness of medicinal leech therapy in relieving pain (6,19,21,24-26). Leech therapy has been recommended in patients with knee osteoarthritis because of the decrease in pain after HT and few side effects after the procedure (6,18,20,27-29). In this study, the 100% success of HT applied against joint pain due to osteoarthritis reveals that leech therapy can be an effective method in managing and reducing or completely eliminating pain. Although no side effects were determined in HT for relieving osteoarthritis, pain shows similar results with Lauche et al. (18).

Many researchers have reported positive results from HT on varicose veins. In particular, it has been stated that it is effective in normalising the colour change in the vein structure, relieving pain, relieving inflammation and oedema (30-35). In a study conducted for the treatment of varicose veins in the lower legs, 8-10 leeches were applied to 20 patients every 10 days for 60 days, and the patients were followed up for one year to evaluate the effectiveness of HT. It was observed that leech therapy significantly reduced venous fullness by removing oedema, inflammation and venous congestion (32). In our study within the framework of our protocol, similar results were obtained with the research of Iqbal et al. (32). One study reported that ulcers entirely healed in 3 out of

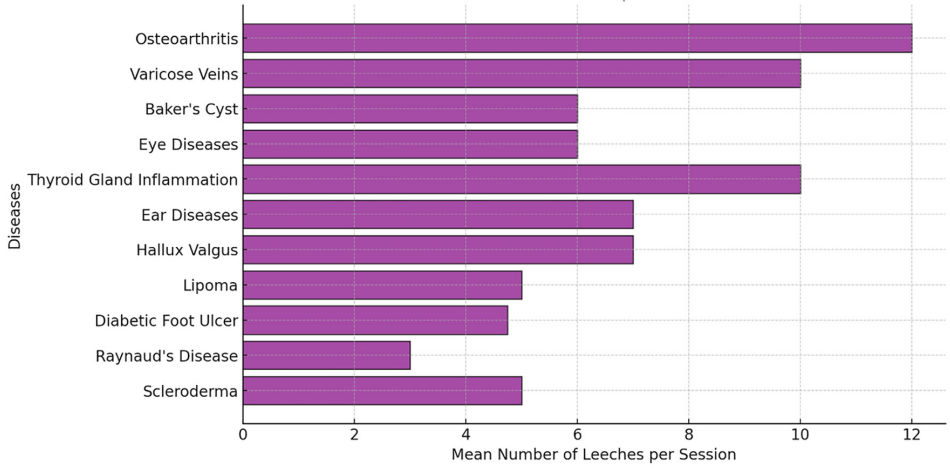

Figure 3. Average number of leeches used per session acros disease

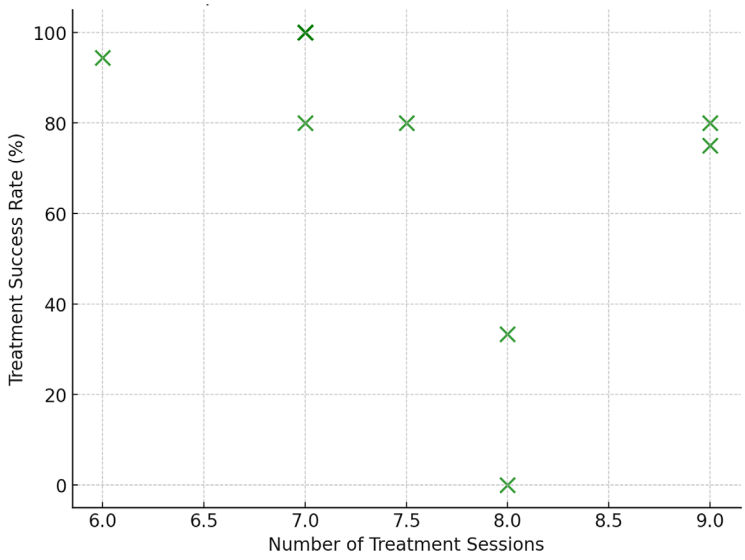

Figure 4. Relationship between number of sessions and success rate

4 cases against varicose vein ulcers, and significant improvements in other variables were also observed (36). Within the scope of this study conducted on varicose veins in Herba Medical Center in Azerbaijan, it was found that the symptoms of all 30 patients were visibly improved, primarily when the pain was eliminated. However, it was determined that the appearance of the veins did not return to its standard structure. HT studies on varicose veins have shown that similar results are generally obtained (20,36,37). Compared to similar studies conducted in regions like Eastern Europe and South Asia, the success rates observed in this study are consistent with findings on conditions such as osteoarthritis and varicose veins.

Clinical practice in patients with Baker's cysts has shown that leeches often significantly reduce cyst size and improve symptoms when application sites are used proximal to or directly above the cyst (20). In one study (38), five leeches were applied locally to the swelling in the popliteal fossa five times every ten days. It was observed that the cysts in all patients entirely resolved and did not recur even after two years of follow-up. In our study, the observation that the applications made directly on the cyst significantly reduced the pain and the size of the cyst in Baker's cyst patients and provided complete healing in some patients was similar to the findings of the researchers.

Excellent results have been obtained in treating severe periorbital haematoma with medicinal leeches (39). HT has been reported to be successfully used in many branches of ophthalmology, ophthalmosurgery, ophthalmo-oncology and emergency ophthalmology, including inflammatory diseases of the eye (keratitis, iridocyclitis, uveitis, etc.), traumatic injuries and vascular pathology of the organ of vision, cataract, glaucoma, etc. Moreover, in Eastern Europe, Russia and some South Asian countries (Indian subcontinent, Sri Lanka) HT is officially recognised as a classical alternative treatment for glaucoma (2,40,41). It has been reported that in cases where surgical treatment methods are insufficient to cure an acute glaucoma attack, HT may be the only way to save the eye (42). Just as researchers have successfully treated a wide range of eye diseases, successful results were obtained in this study for five different ophthalmological problems (glaucoma, eye allergy, blood leakage into the fundus of the eye and eye trauma), excluding diabetic retinopathy and macular disease.

It has been reported that Hashimoto's thyroiditis, an autoimmune disease, can be treated by using multiple TCM methods together, including HT (43). There are almost no scientific references for the treatment of thyroiditis disease with medicinal leeches. However, within the scope of this study, a high rate (94.44%) of positive results were obtained from the leech treatment applied to patients with thyroid gland inflammation (thyroiditis).

It has been reported that leech therapy has been used successfully in the treatment of tinnitus, acute and chronic otitis, outer ear and chronic ear diseases (42,44-46). This study obtained similarly successful results with leech therapy in acute and chronic otitis and otitis external. It has also been determined that leeches have significant effects after ear flap surgery (47) and in cases of tinnitus (1,46). This study also achieved significant success in patients treated with tinnitus complaints.

It has been reported that HT has successfully treated patients diagnosed with lipoma (48). This study, parallel to previous studies, achieved 100% successful leech treatment in 8 patients suffering from Lipoma.

This study obtained successful results in four out of five patients treated with leeches for diabetic foot ulcer disease. In one patient, it was observed that although the ulcer did not close completely, the wound became significantly stable, and the progression of the disease stopped. These findings were similar to many studies using HT against diabetic foot ulcer disease (3,49-52).

Raynaud's disease (53) is a disease characterised by colour changes or bruises due to insufficiency in blood flow in the vessels, especially in the tips of the toes, fingers, and the tip of the ear. Although no article was found on the effect of leech therapy against scleroderma, this study determined that skin thickening improved in two patients who received leech therapy.

Study Limitations

This study demonstrates the significant potential of medicinal leech therapy (HT) in treating various medical conditions, achieving high success rates, particularly in osteoarthritis pain, lipoma, Raynaud's disease, and scleroderma. However, the study also highlighted the limitations of HT, particularly in treating chronic and advanced conditions such as varicose veins and certain eye diseases, where the success rates were lower.

CONCLUSION

These results suggest that while HT can be highly effective, its efficacy may vary depending on the specific condition and the chronicity of the disease. The need for personalized treatment protocols tailored to individual patient conditions was evident, emphasizing the complexity and variability of responses to HT. Further research is necessary to explore the mechanisms underlying HT's effects and to optimize treatment protocols for broader clinical applications. This study contributes to the growing body of evidence supporting the use of HT as a complementary treatment modality, offering a promising alternative for managing pain and various chronic conditions with minimal side effects.

*Ethics

Ethics Committee Approval: According to Azerbaijani laws, an ethics committee approval certificate is not required since the study data are published with the patient's consent and are the results of clinical cases treated after the approval of Herba Medical Center management and physicians.

Informed Consent: All patients were informed about the purpose, procedures, potential risks, and benefits of medicinal leech therapy.

Footnotes

*Authorship Contributions

Surgical and Medical Practices: S.Y., F.H., Concept: S.Y., F.H., N.S., Design: S.F., S.Y., F.H., N.S., Data Collection or Processing: S.F., S.Y., F.H., A.M., N.S., Analysis or Interpretation: A.M., N.S., Literature Search: A.M., N.S., Writing: S.F., S.Y., N.S.

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Case Report: Hydatid Disease as a Potential Cause of Leukocytoclastic Vasculitis in Endemic Regions

Olgu Sunumu: Endemik Bölgelerde Lökositoklastik Vaskülitin Potansiyel Nedeni Olarak Hidatik Hastalık

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ABSTRACT

Leukocytoclastic vasculitis (LCV) is a type of vasculitis that affects small vessels and is commonly associated with infections, malignancies, drugs, and autoimmune diseases. In this case, a 75-year-old female patient presented with clinical signs of LCV, and after ruling out common etiologies, hydatid disease (HD) emerged as a potential cause. This case highlights the importance of considering parasitic infections, particularly HD, in the differential diagnosis of LCV, especially in regions where these infections are endemic and in patients exposed to relevant environmental risk factors.
Keywords: Hydatid cyst, leukocytoclastic vasculitis, parasitic infections

ÖZ

Leukositoklastik vaskulitis (LCV), küçük damarları etkileyen ve genellikle enfeksiyonlar, maligniteler, ilaçlar ve otoimmün hastalıklarla ilişkilendirilen bir vaskulit türüdür. Bu olguda, LCV kliniği ile başvuran 75 yaşındaki kadın hastada, yaygın etiyolojik faktörler dışlandıktan sonra kist hidatik hastalığı olası bir neden olarak ortaya çıkmıştır. Bu olgu, LCV'nin ayırıcı tanısında, özellikle bu enfeksiyonların endemik olduğu bölgelerde ve çevresel risk faktörlerine maruz kalan hastalarda, paraziter enfeksiyonların, özellikle kist hidatik hastalığının göz önünde bulundurulmasının önemini vurgulamaktadır.
Anahtar Kelimeler: Kist hidatik, leukositoklastik vaskülitis, paraziter enfeksiyonlar

INTRODUCTION

Leukocytoclastic vasculitis (LCV) is a form of small-vessel vasculitis commonly manifested by palpable purpura, especially in the lower extremities. It has a broad differential diagnosis, with known associations to autoimmune diseases, infections, malignancies, and medications (1,2). Hydatid disease (HD), caused by *Echinococcus* tapeworms, is a zoonotic infection primarily affecting the liver and prevalent in certain endemic regions. While the link between HD and LCV has been scarcely documented (3), this case suggests the need to consider HD as a potential etiological factor for LCV, especially in regions where both conditions are endemic.

CASE REPORT

A 75-year-old woman presented with a 15-day history of palpable purpura on both lower extremities (Figure 1). She reported a similar episode four years ago, which resolved spontaneously. Her medical history was unremarkable, and she was not on any medications. The patient had a history of livestock farming and owned cats and dogs. No recent infections or significant health events were reported. Physical examination revealed palpable purpura on the lower extremities but no other significant findings. A skin biopsy confirmed the diagnosis of LCV, prompting further investigation of its underlying cause.

Autoimmune screening showed a weakly positive antinuclear antibody at a titer of 1:100 and positive anti-Ku antibodies. However, tests for rheumatoid factor, anti-cyclic citrullinated peptide, anti-double-

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stranded DNA (anti-dsDNA), and antineutrophil cytoplasmic antibodies (ANCA) were all negative. Urinalysis was normal, and C-reactive protein was elevated at 31.1 mg/L. These findings did not support a diagnosis of any specific connective tissue disorder.

Further investigations to identify potential infectious causes, including hepatitis, tuberculosis, and *Brucella*, were negative. A malignancy workup, including tumor markers, fecal occult blood test, mammogram, and imaging studies [computed tomography (CT) of the neck, pelvis, and thorax], also yielded negative results. However, an abdominal CT scan revealed a type 5 hydatid cyst in segments 7 and 8 of the liver (Figure 2). Indirect hemagglutination testing for echinococcal infection was positive with a titer of 1/2560. The patient was diagnosed with hydatid cyst disease, and consultations with infectious diseases and general surgery confirmed that no immediate treatment was necessary.

Given the patient's environmental exposure to livestock and pets, along with the absence of other underlying conditions and negative infectious, malignancy, and autoimmune screening, HD was considered a likely cause of her LCV. The patient was started on a short-term course of prednisone, resulting in significant improvement in the purpura.

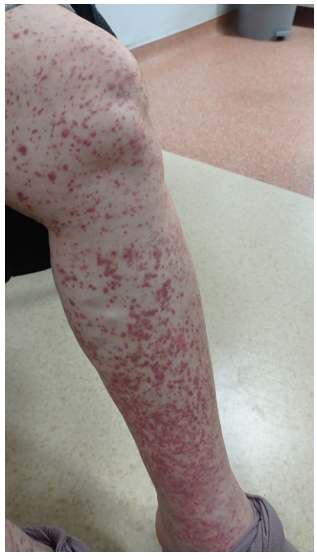

Figure 1. Palpable purpura on the left lower extremity

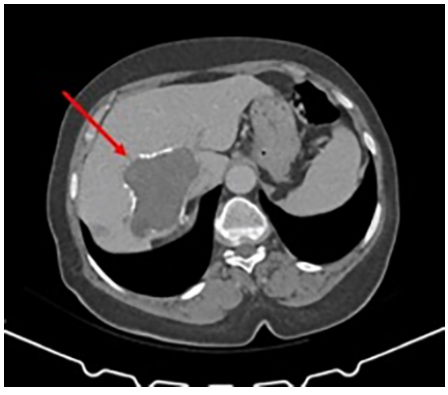

Figure 2. Abdominal CT scan showing a type 5 hydatid cyst measuring 79x73 mm, located in liver segments 7-8
CT: Computed tomography

DISCUSSION

LCV is a common type of cutaneous small vessel vasculitis (CSVV), manifested by palpable purpura, especially in the lower extremities (4). According to the nomenclature of vasculitis provided by the 2012 Revised International Chapel Hill Consensus Conference, CSVV is categorized as a single-organ vasculitis (5). The etiology of LCV is multifactorial, with several potential underlying causes (1). It can be seen in immune-mediated vasculitis (e.g., ANCA-associated vasculitis, Henoch-Schönlein purpura) (2). Additionally, LCV may arise as a complication of connective tissue disorders such as rheumatoid arthritis and systemic lupus erythematosus, Sjögren syndrome (6). It is also associated with malignancies and can be triggered by infectious agents (6). Finally, various medications (7), vaccines (8), and other factors have been recognized as potential contributors to the development of LCV.

HD remains a significant health issue in endemic regions, transmitted through the fecal-oral route, often from dogs or other canids (9). Although the liver is the most frequently affected organ, other organs such as the heart, lungs, spleen and brain, may also be involved (10,11). In many cases, hydatid cysts remain asymptomatic and are incidentally discovered during imaging studies, as seen in our patient (11,12).

Parasitic infections can act as environmental triggers for autoimmune diseases by disrupting immune balance. A striking example is a reported case of polymyalgia rheumatica and giant cell arteritis occurring alongside cystic echinococcosis, suggesting a potential causal link (13). Similarly, LCV may also emerge in the context of parasitic infections, though this association is extremely rare. In the literature, only one case report describes a 10-year-old patient who presented with LCV and necrotizing pneumonia, and was ultimately diagnosed with pulmonary HD (3). The report emphasizes that echinococcal infection should not be overlooked as a potential cause of LCV (3). In light of our patient's exposure to livestock and pets, the presence of a cyst on abdominal CT, serologic evaulation, and the negative findings from autoimmune, malignancy, and other infectious screenings, HD was considered a contributing factor to the development of LCV. Our case is notable as the second reported instance of LCV associated with HD, this time involving the liver.

Although direct evidence linking cystic HD with LCV remains limited, molecular interactions between the human host and the parasite suggest a possible connection. During the early stages of hydatid cyst formation, a cell-mediated immune response involving macrophages, neutrophils, and eosinophils is triggered. *Echinococcus granulosus* stimulates both TH1 and TH2 immune responses (14-16). After treatment or the natural death of the cyst, the TH2 response decreases rapidly, and the TH1 response becomes predominant (12,17). This immune reaction can persist even in the inactive stage, especially in individuals with a history of previous active infection. Additionally, residual immune complexes from prior infections may remain in circulation, potentially sensitizing the immune system and causing it to react to the cyst, even without active parasitic involvement (18).

CONCLUSION

This case emphasizes the importance of considering parasitic infections such as HD in the differential diagnosis of LCV, especially in endemic regions. While the direct link between

cystic HD and LCV remains under study, this case contributes to the growing body of evidence supporting HD as a potential cause of vasculitis. Further research is needed to better understand the underlying mechanisms.

*Ethics

Informed Consent: Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Footnotes

*Authorship Contributions

Concept: G.K., F.N.A.B., Z.B.K., Design: G.K., F.N.A.B., Z.B.K., Data Collection or Processing: G.K., F.N.A.B., Z.B.K., Analysis or Interpretation: G.K., F.N.A.B., Z.B.K., Literature Search: G.K., F.N.A.B., Z.B.K., Writing: G.K.

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