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Sono-photodynamic Therapy-a New Method in the Treatment of Cutaneous Leishmaniasis: an in Vitro Study

Sonofotodinamik Tedavi - Kutanöz Leishmaniasis Tedavisinde Yeni Bir Yöntem: Bir in vitro Çalışma

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ÖZET

AMAÇ: Bu çalışmada, kurkumin aracılı sonodinamik (SDT), fotodinamik (FDT) ve sonofotodinamik (SFDT) tedavilerin Leishmania tropica (L. tropica) promastigotlarına karşı olası bir mekanizma ile anti-leishmanial etkinliğini araştırmayı amaçladık. SFDT, sonodinamik ve fotodinamik tedavileri birleştiren Leishmaniasis tedavisine yeni bir yaklaşımdır. Kurkumin, uzun yıllardır tıbbi durumlarda da kullanılan doğal bir anti-inflamatuvar ajandır. Kurkumin, bu çalışmada PDT, SDT, SPDT'nin L. tropica promastigotları üzerindeki etkisini karşılaştırmak için hem sonosensitizer hem de fotosensitizör olarak kullanılmıştır.

GEREÇ VE YÖNTEM: Hücreler, farklı konsantrasyonlarda (0.25, 1,4,16 ve 64μM) kurkumin ile bir saat süreyle inkübe edildi, 1 MHz frekansında 3 W/cm^2 yoğunluklu ultrasona ve/veya 30 dakika süreyle 1.32 J/cm^2 ışık ışımasına maruz bırakıldı. Aynı zamanda parazit hücreleri sadece ultrason ve ışık ile ve her ikisi de kurkumin varlığında veya yokluğunda SFDT için maruz bırakıldı. Hücre canlılığını değerlendirmek için XTT ve morfolojik değişiklikleri belirlemek için Giemsa boyama kullanıldı.

BULGULAR: Kurkumin ve ultrason, kurkumin ve ışık, kurkumin aracılı ultrason ve ışık kombinasyonu ile L. tropica promastigot canlılığının kontrol, ultrason-kontrol ve ışık kontrol grubuna göre azaldığı bulundu. En büyük azalmanın SPDT grubunda olduğu tespit edildi. Giemsa boyama bulguları, kurkumin aracılı SDT, PDT ve SPDT'nin L. tropica promastigotlarında atipik çeşitli morfolojik değişikliklere neden olduğunu göstermiştir. Bu sonuçlar ile SPDT'nin L. tropica promastigotları üzerinde diğer tedavilerden daha etkili olduğu bulunmuştur.

SONUÇ: Kurkumin aracılı SPDT, L. tropica promastigotları için umut verici bir yaklaşım sağlayabilir.

Anahtar Kelimeler: Leishmania tropica, kurkumin, sonodinamik tedavi, fotodinamik tedavi, sono-fotodinamik tedavi

ABSTRACT

OBJECTİVE: In this study, we aimed to examine of anti-leishmanial effect of curcumin-mediated sonodynamic (SDT), photodynamic (PDT), and sonophotodynamic (SPDT) therapies with a potential mechanism against the Leishmania tropica (L. tropica) promastigotes. SPDT is a new treatment modality for Leishmaniasis that combines photodynamic and sonodynamic therapies. Curcumin is a natural anti-inflammatory agent that has been used for treating medical conditions for many years. Curcumin was used in this study both as a sonosensitizer and photosensitizer to compare the effect of PDT, SDT, SPDT on L. tropica promastigotes.

MATERIALS AND METHODS: The cells were incubated with different concentrations (0.25, 1,4,16 and 64μM) of curcumin for 1 hour, were exposed to 3 W/cm^2 intensity ultrasound for 1MHz frequency and/or subjected to 1.32 J/cm^2 light irradiation for 30 minutes. Also, parasite cells were exposed for SPDT with ultrasound and light only and both in the presence or absence of curcumin. XTT was used to evaluate cell viability and Giemsa staining was used to determine morphological changes.

RESULTS: With the combination of curcumin and ultrasound, curcumin and light, curcumin mediated ultrasound and light, L. tropica promastigote viability was found to be decreased compared to the control, ultrasound-control and light-control group. The greatest reduction was found in the SPDT group. Giemsa staining findings showed that curcumin-mediated SDT, PDT and SPDT induced several morphological alterations in L. tropica promastigotes atypical. These results showed that SPDT is more effective than other therapies on L. tropica promastigotes

CONCLUSIONS: Curcumin-mediated SPDT may provide a promising approach for L. tropica promastigotes.

Key Words: Leishmania Tropica, curcumin, sonodynamic therapy, photodynamic therapy, sono-photodynamic therapy.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a significant public health problem characterized by various skin lesions. Cutaneous leishmaniasis exhibits an increasing trend in Turkey as well as in the rest of the world due to different local and global factors (1,2). Treatment of CL involves topical therapy, systemic drug therapy, and intralesional therapy, alone or in combination, depending on the location, number,

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severity of the lesion, the type of leishmania, and the immune response (3). Meglumine antimonate compounds are used as the first-line treatment in Turkey. However, studies have reported increased resistance to meglumine antimonate. In addition, there are other drugs used in treatment such as amphotericin B, which is associated with side effects, liposomal amphotericin B, which has low side effects but it is expensive, and miltefosine, which is known to exert teratogenic effects in pregnant women (4,5). Today, treatment methods vary depending on the clinical picture, immune response, and the type of Leishmania spp. There is still no standard treatment scheme for side effects in the treatment of Leishmaniasis. An ongoing search is in question for alternative treatment and, therefore, alternative anti-leishmanial therapies based on physical mechanisms such as ultrasound and light are being further looked into.

Sound and light have both been used as sources of energy for minimally-invasive treatment in clinics, such as correction of refractive errors, interstitial laser thermotherapy, and high-intensity focused ultrasound (HIFU) ablation of tumors (6,7).

Photodynamic therapy (PDT) is used as a minimally invasive treatment for many diseases such as keratoconus (8), age-related macular degeneration (9), malignant diseases (10), acne, non-melanoma skin cancer, chronic ulcers, and cutaneous leishmaniasis (11,12). PDT requires the presence of three basic components: light, O_2, and a photosensitizer. An alternative treatment method targets cell death with apoptosis or necrosis using a photosensitizer activated in the presence of light and molecular oxygen to produce reactive oxygen radicals. However, the penetration depth of light in the skin is approximately 1.5, 1.8, 2.2, and 2.4 mm for wavelengths of 600, 660, 750, and 850 nm, respectively (13). It places a serious limitation on traditional Photodynamic Therapy in terms of transmitting sufficient energy to deep targets. To eliminate this limitation, researchers have investigated the possibility of photosensitizer modification. Ultrasound has the ability to deliver to a much greater depth in biological tissues and has also been found to stimulate the same types of photosensitizers and to produce similar therapeutic effects with PDT. Sonodynamic therapy (SDT) is a treatment method based on killing tumor cells by triggering the overproduction of reactive oxygen species activated by the combination of ultrasound and sonosensitizer. Because of the inherent characteristics of ultrasound, SDT is an effective therapy for deep-seated tumors (14-16). Ultrasound itself can also induce cell death in localized region with the help of sonosensitizers through thermal effects and mechanical stress. Such therapeutic properties of ultrasound may synergistic effects by enabling combined therapy. For sensitizers that respond to both light and ultrasound stimulation, the effect of sono-photodynamic therapy (SPDT) has proven to be even superior to single-source irradiation (17,18). However, despite that, SDT and PDT are frequently considered individual therapies; it is recommended that combined SPDT may have even more clinical translation potential with its lowered incident energy levels.

Curcumin, which is obtained from turmeric (Curcuma longa), is a safe, non-toxic natural polyphenol with anti-inflammatory and antioxidant properties, and is being used in food coloring, and traditional medicine (19). Curcumin is used as a medicine for the treatment of inflammatory diseases for many years. It is used as a sensitizer in photo-sonodynamic therapy (20,21). In addition, several studies have reported that curcumin exerts anti-bacterial, anti-fungal and antitrypanosomal activity while exerting antiparasitic activity against Leishmania spp (22-25).

In this study, it was aimed to determine the efficacy of curcumin-mediated sonodynamic, photodynamic and sono-photodynamic therapy, which acts as a sonophotosensitizer on *L. tropica* promastigotes, in terms of cell viability and morphology.

MATERIAL & METHODS

Parasites: *L. tropica* promastigotes were obtained from Prof. Dr. Hatice ERTABAKLAR from Aydın Adnan Menderes University, Faculty of Medicine, Department of Parasitology. Promastigotes were stored in liquid nitrogen until used. *L. tropica* promastigotes removed from liquid nitrogen tank were dissolved in a 37°C hot water bath for 2 minutes and then maintained in RPMI-1640 (10% FBS + 1% penicillin/streptomycin + 1% gentamicin). Parasites were incubated at 26 °C and a new medium was added to the flasks every three days. Promastigotes were counted on the hemocytometer and promastigotes suspension was prepared as $1x10^7$ promastigotes/ml.

Curcumin Preparation: Curcumin (Thermo Fisher Scientific, UK) was used as both a sonosensitizer and photosensitizer in this study. The stock solution of curcumin was prepared

in 2% DMSO. Curcumin concentrations of 0.25, 1, 4, 16, and 64 µM were prepared.

Study groups:

Control Group: No Curcumin, No ultrasound and No light

Curcumin Group: Parasite samples with $1x10^7$ promastigotes/ml were exposed to curcumin for one hour with no ultrasound and light

Curcumin + SDT Group: Parasite samples with $1x10^7$ promastigotes/ml were exposed to curcumin for one hour and then free curcumin was removed and samples were exposed to ultrasound with a density of 3 W/cm 2 at a frequency of 1MHz at a distance of 5 cm for 5 minutes (50% duty cycle).

Curcumin + PDT Group: Parasite samples with $1x10^7$ promastigotes/ml were exposed to curcumin for one hour and then free curcumin was removed and exposed to blue light for 30 minutes at a distance of 10 cm.

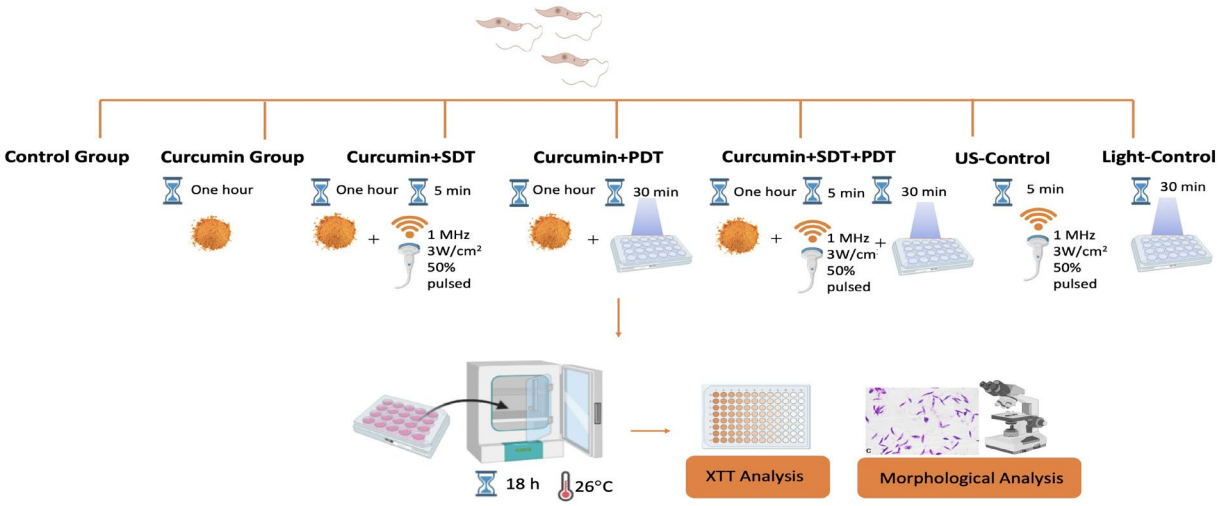
Curcumin + SPDT Group: Parasite samples with $1x10^7$ promastigotes/ml were exposed to curcumin for one hour and then free curcumin was removed and samples were exposed to ultrasound at a distance of 5 cm with a density of 3 W/cm^2 at a frequency of 1MHz for 5 minutes (50% duty cycle) and exposed to blue light for 30 minutes at a distance of 10 cm.

Ultrasound Group: Samples were exposed to ultrasound at 1MHz frequency, 3 W/cm^2 intensity at a distance of 5 cm for 5 minutes (50% duty cycle).

Light Group: Samples were exposed to blue light for 30 minutes at a distance of 10 cm.

Determination of the Efficacy of Curcumin-mediated SDT, PDT and SPDT in vitro : Experimental setup for curcumin-mediated SDT, PDT and SPDT is shown in Figure 1. The light source was an LED (O'melon Omega Led) system containing 287 units (in a three panel) that emit blue light, with 450 nm wavelength. The light output was measured by a power meter (Newport, USA) and delivered an irradiance of 0.73 mW/cm^2 and a fluence of 1.32 J/cm^2 at 30 min. The LED system was chosen because curcumin exhibits maximum absorbance at 435 nm light but also absorbs components at longer wavelengths in the blue light ranging 400 to 500 nm. BTL 4710 Sono dual-frequency ultrasound therapy device (BTL, CZ) was used to apply ultrasound to the *L. tropica* promastigotes. After *L. tropica* promastigotes were exposed to curcumin at different doses for one hour, the samples were centrifuged at 1500 rpm for five minutes and free curcumin was removed from the medium. For SDT therapy, ultrasound was applied to the cells in fluid using a frequency of 1 MHz from a distance of 5 cm at an intensity of 3 W/cm^2 and 50% pulse. For PDT therapy, cells were exposed to blue light in the dark for 30 minutes. For SPDT therapy, cells were exposed to light for 30 minutes following 5 minutes of ultrasound application. Then, fresh medium was added to the samples and incubated at 26 °C for 18 hours.

Figure 1. *Experimental setup for curcumin-mediated SDT, PDT and SPDT*



Analysis of cell viability of promastigotes of *L. tropica* by the XTT cell proliferation test: XTT is a water-soluble tetrazolium salt that, if degraded by the dehydrogenase enzyme in viable cell mitochondria, is converted into a soluble formazan. The concentration of the orange color formed by formazan is metabolically proportional to the number of viable cells. Samples were assessed by a spectrophotometric method using XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl] -2H-tetrazolium-5-caboxanilide salt) solution in 96 microplates. Percent cell viability was calculated according to the following formula measured in each well.

Cell Viability % = (Sample OD value- Blank OD value) / (Control OD value- Blank OD value) X 100

Giemsa Staining: Three alliquotes were prepared for each of the study groups, and some Leishmania samples were taken from each group and spread on the slides to dry. Methanol was placed on the slides for fixation of dried cells, and the Giemsa staining solution was dripped onto the slides and let sit for 20 minutes. Followingly, the slides were rinsed under tap water and dried, then immersion oil was dripped on them and cell morphologies were examined under the light microscope with x100 magnification.

Statistical Analysis

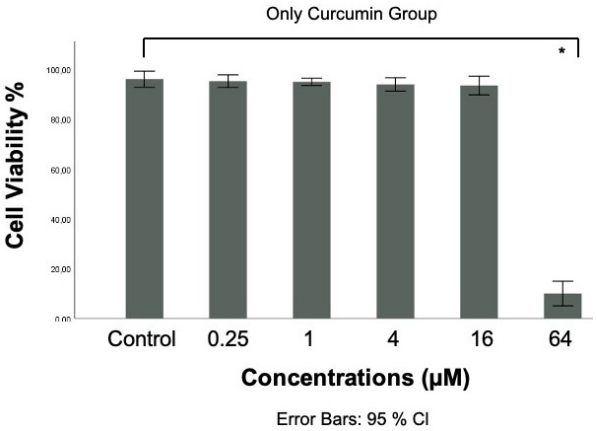
Statistical analyzes were performed using the SPSS 25.0 program. Group comparisons were made using one-way ANOVA of variance followed by Tukey post-hoc test . The values were considered statistically significant when the p value was ≤ 0.05. The degree of signifcance was symbolized by an asterisks (*) for the comparison of all groups with respect to the control group; by a daggers (†) for the comparison of SDT and PDT treated group with respect to SPDT treated group.

RESULTS

The cytotoxicity of curcumin-mediated treatments on L. tropica promastigotes

At 64 μM concentration of curcumin, cell viability decreased by 9.9% (p<0.001). No significant difference was found in the concentrations of 0.25, 1, 4 and 16μM of curcumin compared to the control group. The results of cell viability (XTT assay) showed that curcumin had no cytotoxic effects (all values >90%) and the values of cell viability ranged from 91.2% to 98.4%. Since curcumin alone affects *L. tropica* promastigotes, the 64 μM concentration of curcumin was excluded from the study.No effect of curcumin application on *L. tropica* promastigotes was detected at concentrations of 0.25, 1, 4 and 16μM (Figure 2).

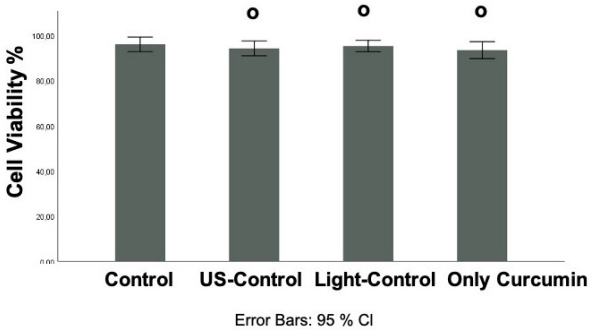
Figure 2. *% survival rate of L. tropica promastigotes treated with curcumin alone.*



Results are presented as means + SD; n = 3 (denotes significant alterations in comparison to the control group, *p<0.001, Error bars 95% confidence interval)*

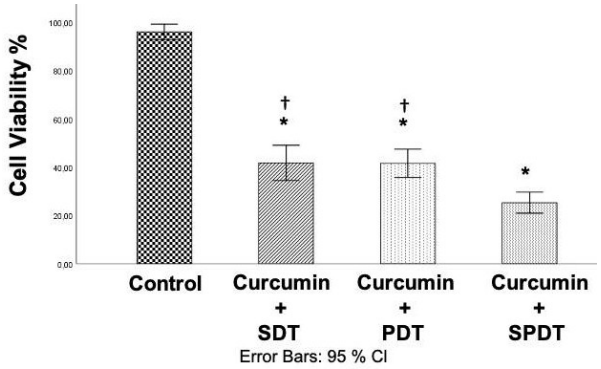
According to the XTT results of the curcumin group alone, the curcumin concentration of the treatment groups was chosen as the medium dose of 4 μM. The cytotoxicity effect of curcumin mediated sonodynamic, photodynamic, and sonophotodynamic therapies were detected by XTT assay, and results were shown in Figure 3 and Figure 4. As shown in Figure 3, the cytotoxic effect of curcumin alone (p=0.149), light alone (p=0.897) and, ultrasound alone (p=0.383) were not observed significantly on *L. tropica* promastigotes. Besides, after sonodynamic, photodynamic and sonophotodynamic therapies, the cell viability was detected at 41.7 ± 2.93%, 41.6± 2.35%, 25.2 ± 1.76% respectively. These results showed that SPDT is more effective than SDT and PDT on *L. tropica* promastigotes (Figure 4).

Figure 3. *The cytotoxic efficiency of untreated control groups*



(Control, Ultrasound-Control, Light Control and Curcumin Control). Results are presented as means + SD; n = 3 (°p> 0.5, Error bars 95% confidence interval)

Figure 4. *The cytotoxicity effect of curcumin mediated sonodynamic, photodynamic, and sonophotodynamic therapies.*

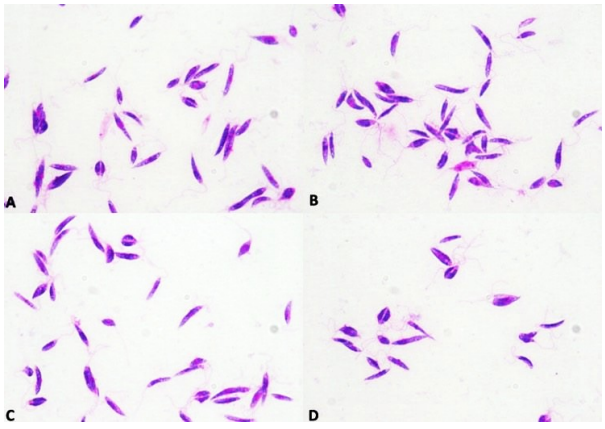


Results are presented as means + SD; n = 3 (denotes significant alterations in comparison to the control group, ***p<0.001; † denotes significant alterations in comparison to the SPDT group, Error bars 95% confidence interval).*

Morphological analysis of Leishmania tropica promastigotes

Determine to treatment's role in morphology, Giemsa stain were used and the results were shown in Figure 5 and Figure 6. The morphological analysis of *L. tropica* promastigotes, revealed that the control groups, US-group, light group and the curcumin group showed no morphological changes, maintaining fusiform appearance, with single nucleus, kinetoplast, narrow body and flagellum. Parasites in all groups had typical morphological features (Figure 5A-D).

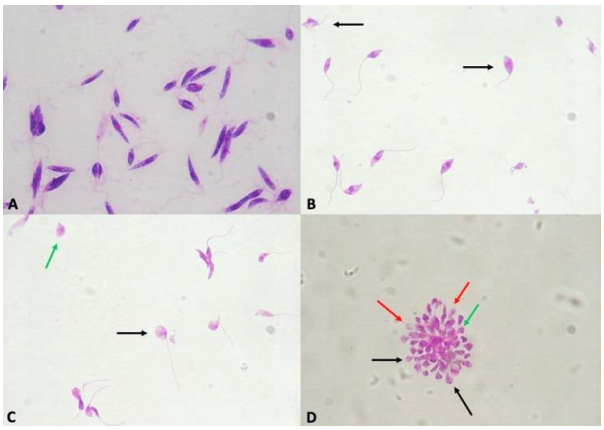
Figure 5. *Morphology of L. tropica promastigotes with Giemsa staining for untreated control groups (x100).*



A. Control B. Ultrasound-Control C. Light-Control D. Curcumin-Control

The damage caused by the action of SDT, PDT and SPDT on the parasites were observed in the morphological analysis presented in Figure 6. Atypical morphological features were increased after treatments. In the SDT group, *L. tropica* promastigotes exhibited alterations like round and altered shape than the control group (Figure 6B). In the PDT group, *L. tropica* promastigotes lost their characteristic morphological features such as fusiform shape and flagellum (Figure 6C). In turn, the morphology of *L. tropica* promastigotes was affected in a much more pronounced way in the SPDT group, since all the cells in the treated group were irregular, round and altered shape, no nucleus, and presenting a absence of the flagellum (Figure 6D).The most increased atypical cells were observed in the SPDT group.

Figure 6. *Morphology of L. tropica promastigotes with Giemsa staining for treatments groups (x 100)*



A. Control B. Curcumin mediated SDT C. Curcumin mediated PDT D. Curcumin mediated Sono-photodynamic therapy. ( ) irregular shape, round structures, ( ) No nucleus, ( ) No flagellum

DISCUSSION

SDT and PDT have been used as individual therapies for many years. There are many published articles on their potential use in treatment. Using light and sound at a certain wavelength and frequency, sonophotodynamic therapy selectively binds to the target cells and activates a light- and sound sensitizer that damages the cells. The basis of SPDT is to stimulate a sensitizer with light and sound to initiate photochemical and sonochemical events that produce cytotoxicity in cells (26). SDT has been widely reported to integrate with PDT due to the fact that most sonosensitizers are also photosensitizers (27).

There are recent studies reporting the use of curcumin as a photosensitizer and sonosensitizer on Leishmaniasis . Four different studies in the literature have focused on testing the efficiency of PDT on Leishmania using curcumin in vitro and the investigated Leishmania species were *L. amazonensis, L. major*, and *L. braziliensis*. However, no studies have been focused on *L. tropica* so far. Marcolino et al. (28) examined the photodynamic therapy of curcumin in *L. braziliensis* and *L. major* promastigotes. Furtermore, cell

viability decreases depending on curcumin concentration and they demonstrated the morphological changes with SEM analyses, and they detected that as the curcumin concentrations increased, irregular flagellum, its shape alters, or there is a shortened or absence of the flagellum. Pinto et al. (29) investigated the photodynamic activity of curcumin on *L. major* and *L. braziliensis* promastigotes. They reported that the morphology of L. major and *L. braziliensis* promastigotes was highly affected by PDT. Maciel et al. (30) reported that curcumin-mediated PDT was effective in inducing the mortality of promastigotes of *L. braziliensis* and *L. amazonensis* in vitro. Pereira et al. (31) reported that curcumin-mediated PDT has the potential to inactivate infected macrophages even at the lowest concentration. A study in the literature, including our group have focused on testing the efficiency of SDT on Leishmania using curcumin in vitro and the investigated Leishmania species was *L. tropica* promastigotes. Caliskan-Ozlem et al. (32) detected that with the combination of curcumin and ultrasound, *L. tropica* promastigote viability was significantly reduced compared to the control group. Giemsa staining findings showed that curcumin-mediated SDT induced morphological changes typical for apoptosis. However, to the best of our knowledge, there are no studies using two therapies in combination for Leishmaniasis. SPDT studies have focused mostly on cancer and antibacterial studies in recent years.

The study of De melo et al.(33), in which the effect of curcumin-mediated SPDT on S. mutans was examined, reported that 55s and 7 minutes of 15J light application and 42 kHz frequency and 5 minutes of ultrasound application with an intensity of 757 mW/cm^2 resulted in a decrease in bacterial viability and that curcumin showed promising results as a sensitizer for sono-photodynamic therapy. Zongfang et al. (34) demonstrated that hematoporphyrin monomethyl ether-mediated SPDT, which they integrated with nanoparticles on E. coli provided higher ROS generation and antibacterial activity compared to PDT alone or SDT alone (1 W cm^2 laser, 2 W cm^2 ultrasound, 10 minutes). Bhavya and Hebbar studied curcumin-mediated SDT, PDT, and SPDT on E. coli and S. aureus. They observed that combined therapy was more effective on E. coli than individual application, while curcumin-mediated PDT exerted greater efficacy than SDT and SPDT on S. aureus (35). The study of Zang et al. (20) on Listeria monocytogenes reported that curcumin-mediated SPDT induced excessive ROS generation whereas combined therapy lead to membrane rupture. Niavarki et al. (36) aimed to compare the relationship between methylene blue-mediated ultrasound and light to inactivate Enterococcus faecalis biofilms formed in root canals. They observed that methylene blue exhibited higher penetration depth when applied with ultrasound and light. Pourhajibagher et al. (37) evaluated the efficacy of PDT, SDT and SPDT mediated by nanoparticles-indocyanine green (CNPs-ICG) against bacterial biofilms on the surfaces of titanium dental implants. They showed that SPDT was more effective than SDT and PDT in reducing bacterial biofilm and was as effective as chlorhexidine, which was used as a standard.

Sono-photodynamic therapy was developed to overcome problems with SDT and PDT. The mechanism of action of sono-photodynamic therapy stems from the benefits of both light source and ultrasound energy. As a biological therapeutic approach, ultrasound energy can produce microbubbles, and their oscillations have the ability to increase the permeability of photosensitizers. This increased permeability leads to increased cellular uptake of molecules, therapeutic agents and nanoparticles (38). Therefore, SPDT can be a promising treatment method for eradicating *L. tropica* parasites and causing cell damage. SPDT is largely used for the treatment of cancer cells (39, 40), and there is no information on the use of SPDT in parasites. Also, there are few studies investigating possible suitable sensitizers for SPDT.

In this study, it was observed that curcumin-mediated SDT, PDT and SPDT cause a decrease in the proliferation of *L. tropica* promastigotes, a more effective result was obtained with SPDT than SDT and PDT, and changes occurred in cell morphology following treatments. Compared with the control group, our results showed that SPDT (~3.8-fold decrease, $p < 0.001$) showed a greater antileishmanial effect than PDT (~2.3-fold decrease, $p < 0.001$) and SDT (~2.3-fold decrease, $p < 0.001$).

This study has a main limitation which is an in vitro-based study of curcumin-mediated SPDT against *L. tropica* promastigotes. There are no intracellular amastigotes or experimental animal models. Yet, our study has some strengths as it enabled the evaluation of a treatment regime in a specific *L. tropica* promastigotes by curcumin. The results obtained in this study are further important in the determination of Leishmaniasis-specific treatment

which may lead to the discovery of novel therapeutics based on SPDT.

In conclusion, the cytotoxicity and changes in cell morphology (round, no flagellum, large, nucleus and kinetoplast-free structures) observed following curcumin-mediated SPDT therapy offer significant potential as an inexpensive, non-toxic, and non-invasive treatment for *L. tropica* promastigotes. In this approach, the fact that each component (curcumin, ultrasound, and light) has been previously used safely in humans will provide a significant opportunity for clinical applications. We anticipate a higher number of prospective studies will focus on the use of curcumin-mediated SPDT in biomedical applications in line with the promising results in various fields and the interesting insights curcumin-mediated SPDT offers. Nevertheless, further in situ and in vivo studies are needed to verify these results in a clinical setting.

Etik: Çalışmanın metodolojik yapısının "hücre kültürü çalışması" olması nedeniyle Dünya Tabipleri Birliği Helsinki Bildirgesi "İnsanlar Üzerinde Yapılan Tıbbi Araştırmalarla İlgili Etik İlkeleri" gereğince etik kurul onayı gerektirmemektedir.

Since the methodological structure of the study is a "cell culture study", it does not require ethics committee approval in accordance with the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research on Humans".

Yazar katkı durumu; Çalışmanın konsepti; SOC, HTY, dizaynı; SOC, Literatür taraması; SOC, HTY, verilerin toplanması ve işlenmesi; SOC, HTY, istatistik; SOC, yazım aşaması; SOC, HTY,

Author contribution status; The concept of the study; SOC, HTY, design; SOC, literature review; SOC, HTY, collecting and processing data; SOC, HTY, statistics; SOC, writing phase; SOC, HTY

Yazarlar arasında çıkar çatışması yoktur.

The author declares no conflict of interest.

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