Int. J. Plant Bas. Pharm. 1(1), 48-51

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

https://ijpbp.com

RESEARCH ARTICLE

OPEN ACCESS

Investigation of the anti-parasitic effect of the water extract of *Thymbra spicata* on *Acanthamoeba castellani* (L.) trophozoites and cysts

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ARTICLE INFO

Article History:

Received: 08 July 2021
Revised: 16 July 2021
Accepted: 17 July 2021
Available online: 19 July 2021

Edited by: B. Tepe

Keywords:
Thymbra spicata
Acanthamoeba castellani
Trophozoites
Cysts
Extract

ABSTRACT

This study aimed to determine the anti-parasitic activity of the water extract obtained from *Thymbra spicata* (L.). The plant material was extracted with methanol in a Soxhlet apparatus. The extract was then fractionated with water and chloroform. The water phase was frozen and freeze-dried. Afterward, this extract was applied on *A. castellani* trophozoites and cysts at various concentrations, and the viability rates were determined by counting under the microscope. At the end of the experimental process, it was determined that there was a strong correlation between the increasing extract concentration and the anti-parasitic effect. *T. spicata* extract was not effective enough to neutralize all cysts at any of the concentrations examined. *T. spicata* extract, at concentrations of 16.0 and 32.0 mg/ml, removed all trophozoites in the medium from the 24th h of the experiment. Based on this result, it was determined that the plant species evaluated here could be used to treat *A. castellani* infections. It will be possible to achieve a good effect on the cyst forms of the parasite species by testing higher extract concentrations, which cannot be evaluated during the experimental process. In addition, with advanced analyzes, it will be possible to reveal the chemical substance responsible for the activity in the plant species in question and to perform further analyzes on this substance.

1. Introduction

Plants have been used as medicine for thousands of years (Samuelsson, 2004). Medications were initially used in herbal teas, poultices, powders, and other herbal formulations (Balick and Cox, 2020; Samuelsson, 2004). Some specific plants used and the treatment methods against various diseases were transferred from language to language and brought to the next generations. If we look at the recent past, it is seen that the use of plants as medicine has come up to the isolation of the active substances. The first active substance obtained from plants was morphine, which was was followed by cocaine, codeine, digitoxin, and quinine, which are isolated from the poppy plant in the early 19th century (Kinghorn, 2001; Sam-uelsson, 2004). In the following years, this development still in use today (Butler, 2004; Newman et al., 2000; Samuelsson, 2004). The isolation and identification of compounds with pharmacological activity from medicinal plants continue today.

Acanthamoeba is a parasite that causes painful and often progressive cases of keratitis (Jones, 1986). This organism is also the causative agent of the disease, also known as granular amoebic encephalitis (GAE), which causes severe immune system deficiencies. It is possible to collect the genus Acanthamoeba under three main morphological groups. Most of the pathogenic species are in the morphological group II. On the other hand, there are reports that some members of group III are also responsible for diseases (Pussard, 1977).
Unfortunately, there is no practical method that can be used in the treatment of *Acanthamoeba* infections today. In only a minority of

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e-ISSN: 2791-7509
doi: https://doi.org/10.62313/ijpbp.2021.4

Please cite this article as: Gulec, B., Tore, B., Sihoglu Tepe, A., Kilic, I.H., 2021. Investigation of the anti-parasitic effect of the water extract of Thymbra spicata on Acanthamoeba castellani trophozoites and cysts. International Journal of Plant Based Pharmaceuticals, 1(1), 48-51, https://doi.org/10.62313/ijpbp.2021.4.

cases, patients respond positively to treatment. *Acanthamoeba* keratitis is treated with a combination of cationic antiseptics such as olyhexamethylene biguanide, which inhibits membrane function, aromatic diamines, and propamidine isethionate (Brolene), which inhibits DNA synthesis. This treatment is given to the patient every hour for three days, and the dose can be increased up to 6 times a day (Duguid et al., 1997; Pussard, 1977). However, almost half of the patients show improvement, while others develop resistance to propamidine (Ficker et al., 1990). Another substance used as an alternative to this substance is chlorhexidine, which is used together with propamidine isethionate (Hay et al., 1994). Chlorhexidine is one of the substances successfully applied alone today (Kosrirukvongs et al., 1999). However, all these treatment methods need to be applied for a few days and hourly. Therefore, in the majority of cases, patients must be in stable conditions.

Most of the chemical agents used to treat parasitic infections today are highly toxic to humans and cause undesirable side effects. For this reason, intense efforts are being made to find alternative methods in the treatment of such infections, where serious problems and sometimes deaths occur. Herbal medicines are currently used to treat parasitic infections caused by agents other than *Acanthamoeba (Arrieta et al., 2001; Fernández et al., 2005; Kayser et al., 2003)*.

In light of the information obtained within the current study, if it is determined that the related plant species has anti-parasitic activity, it aims to recommend this information to industrial areas such as pharmacology, which need new active molecules used in product development studies. This study aimed to investigate the anti-parasitic activity of the water extract obtained from *Thymbra spicata*.

2. Materials and methods

2.1. Plant material and extraction processes

The aerial parts of the plant material were dried in a room where there is no direct sunlight and airflow. Following the literature studies, the extraction processes of all plants were carried out according to the following methods.

A 100 g sample was ground by using a blender. The sample was then extracted with methanol in a Soxhlet apparatus for 6 h. The extract was then evaporated to remove the methanol. The medium to be used in anti-parasitic activity studies is an aqueous medium. Therefore, substances that can dissolve in this medium are also polar.
For this reason, the extract, which became viscous, was shaken by adding water and chloroform to separate polar and nonpolar substances from each other. While chloroform remained in the lower part, the water remained in the upper part so that nonpolar substances in chloroform and polar substances in water were dissolved and separated from each other. The water phase was separated, frozen, and lyophilized into powder. The extract was stored in a refrigerator at +4 °C until testing (Sokmen et al., 1999).

2.2. Determination of anti-parasitic activity

In the determination of anti-parasitic activity, the method used by Tepe et al. (2011) was followed.

2.2.1. Trophozoites

A. castellani was incubated on non-nutritive agar plates and covered with *Escherichia coli* at 26 °C. Trophozoites in the exponential growth stage (72-96th h) were isolated from the plates using a sterile cell scraper. The trophozoites in the plates were washed twice with sterile Page's salt [(PS) (0.12 g NaCl, 0.004 g $MgSO_4.7H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.142 g Na_2HPO_4, and 0.136 g KH_2PO_4 per 1 liter distilled water]. Afterward, centrifugation increased its concentration (1500g, 5 min) (Garcia, 2001). Viable trophozoites were counted using a hemocytometer.

2.2.2. Cysts

In this part of the study, three-week cultures of *A. castellani* were used. Cysts were isolated using sterile Page's salt, and the final concentration was adjusted to $10x10^5$ cysts/ml. The viability of the cysts was determined by the trypan blue method.

2.2.3. Anti-parasitic activity

In this study, 1.5 ml microcentrifuge tubes were used. 200 µl of calibrated trophozoite and/or cyst solutions were mixed with the same volume of test solutions (1, 2, 4, 8, 16, and 32 mg/ml) in microcentrifuge tubes. Then, the tubes were incubated at 26 °C in a bacterial incubator (Electro-Mag) for periods of 1, 3, 6, 8, 24, 48, and 72 h. The exact process was applied to control tubes containing only sterile distilled water and trophozoite/cyst.

2.2.4. Determination of the effects of the extract on the trophozoite stage

After incubation at 26 °C, 25 µl of parasite solution was mixed with the same volume of 0.05% trypan blue and taken into the counting chamber. This mixture was left to stabilize for 3 min at room temperature, and live and dead trophozoites were counted separately in the hemocytometer. Approximately one hundred *A. castellani* trophozoites were counted each time, and this process was repeated 3 times.

2.2.5. Determination of the effects of the extract on the stage of the cyst

After each incubation period, 25 µl of parasite solution was mixed with the same volume of 0.05% trypan blue as stated above and transferred to the counting chamber. This mixture was left to stabilize for 3 min at room temperature, and live and dead cysts were counted separately in the hemocytometer. Approximately one hundred *A. castellani* cysts were counted each time, and this procedure was repeated 3 times. In addition, cultures without live cysts were plated with *E. coli* on non-nutritive agar plates, and the results were confirmed. Parasite growth was observed daily for 14 days using a light microscope (Nikon, Eclipse E 200) at 26 °C.

2.2.6. Statistical analysis

All tests were carried out in triplicate. To determine the degree of statistical difference, using SPSS v. 22.0, Tukey's test was used.

3. Results and discussion

The extract obtained from *T. spicata* was tested by following the protocol detailed in the method section to determine its anti-parasitic activity. The anti-parasitic effect was determined by testing the lethal effect on trophozoites and cysts periodically between 1-72nd h of increasing extract concentrations. The results were given in Tables 1 and 2. At the end of the experimental process, it was

determined that there was a strong correlation between the increased extract concentration and the anti-parasitic effect. The water extract of the *T. spicata* was not effective enough to neutralize all cysts at any of the concentrations tried. As can be seen from Table 1, *T. spicata* extract eliminated all trophozoites in the environment from the 24th hour of the experiment at concentrations of 16.0 and 32.0 mg/ml (Figure 1).

Table 1. Effect of extract from *T. spicata* on *A. castellani* trophozoites[1]

Dose (mg/ml)	Duration						
	1 h	3 h	6 h	8 h	24 h	48 h	72 h
32.0	72.0 ± 2.6^{a}	59.3 ± 1.2^{a}	42.7 ± 0.6^{a}	24.0 ± 1.0^{a}	0	0	0
16.0	82.3 ± 1.5^{b}	70.7 ± 2.5^{b}	59.7 ± 1.5^{b}	44.0 ± 2.0^{b}	0	0	0
8.0	87.3 ± 2.5^{bc}	81.7 ± 2.1^{c}	73.3 ± 2.1^{c}	62.3 ± 2.1^{c}	47.3 ± 2.5^{a}	34.0 ± 1.7^{a}	21.0 ± 1.0^{a}
4.0	91.3 ± 1.2^{d}	86.7 ± 2.1^{c}	82.3 ± 1.2^{d}	72.7 ± 3.1^{d}	60.3 ± 1.5^{b}	46.0 ± 1.7^{b}	36.7 ± 1.5^{b}
2.0	93.0 ± 1.0^{d}	90.7 ± 1.2^{d}	86.3 ± 1.2^{d}	77.7 ± 2.5^{d}	65.0 ± 1.0^{b}	57.3 ± 1.5^{c}	42.7 ± 2.9^{c}
1.0	96.7 ± 1.2^{d}	93.3 ± 1.5^{d}	90.7 ± 1.2^{d}	84.0 ± 1.7^{de}	77.7 ± 2.5^{c}	71.1 ± 1.0^{d}	64.3 ± 2.5^{d}
Control	97.3 ± 0.6^{d}	94.7 ± 0.6^{d}	94.7 ± 1.2^{de}	94.0 ± 1.7^{e}	93.3 ± 1.5^{d}	92.3 ± 1.5^{e}	91.0 ± 1.7^{e}

[1]The values indicated by the same superscripts within the same column are not different according to Tukey's honestly significant difference post hoc test at a 5% significance level.

There is no study in the literature on the effect of *T. spicata* on *A. castellani* trophozoites and cysts. Therefore, the data presented in the present study is the first record for literature. However, there are some literature data regarding the effect of some closely related species on the parasite in terms of taxonomical point of view. It has been reported that *Satureja cuneifolia* (1.0-32.0 mg/ml), which has similar phytochemical content to *T. spicata*, exhibited a time and dose-dependent activity on *A. castellani* torphozoites and cysts. In the study, as mentioned earlier, the methanol extract of *S. cuneifolia* killed all trophozoites at a concentration of 32.0 mg/ml within 24 h of the experiment (Malatyali et al., 2012).

Table 2. Effect of extract from *T. spicata* on *A. castellani* cysts[1]

Dose (mg/ml)	Duration						
	1 h	3 h	6 h	8 h	24 h	48 h	72 h
32.0	86.0 ± 0.0^{a}	79.7 ± 2.5^{a}	78.3 ± 1.5^{a}	63.3 ± 2.9^{a}	51.3 ± 1.2^{a}	42.3 ± 2.5^{a}	28.3 ± 2.9^{a}
16.0	89.3 ± 1.5^{a}	87.7 ± 0.6^{b}	84.0 ± 2.0^{b}	73.3 ± 1.5^{b}	65.7 ± 1.2^{b}	60.0 ± 2.0^{b}	46.3 ± 1.5^{b}
8.0	91.0 ± 1.0^{b}	89.0 ± 1.0^{b}	88.7 ± 2.1^{c}	84.3 ± 1.2^{c}	82.3 ± 0.6^{c}	75.3 ± 1.5^{c}	67.7 ± 2.5^{c}
4.0	93.7 ± 0.6^{b}	92.3 ± 0.6^{c}	90.0 ± 2.0^{c}	87.0 ± 2.0^{d}	86.3 ± 0.6^{cd}	83.7 ± 1.5^{d}	81.3 ± 1.2^{d}
2.0	94.3 ± 1.2^{b}	93.0 ± 1.0^{c}	90.3 ± 0.6^{c}	89.0 ± 1.0^{d}	88.0 ± 0.5^{d}	85.7 ± 2.3^{d}	84.3 ± 1.2^{d}
1.0	94.7 ± 0.6^{b}	93.7 ± 1.5^{c}	93.0 ± 2.6^{d}	92.3 ± 0.6^{e}	91.0 ± 1.0^{de}	90.3 ± 0.6^{e}	89.7 ± 1.5^{e}
Control	95.7 ± 0.6^{b}	95.0 ± 1.0^{cd}	94.0 ± 1.0^{d}	94.0 ± 2.0^{e}	93.3 ± 0.6^{de}	93.0 ± 1.0^{e}	92.3 ± 0.6^{e}

[1]The values indicated by the same superscripts within the same column are not different according to Tukey's honestly significant difference post hoc test at a 5% significance level.

Many researchers agree that *T. spicata* has a high carvacrol content (Hancı et al., 2003; Markovic et al., 2011; Ozel et al., 2003). This suggests that the phytochemical responsible for the activity might be carvacrol, which is in the oxygenated monoterpene. Quintanilla-Licea et al. (2014) support this idea. As mentioned earlier, the study determined that *Lippia graveolens* and *Ruta chalepensis* exhibited significant antiprotozoal activity on *Entamoeba hystolytica* (91.54% and 90.50% growth inhibition at a concentration of 150 µg/mL, respectively). As a result of bioactivity-guided fractionation, carvacrol was determined as the compound responsible for this activity.

4. Conclusions

Based on this result, it was determined that the plant species (*T. spicata*) discussed here can be used in the treatment of *A. castellani* infections. It will be possible to achieve a good effect on the cyst forms of the parasite species by testing higher extract concentrations, which cannot be evaluated during the experimental process. In addition, with advanced analyzes, it will be possible to reveal the chemical compounds responsible for the activity in the plant species in question.

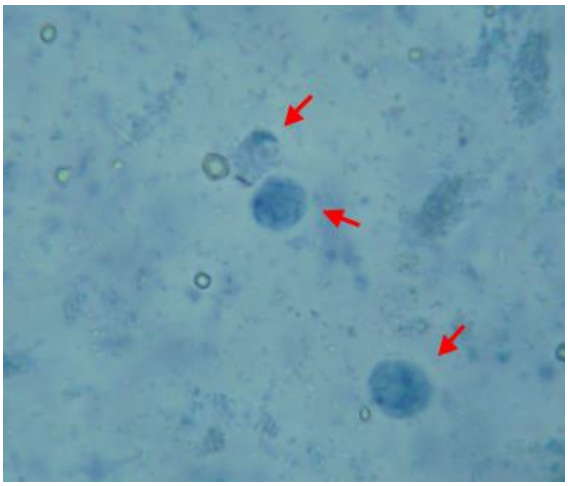

Figure 1. 24-hour image of *A. castellani* trophozoites treated with 16.0 mg/ml of*T. spicata* extract (optical microscope magnification: 40X)

Acknowledgments

The authors thank Dr. Serpil DEGERLI from Sivas Cumhuriyet University for her kind help in obtaining *A. castellani*.

Conflict of Interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Baris Gulec: Conceptualization, Investigation, Methodology, Data curation.
Duhan Tore: Resources, Conceptualization, Visualization, Formal analysis, Methodology.
Arzuhan Sihoglu Tepe: Investigation, Methodology, Supervision, Writing - original draft.
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Supplementary file

None.

References

Arrieta, J., Reyes, B., Calzada, F., Cedillo-Rivera, R., Navarrete, A., 2001. Amoebicidal and giardicidal compounds from the leaves of *Zanthoxylum liebmannianun*. *Fitoterapia*, 72, 295-297.

Balick, M.J., Cox, P.A., 2020. Plants, people, and culture: the science of ethnobotany. Garland Science.

Butler, M.S., 2004. The role of natural product chemistry in drug discovery. *Journal of Natural Products*, 67, 2141-2153.

Duguid, I.G.M., Dart, J.K., Morlet, N., Allan, B.D., Matheson, M., Ficker, L., Tuft, S., 1997. Outcome of *Acanthamoeba* keratitis treated with polyhexamethyl biguanide and propamidine. *Ophthalmology*, 104, 1587-1592.

Fernández, S.S., Guerra, M.C.R., Cárdenas, B.D.M., Villarreal, J.V., Trevino, L.V., 2005. *In vitro* antiprotozoal activity of the leaves of *Artemisia ludoviciana*. *Fitoterapia*, 76, 466-468.

Ficker, L., Seal, D., Warhurst, D., Wright, P., 1990. *Acanthamoeba* keratitis—resistance to medical therapy. *Eye*, 4, 835-838.

Garcia, L.S., 2001. Diagnostic medical parasitology. IN: Manuel of Commercial Methods in Clinical Microbiology, Wiley Online Library.

Hancı, S., Sahin, S., Yılmaz, L., 2003. Isolation of volatile oil from thyme (*Thymbra spicata*) by steam distillation. *Food/Nahrung*, 47, 252-255.

Hay, J., Kirkness, C.M., Seal, D.V., Wright, P., 1994. Drug resistance and *Acanthamoeba* keratitis: the quest for alternative antiprotozoal chemotherapy. *Eye*, 8, 555-563.

Jones, D.B., 1986. Acanthamoeba-the ultimate opportunist? *American Journal of Ophthalmology*, 102, 527-530.

Kayser, O., Kiderlen, A., Croft, S., 2003. Natural products as anti-parasitic drugs. Parasitology Research, 90, S55-S62.

Kinghorn, A.D., 2001. Pharmacognosy in the 21st century. *Journal of Pharmacy and Pharmacology*, 53, 135-148.

Kosrirukvongs, P., Wanachiwanawin, D., Visvesvara, G.S., 1999. Treatment of *Acanthamoeba* keratitis with chlorhexidine. *Ophthalmology*, 106, 798-802.

Malatyali, E., Tepe, B., Degerli, S., Berk, S., 2012. *In vitro* amoebicidal activities of *Satureja cuneifolia* and *Melissa officinalis* on *Acanthamoeba castellanii* cysts and trophozoites. *Parasitology Research*, 110, 2175-2180.

Markovic, T., Chatzopoulou, P., Šiljegović, J., Nikolić, M., Glamočlija, J., Ćirić, A., Soković, M., 2011. Chemical analysis and antimicrobial activities of the essential oils of *Satureja thymbra* L. and *Thymbra spicata* L. and their main components. *Archives of Biological Sciences*, 63, 457-464.

Newman, D.J., Cragg, G.M., Snader, K.M., 2000. The influence of natural products upon drug discovery. *Natural Product Reports*, 17, 215-234.

Ozel, M.Z., Gogus, F., Lewis, A.C., 2003. Subcritical water extraction of essential oils from *Thymbra spicata*. *Food Chemistry*, 82, 381-386.

Pussard, M., 1977. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). *Protistologica*, 13(4), 557-598.

Quintanilla-Licea, R., Mata-Cárdenas, B.D., Vargas-Villarreal, J., Bazaldúa-Rodríguez, A.F., Kavimngeles-Hernández, I., Garza-González, J.N., Hernández-García, M.E., 2014. Antiprotozoal activity against *Entamoeba histolytica* of plants used in northeast Mexican traditional medicine. Bioactive compounds from *Lippia graveolens* and *Ruta chalepensis*. *Molecules*, 19, 21044-21065.

Samuelsson, G., 2004. Drugs of natural origin: a textbook of pharmacognosy, 5th Swedish Pharmaceutical Press. Stockholm, Sweden.

Sokmen, A., Jones, B.M., Erturk, M., 1999. The *in vitro* antibacterial activity of Turkish medicinal plants. *Journal of Ethnopharmacology*, 67, 79-86.

Tepe, B., Degerli, S., Arslan, S., Malatyali, E., Sarikurkcu, C., 2011. Determination of chemical profile, antioxidant, DNA damage protection and antiamoebic activities of *Teucrium polium* and *Stachys iberica*. *Fitoterapia*, 82, 237-246.