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Research Note

Exploring the anthelmintic activity of *Olea europaea* L (Olive) leaves extract and oleuropein in mice naturally infected with *Aspiculuris tetraptera*

M. A. KAMRAN[1]*, H. S. YILDIRIMHAN[1], B. ŞENLIK[2]

[1]Uludağ University, Faculty of Science and Literature, Department of Biology, 16059, Bursa, Turkey, E-mail: **asadkamran32@gmail.com*, *yhikmet@uludag.edu.tr*; [2]Uludağ University, Faculty of Veterinary Sciences, Department of Parasitology, 16059, Bursa, Turkey, E-mail: *bsenlik@uludag.edu.tr*

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Summary

Oxyuriasis, caused by the nematode *Enterobius vermicularis,* is one of the cosmopolitan intestinal infections of humans. *Aspiculuris tetraptera* commonly infects mice and it is morphologically similar to *E. vermicularis*. Parasitic resistance reduces the efficiency of synthetic drugs and poses economic impacts on the dairy sector, thus necessitating novel anthelmintic agents. *Olea europaea* L. (Olive) is a bioactive plant with potent pharmacological activities. However, its effects on oxyurids are poorly known, and no studies are currently exploring olives' anthelmintic potential. In this study, we investigated the pharmacokinetic behaviors of *O. europaea* leaves extract (OLE) and its phenolic compound oleuropein in mice infected with *A. tetraptera*, in comparison with Albendazole (ABZ), a standard drug used to treat parasitic worms. Fecal flotation method was used to identify the infestation with *A. tetraptera* eggs by examining the stool samples from mice. Infected animals were divided into 7 groups. 250 mg/kg, 500 mg/kg, and 1000 mg/kg doses of OLE, 5 mg/kg and 20 mg/kg doses of oleuropein, 10 mg/kg of ABZ and tap water were orally administered by gavage for 7 days during treatments. Drug efficacies and statistical differences between the treatments and controls were evaluated. Our results revealed 92.43 % efficacy of ABZ, similar to 92.19 % efficacy of 1000 mg/kg of OLE. At the same time, 250 mg/kg and 500 mg/kg concentrations of OLE remained 70.03 % and 63.18 % effective in reducing worm counts. Efficacy percentages of 5 mg/kg and 20 mg/kg of oleuropein were 9.27 % and 70.56 %, respectively. Statistical analysis of ABZ was significant compared to 1000 mg/kg of OLE, which was almost equal but insignificant. In general, our results confirm the anthelmintic potential of OLE and oleuropein against mice pinworms and open the way for targeted extraction of bioactive compounds from plants to optimize its use in human and veterinary medicine.
Keywords: *Olea europaea*; Oleuropein; *Aspiculuris tetraptera*; Anthelmintic effect; Olive leaves extract; Albendazole

Introduction

Helminths, commonly known as parasitic worms, are multicellular invertebrate organisms characterized by elongated, round, flat or cylindrical bodies (Castro, 1996). Parasitic infections are particularly prevalent in developing and underdeveloped countries with inadequate hygienic conditions. Approximately 1.7 billion people worldwide are infected with *Ascaris* (common roundworm), *Trichuris* (whipworm) and hookworm. Schistosomiasis is endemic in 70 countries, with millions suffering due to a lack of clean water and sanitation facilities (Hedley, 2015).

In endemic areas, these infections are treated periodically with

* – corresponding author

anthelmintic medicines. Piperazine hydrochloride, albendazole, mebendazole, ivermectin and levamisole are the approved drugs widely used to treat human and animal parasites (Yadav *et al*., 2017). For example, albendazole inhibits parasite microtubule polymerization by binding to the β-tubulin. This process disrupts the glucose uptake in nematodes, limiting them only to glycogen stores. As a result, it causes energy loss, consequently leading to the mortality of parasites (Venkatesan, 1998). Frequent treatment of helminths through anti-parasitic drugs has allowed parasites to develop resistance against these medicines. Resistance, as in other microorganisms, stems from the genetic mutations caused by deleting one or more alleles (Martin, 1997). If diseases are part of nature affecting humans, plants and animals, so do cures, as remedies are hidden in the very nature waiting to be explored. Before the industrial and scientific revolution, primeval people used plants, herbs, and shrubs to cure diseases and treat wounds. Therefore, scientists are now focusing on harnessing plants and their compounds to develop alternative medicines to overcome parasitic resistance with less adverse effects compared to pharmaceutical drugs.

Olea europaea L. (Olive) is a plant widely used in the Mediterranean region. Olive leaves, fruit, and oil are extensively consumed due to their tremendous pharmacological potential. Olives are rich in vitamins K, C and other antioxidants that can reduce the risks of cardiovascular diseases, cancer, inflammation caused by reactive oxygen species, diabetes, and high blood pressure (Soler-Rivas *et al*., 2000). Oleuropein is generally the most prominent phenolic compound found in olives. It belongs to secoiridoids, a specific group of coumarin-like compounds usually glycosidically bound and produced from the secondary metabolism of terpenes. Basically, oleuropein is an ester of hydroxytyrosol and oleosidic skeleton. Hydroxytyrosol is found in olives and olive oil, in the form of its elenolic acid ester, known as oleuropein (Soler-Rivas *et al*., 2000). Anticancer, antidiabetic, antimicrobial, antioxidant, antihypertensive and cardioprotective, enzyme inhibition, anti-inflammatory and antinociceptive, gastroprotective, neuroprotective, and other predominant biological activities of olives are also associated with oleuropein (Hashmi *et al*., 2015).

Aspiculuris tetraptera belongs to the Oxyuridae family. It is a natural and common parasite that inhabits the colon and cecum of mice and other rodents (Moulia *et al*., 1993; Kozan *et al*., 2006). *A. tetraptera* has a direct life cycle and infection occurs after ingestion of eggs by the host from environmental contaminants, which takes approximately 23 – 25 days. *A. tetraptera* eggs can be easily detected in feces (Behnke, 1974). Short embryonic period, direct development and incidence of autoinfection may also contribute to the pervasiveness of pinworms. Nevertheless, pinworm helminths are considered relatively non-pathogenic, and infections are known to be asymptomatic (Plachý *et al*., 2016). Therefore, *A. tetraptera* is widely used to determine the efficacies of various chemotherapeutic agents.

Given the above information and published literature, *A. tetraptera* was established as a model parasite in determining the unexplored anthelmintic potential of OLE and oleuropein.

Materials and Methods

Collection of the O. europaea leaves extract, Oleuropein and Albendazole

Aqueous extract of olive leaves was procured from Kale Natural Herbal Products, Foods Cosmetics, and Agricultural Products LTD. Co, Balıkesir, Turkey. Oleuropein content in the extract was 13 %, as described. Oleuropein, >98 % HPLC of analytical grade, was purchased from Gül Laboratory Equipment and Chemical substances and Bio Medical Scientific Products LTD. Co, Istanbul, Turkey. Albendazole was provided by the Faculty of Veterinary Sciences, Uludağ University, Bursa, Turkey.

Animals

Male and Female Balb/c albino mice (25 – 30g) were purchased from Uludağ University experimental animals center, Bursa, Turkey. Animals were housed in standard cages, kept at recommended room temperature (20 – 22°C) with 12 hrs artificial light and 12hrs dark cycles for 7 days prior to pharmacological studies with free access to standard laboratory feed and tap water. All animals were deprived of food overnight before dosing, except the tap water was available *ad libitum*.

Fecal Flotation Method

The saturated NaCl (salt) flotation method was used to diagnose *A. tetraptera* eggs from stool samples collected from each cage (Sueta *et al*., 2002).

In vivo experimental assay

Animals were exposed to pinworm-contaminated bedding from an in-house colony of naturally infected mice with *A. tetraptera*. The stool samples from approximately 200 mice were microscopically examined for endoparasite eggs of *A. tetraptera* using a fecal flotation technique for detecting infected mice. Infected animals were randomly distributed into 7 groups (n=13). OLE, oleuropein, and ABZ were orally administered for 7 days. Groups 1, 2, and 3 (experimental mice) were treated with 250 mg/kg, 500 mg/kg and 1000 mg/kg concentrations of OLE, and groups 4 and 5 (experimental mice) received 5 mg/kg and 20 mg/kg of % 98.0> oleuropein at body weight. Animals in Group 6 were constituted as untreated control and were given tap water. Group 7 was given a 10 mg/kg dose of a reference drug, ABZ. 21 animals randomly died during oral administrations, leaving 70 animals for autopsy, as mentioned in Table 1.

Mice were euthanized by cervical dislocation for necropsy on the 8th-day post-treatment. Gastrointestinal tract was removed and washed with normal saline. The contents were examined under a stereomicroscope for adult *A. tetraptera* parasites. *Syphacia* spp. was also found during necropsies. The numbers of these parasites

were determined by keeping records. Despite *A. tetraptera* being a prime target of our study, data about *Syphacia* spp was also included in Table 2 to increase the robustness of this study.
Efficacy was calculated from the geometric means of *A. tetraptera* counts as mentioned below:

$$\%\ Efficacy = \frac{a - b}{a} \times 100$$

"a" is the geometric mean number of *A. tetraptera* in the control group and "b" is the geometric mean number of *A. tetraptera* in the treated group.

Statistical analysis of data
Data obtained from experimental groups were expressed as statistical mean ± standard error of the mean (SEM). Statistical differences between the treatments and the control groups were evaluated by Minitab (Version 15.0: Minitab Inc., State College, PA, USA) and Mann-Whitney U tests. P<0.05 was considered to be statistically significant.

Ethical Approval and/or Informed Consent

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on the care and use of laboratory animals. Experiments followed the ethical, care and non-unnecessary suffering regulations mandated by the Turkish Animal Ethics Committee of Bursa Uludağ University (Ethical council number: UÜHADYEK-2020-05/02).

Results

During the study, no adverse effects and reactions were observed clinically in any of the mice treated with olive leaves extracts and oleuropein. The number of adult *A. tetraptera* collected from the gastrointestinal tracts of control and experimental groups, min-max values, geometrical means, and percentages of efficacy are given in Table 1. Total *A. tetraptera* in animal groups administered with 250 mg/kg, 500 mg/kg and 1000 mg/kg of OLE were; 1,608, 1,976, and 419, while mice treated with 5 mg/kg and 20 mg/kg of oleuropein had 4,869 and 1,580 parasites respectively. Control and ABZ-treated groups contained 5,367 and 406 worm counts. On the other hand, a total of 5,342 *Syphacia* spp. counts were observed during necropsies in control and all the treated groups. The numbers of these species were much lower as compared to *A. tetraptera*. Hence, it is most unlikely that *Syphacia* spp. parasites have interfered with the results. The geometric means of adult *A. tetraptera* obtained in the control group were significantly higher, followed by 5 mg/kg oleuropein and 500 mg/kg OLE. The remaining groups had comparatively lower geometric mean values. Efficacies of 500 mg/kg and 5 mg/kg oleuropein were low. 1000 mg/kg concentration of OLE and 10 mg/kg ABZ illustrated high efficacy percentage, while 250 mg/kg OLE and 20 mg/kg dose of oleuropein displayed moderate anthelmintic effects. Statistical analysis of ABZ was found to be significant (p<0.05), while that of 1000 mg/kg OLE was equal but not significant (p=0.05). Statistical analysis of remaining concentrations was not significant (p>0.05). In the case of oleuropein, anthelmintic effects increased in a dose-dependent manner. Since oleuropein is the most prominent phenolic compound in olive cultivars, the anthelmintic activity of *O. euro-*

Table 1. The number of *A. tetraptera* collected from autopsied animals in treatment and control groups and descriptive statistical data (n=13).

Groups	The number of animals autopsied post-treatment (n)	The number of minimum and maximum ***A. tetraptera*** collected in necropsy (Min.-Max.)	Mean ± SEM	Median	Q1	Q3	Efficacy (%)
Olea europaea 250 mg/kg	11/13	0 – 1055	146.2 ± 92.9	48	1	115	70.03
Olea europaea 500 mg/kg	6/13	1 – 863	329 ± 153	258	2	661	63.18
Olea europaea 1000 mg/kg	6/13	0 – 365	69.8 ± 59.3	9	0	365	92.19
Oleuropein 5 mg/kg	13/13	2 – 959	374.5 ± 88.7	286	92.5	665.5	9.27
Oleuropein 20 mg/kg	11/13	1 – 397	143.6 ± 45.5	56	19	299	70.56
Tap Water (control group)	10/13	0 – 1661	537 ± 180	381	35	950	0
Albendazole 10 mg/kg	13/13	0 – 153	31.2 ± 12.9	4	0	58	92.43

paea can be attributed to this compound. The results of the *in vivo* anthelmintic assay and related statistical data are presented in Table 1.

Discussion

Today, it is estimated that approximately one-third of three billion people living in underdeveloped and developing regions of Asia, sub-Saharan Africa, and America are directly or indirectly exposed to co-infestations. Ascariasis, trichuriasis, hookworm, tapeworm, intestinal helminths, and schistosomiasis infections are most prevalent (Hotez *et al*., 2006; Hotez *et al*., 2007). Hookworm and schistosomiasis can induce complications during pregnancy, resulting in reduced neonatal birth weight, premature birth, and increased maternal morbidity as well as mortality (Christian *et al*., 2004).

A plethora of anthelmintic drugs is used to treat helminth infections worldwide. Approved anthelmintic medicines are becoming inadequate in the face of resistance exhibited by the parasites. Kingdom *Plantae* has always been a tremendous source of natural remedies against infections, inflammation, gastrointestinal and cardiovascular diseases. Gürağaç Dereli *et al*. (2019) reported that methanolic extract of aerial parts of *Polygonum cognatum* reduced *S. obvelata* egg count by 66.8 % and *A. tetraptera* by 73.4 % as compared to doramectin (100 %). It was deduced that the anthelmintic activity of *P. cognatum* could be related to phenolic compounds and tannins present in the plant (Gürağaç Dereli *et al*., 2019). In another study, 250 µL methanolic extract of *Plantago major* leaves showed 27.62 % and 250 µL aqueous extract illustrated 39.25 % efficacy, much lower than 0.2 mg kg^{-1} dose of ivermectin, which exhibited 88.57 % anthelmintic efficacy against *A. tetraptera* respectively (Türel *et al*., 2013). In another study, aqueous and ethanol extracts of *Cucurbita maxima* showed lower efficacy than ivermectin (91 %) against *A. tetraptera* (Ayaz *et al*., 2015). The studies mentioned above suggest that specific plants and their compounds can possess anti-parasitic activities against helminth infections. Unexpected results have also been reported in another *in vivo* anthelmintic assay in which 250 mg/kg, 500 mg/kg and 1000 mg/kg of *Rosmarinus officinalis* (Rosemary) plant extracts showed low anti-parasitic activities; 25.44 %, 46.54 %, and 7.16 %, against *A. tetraptera* infected mice. On the contrary, rosmarinic acid, a polyphenol constituent of the rosemary plant, increased worm burden by -8.17 % as compared to ABZ, which caused an 89.1 % reduction in *A. tetraptera* counts (Eylek, 2021). Polyphenols-rich and tanniferous plant extracts or isolated tannins have not been proven to exert anthelmintic activities equal to that of the readily available drugs in the sense of half maximal effective concentration (EC_{50}) values (Spiegler *et al*., 2017). However, there is still a huge difference between *in vitro* or low-impact animal studies and evidence regarding the unambiguous efficacy of polyphenols and polyphenols–enriched extracts.

A safety assessment study of ethanolic extract of *O. europaea* leaves in Winstar rats was conducted to investigate OLE's acute and subacute oral toxicity. Acute toxicity was assessed using a single dose of 2000 mg/kg of ethanolic extract of olives (EEO) and subacute toxicity was evaluated during 28 days using 100 mg/kg, 200 mg/kg and 400 mg/kg doses of EEO, administered by oral gavage. According to the results, neither single nor three different concentrations of EEO induced mortality or any sign of abnormalities and toxicities among the treated animals (Guexa *et al*., 2018). This study implied that OLE and oleuropein could be used at higher concentrations to study their anthelmintic potential in animals or humans. Since indigenous people in the Mediterranean region consume olives and related products, no fatal side effects among humans stemming from olive consumption have been reported till today. Bhattacharjee *et al*. (2016) investigated the anthelmintic effects of crude aqueous, petroleum ether, chloroform, and methanol extract of *O. europaea* leaves on *Pheretima posthuma* (earthworm). According to the study, various extracts of *O. europaea* showed significant worm reduction compared to piperazine citrate (Bhattacharjee *et al*., 2016). The study and its' result seem ambiguous because anthelmintic effects were analyzed *in vitro* in free-living and soil-dwelling worms, and no efficacy percentage was evaluated to support the findings. *O. europaea* leaves contain immense antioxidant properties. Lins *et al.* (2018) suggested in a recent study that OLE inhibits the action of reactive oxygen species (ROS) and protects human red blood cells against oxidative damage.

Oleuropein has low anthelmintic validity because it has not been studied thoroughly for its anti-parasitic potential (Bisignano *et al*., 1999). However, oleuropein has several potent pharmacological properties, including antioxidant, anti-inflammatory (Moa *et al*., 2019), hypotensive (Tsoumani *et al*., 2021), anti-neuropathic, anticancer, antimicrobial (Gamli, 2016), and hypoglycemic (Cristiano *et al*., 2021). Oleuropein also decreases low-density lipoprotein (LDL) levels in the blood (Ahamad *et al*., 2019). In addition, the cardioprotective activity of *O.europaea* can be attributed to

Table 2. Efficacy percentages (%) of *Olea europaea*, Oleuropein, and Albendazole used in different concentrations against *Syphacia* spp. compared to the control group (water).

Control groups	Efficacy Percentage (%)						
	Parasite species	Oleuropein 5 mg/kg	Oleuropein 20 mg/kg	*Olea europaea* 250 mg/kg	*Olea europaea* 500 mg/kg	*Olea europaea* 1000 mg/kg	Albendazole 10 mg/kg
Water (Control) Group	*Syphacia spp.*	-83.70	-218.84	-413.73	-531.30	-110.54	2.23

oleuropein (Andreadou *et al*., 2007). Oleuropein also exhibits anti-ischemic and hypolipidemic activities (Andreadou *et al*., 2006). Several compounds, like polyphenols, alkaloids, flavonoids and terpenes etc., may be causative agents for the anthelmintic activity of the plants, particularly; polyphenols compounds are known to have anti-parasitic potential. Phenolic compounds may exhibit their anthelmintic activity by uncoupling oxidative phosphorylation or binding to free proteins in the gastrointestinal tract of host animals (Mali & Wadekar, 2008).

Polyphenols or tannins extracted from plants can be used for long-term chronic therapeutics rather than for treating acute parasite infections in animals and humans. Hence further research is required to elucidate the mode of action of polyphenol or tannin-enriched plant extracts, which could possibly lead to the identification of potential drug targets.

Conclusion

In light of the derived results, it can be concluded that the anthelmintic activity of *O. europaea* has stemmed from oleuropein. Our study illustrates the high anthelmintic potential of OLE against *A. tetraptera*. Anthelmintic activity of 250 mg/kg, 1000 mg/kg, 5 mg/kg and 20 mg/kg concentrations of OLE and oleuropein increased in a dose-dependent manner except that of 500 mg/kg. This unexpected deviation might have resulted from mishandling and random placement of oral gavage into the trachea of mice instead of the esophagus during the treatment, which led to the suffocation and instant death. Although 1000 mg/kg of OLE reduced a significant amount of parasite counts, it must be kept in mind that the number of animals in this treatment group also declined to 6 compared to ABZ, which remained 13. Since olive is known to be non-toxic, further studies with higher doses of OLE and oleuropein are required to explore different dimensions of our findings. Oleuropein holds potential anthelmintic effects that can be used either as an alternative drug or a supplementary diet to treat parasitic resistance. We also suggest determining Eggs per gram (EPG) values during the formation of the groups in drug efficacy trials against *A. tetraptera* infections in mice to consolidate the results in future studies. When ingested, oleuropein metabolizes into hydroxytyrosol. Therefore, various pharmacological aspects of olives may be attributed to hydroxytyrosol and more comparative studies are required to test this hypothesis.

Conflict of Interest

The authors state no conflict of interest.

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