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Antimicrobial evaluation of *Tripleurospermum callosum* (Boiss. & Heldr.) E. Hossain extracts using *in vitro* and *in vivo Caenorhabditis elegans* model against urinary system pathogens

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ABSTRACT

Tripleurospermum callosum (Boiss. & Heldr.) E. Hossain was recorded in Turkish ethnobotanical data for its use against urinary and respiratory system ailments. Infusion, decoction and 96% ethanol extracts of *T. callosum* aerial parts were prepared for in vitro antimicrobial activity against urinary system pathogens *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella aerogenes* ATCC 1348 and *Candida albicans* ATCC 10231. The non-toxic concentrations of extracts and *in vivo* antimicrobial assay were performed using *C. elegans*. The extracts were analysed by Liquid Chromatography Mass Spectrometry (LC-MS/MS) for phytochemical composition. The water extracts were non-toxic at between 5000 and 312 µg/mL, while 96% ethanol extract at 312 µg/mL for *C. elegans*. The infusion extract showed *in vivo* anti-infective effect 5000–312 µg/mL against Gram-negative strains. The results indicate a potential role of plant extracts with relatively non-toxic and anti-infective effects against urinary system pathogens.

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Tripleurospermum callosum; antimicrobial effect; urinary system pathogens; *Caenorhabditis elegans*

Introduction

Microbial infections are one of the most important health concerns for humans, one of which is urinary system infections (USI) caused by *Escherichia coli* and various members of the pathogenic genera *Proteus, Pseudomonas, Klebsiella, Enterobacter* and *Staphylococcus*. USIs are the most common bacterial infections encountered and show differences depending on the age and gender of the affected people. The eradication of USI relies on antibiotic use, both on an individual and clinical therapeutical basis, which may also be frequently associated with resistance cases (Sheerin 2011). According to international (Van Wietmarschen et al. 2022) and national traditional data of Türkiye (Bulut et al. 2017) herbal medicinal drugs are still used for the treatment and prophylaxis of many infections, including USI.

According to the relevant botanical data, more than 40 species of the genus *Tripleurospermum* Schultz Bip. within Asteraceae is documented in Europe, North Africa,

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Asia, and North America (Himmelreich et al. 2008). Enayet Hossain (1975) revised the genus for the Flora of Turkey and the East Aegean Islands, describing 26 taxa (İnceer 2012). Recently, five new taxa from Turkey were identified, and two new records were added, increasing the number of *Tripleurospermum* taxa in Turkey to 33 (29 species and 4 varieties) of which 17 are endemic to the country (Tekşen et al. 2022).

Ethnobotanical data for the endemic species *Tripleurospermum callosum* (Boiss. & Heldr.) E. Hossain was documented for the first time in Çamlıdere, Ankara, Türkiye. Its flowers are used as herbal tea against urinary infections, for shortness of breath, common cold, asthma, bronchitis, and against kidney Stones (Günbatan et al. 2016).

Some *Tripleurospermum* species were reported to show antimicrobial activity, and cytotoxic effects (Erdoğan et al. 2013; Tofighi et al. 2015). The antimicrobial activity of *T. parviflorum* (Willd.) Pobed. was investigated by Erdoğan et al. (2013). The antimicrobial effects of aerial part extracts of *T. disciforme* (C.A. Mey) Schultz Bip were evaluated against the Gram-positive, Gram-negative and fungal species such as *Candida albicans* and *Aspergillus niger*. According to the literature search, there is only one bioactivity study *on* antiplatelet and hematological activities of *T. callosum*, along with aldose reductase and anti-coagulation activities were determined (Enomoto et al. 2004).

The present study aimed to reveal the antimicrobial effects of aqueous and ethanol extracts of aerial parts of *T. callosum* on selected urinary system pathogens *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, *K. aerogenes* ATCC 1348, and *C. albicans* ATCC 10,231. In addition to the determination of the safe and non-toxic concentrations of the active extracts, the lifespan assay was used *in vivo* using the nematode model *Caenorhabditis elegans*. We used the *C. elegans*-pathogen infection assay to determine antimicrobial activity from non-toxic concentrations of the active extracts by infecting the nematode with the urinary system pathogens. The phytochemical composition of the evaluated extracts was performed by Liquid Chromatography Mass-Spectrometer (LC-MS/MS).

Materials and methods

The plant material

Tripleurospermum callosum was collected in full flowering stage in July 2017 from Isparta, Türkiye (C3, Isparta, Sütçüler, Dedegöl Dağı, Melikler Yaylası, 1735 m, 2017, N 39° 38' 8'', E 31° 57' 21'') identified by YBK. A voucher specimen (ESSE 15,409) of the species is deposited in the Herbarium of the Faculty of Pharmacy at Anadolu University.

Strains

The human urinary system pathogens *Escherichia coli ATCC* 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella aerogenes* ATCC 1348, and *Candida albicans* ATCC 10231 acquired from American Type Culture Collection (ATCC) were used.

Preparation of crude extracts

Infusion, decoction, and ethanol extracts were prepared from the aerial parts. The dried aerial parts were cut into small pieces, and an aqueous extract was made by soaking 2 g of the plant material in 200 mL freshly boiled distilled water for 5 minutes, and allowing it to stand at room temperature. The decoction was also prepared using 2 g plant material set in 200 mL cold distilled water, which was boiled and cooled. After filtration, the water extracts were separately lyophilized. The plant material was ground finely and macerated manually in 96% ethanol at 25°C for 3 days, filtered and evaporated to dryness using vacuum. All the extracts were stored at 2–8 °C until further use.

Phytochemical analysis by Liquid Chromatography Mass-Spectrometer (LC-MS/MS)

An Absciex 3200 Q trap MS/MS detector was used for the LC-MS/MS analyses. A Shimadzu 20A HPLC system was used for the experiments, along with an Applied Biosystems 3200 Q-Trap LC-MS /MS device, with an ESI source working in negative ion mode. A GL Science Intersil ODS 250 4.6 mm i.d., 5 µ particle size, octadecyl silica gel analytical column running at 40°C was employed for the chromatographic separations. The flow rate of the solvent was kept constant at 0.5 mL/min. A PDA detector was used. The mobile phases in the elution gradient were (A) methanol:water: formic acid (10:89:1, v/v/v), and (B) methanol:water:formic acid (89:10:1, v/v/v), respectively. In 40 minutes, B's concentration increased from 10% to 100%. The Analyst 1.6 software was used to collect and analyze data from the LC-MS/MS system.

Minimum inhibitory concentrations (MIC, µg/mL)

Antimicrobial activities of the plant extracts and standard antimicrobials were evaluated according to the previous method (Göger et al. 2018) by a modification of the CLSI (M100-S16) and CLSI (M27-A2) microdilution techniques. Clarithromycin, moxifloxacin, cefuroxime, ampicillin, terbinafine, and fluconazole were used as the standard antimicrobial substances. The extracts were initially diluted twofold, with a final concentration range of 5120–10 µg/mL, whereas the standard antimicrobial substances were used in the range of 64–0.125 µg/mL. The mean MIC values from three independent experimental experiments were calculated.

Maintenance of *C. elegans*

Caenorhabditis elegans glp-4(bn2ts); sek-1(km4) and its food source, *Escherichia coli* OP50 strain, were provided by the Caenorhabditis Genetics Center, University of Minnesota, Minneapolis. Nematodes were maintained on the Nematode Growth Medium (NGM) agar, which was inoculated with an overnight culture of the *E. coli* OP50 strain in Luria-Bertani broth, as described previously (Stiernagle 2006).

Preparation of synchronize *C. elegans*

The previously described bleaching technique was used for synchronizing *C. elegans* (Ikeda et al. 2007). *C. elegans* strain was transferred to *E. coli* OP50 seeded NGM agar plates (in 6 cm Petri plates) by the chunking method, and the plates were incubated at 15°C for 3 days. After incubation, the plates were washed with 2 mL of M9 buffer to enable the transfer of gravid nematodes and eggs into centrifuge tubes. M9 Buffer was added to each tube to reach a final volume of 3.5 mL. Then, 0.5 mL of sodium hydroxide and 1 mL of 5% sodium hypochlorite solutions were added into each tube, and they were mixed gently and incubated at room temperature for 5 min. During incubation, living nematodes died while eggs survived. Following centrifugation of the tubes at 1300 g for 30 s, the supernatant was discarded, and egg pellet was resuspended in 5 mL of M9 buffer for a second centrifugation at 1300 g for 30 s. The pellet was washed twice with M9 buffer, as described above. After the final centrifugation, the pellet was resuspended in 7 mL of M9 buffer, and incubated at 15°C for 18 hours, during when hatched L1 larvae were obtained. These synchronized nematodes were used for further assays.

Evaluation of plant extracts on the life span of *C. elegans*

To determine the effect of the extracts on the life span of *C. elegans*, the broth culture method with some modifications was used (Solis and Petrascheck 2011). To provide the food source for *C. elegans*, one colony of *E. coli* OP50 was put into 5 mL of TB containing 100 g/mL of ampicillin

and 0.1 g/mL of amphotericin B and incubated overnight at 37°C. The bacterial culture was diluted (1:2000) in TB containing 50 µg/mL ampicillin at 37°C for 8–12 hours. The culture was centrifuged at 3500 rpm for 10 minutes after incubation. Bacterial pellets were washed in sterile distilled water and then resuspended at a concentration of 100 mg/mL in the S-medium. The obtained OP50 solution was stored at 4°C for use as a food source for *C. elegans*. L1 larvae collected after synchronization were diluted in the S-medium at 80–100 nematode/S-medium concentration, and amphotericin B and OP50 solutions were added at 0.1 µg/mL and 0.1 µg/mL concentrations, respectively. About 120 µL of the solution containing L1 larvae was transferred to 96-well plates and incubated at 25°C for 2 days. After incubation, the number of nematodes in each well was determined by using an inverted microscope, and wells containing more than 18 nematodes were not included in the experiment. Extracts were added to the wells at a final concentration of 5000–2500-1250-625 and 312 µg/mL. The dead and alive nematode numbers were scored every 24 hours, and the experiment was continued until all nematodes died. During the experiment, 5 µL of the OP50 solution was added to each well on the 5th, 12th and 19th days of the experiment to feed the nematodes. Mortality and survival were analyzed using the GraphPad Prism program. The significant differences between the groups to which different concentrations were added for each plant extract and the control group were determined by the survival analysis (Logrank test).

Pathogenicity of urinary system pathogens using the *C. elegans* model

Synchronized *C. elegans* L1 larvae were obtained as described above. M9 buffer containing L1 larvae was incubated on ice for 15 minutes and then the supernatant was removed. Pellets containing L1 larvae were transferred to *E. coli* OP50-seeded NGM agar, and the plates were incubated at 25°C for 48–52 h. After incubation, the plates were washed with M9 buffer to collect the nematodes into centrifuge tubes. The supernatant was removed after centrifuging the tubes at 1000 rpm for 1 minute, and the pellet was then washed twice with M9 buffer. Then, the nematodes were transferred to NGM agar seeded with pathogenic bacterial strains seeded and incubated at 25°C for 8–12 hours (Moy et al. 2006). Following incubation, infected nematodes were collected and washed with M9 buffer as described earlier. Nematodes that were infected were transferred to media that contained 20% Brain Heart Infusion (BHI) and 80% M9 buffer. About 100 µL of media containing infected nematodes and an equal volume of plant extracts were transferred into the wells of 96-well plates. The plates were covered with gas-permeable membranes and incubated at 25°C under static conditions. Survival was scored every 24 hours until all nematodes died. Living nematodes maintained their sinusoidal postures and were observed to be mobile, while dead nematodes were observed to be flat, immobile, and full of bacteria. The time of death of 50% of the nematodes (TD_{50}) was calculated by using the GraphPad Prism program.

The equation: Y = Bottom + (Top – Bottom)/(1 + 10^[(LogEC_{50} – X)*HillSlope)], where X is the logarithm of days, Y is the percentage of dead worms; the bottom value is 0 and the top value is 100. The calculated EC value was determined as TD_{50}.

Anti-infective effect of extracts using the *C. elegans* model

The broth culture method with some modifications was used as previously described (Moy et al. 2006). Nematodes were infected with the urinary tract pathogens as described above. After incubation, infected nematodes were collected and washed with M9 buffer. Infected nematodes were transferred to media composed of 20% BHI and 80% M9 buffer. About 50 µL of media containing infected nematodes, and an equal volume of the test extracts were transferred into to 96-well plates. The concentration to be used for the extracts was determined according to the non-toxic concentrations determined in the experiments described in Section 2.8. In this experiment, 5000, 2500, 1250, 625 and 312 µg/mL concentrations for infusion and decoction extracts, and 312 µg/mL concentrations for ethanol extracts were used. The plates were

Table 1. Colonization values for pathogens in the nematode-interacted extract concentrations (µg/mL).

Strain	Extract concentration(µg/mL)	log cfu/nematod (mean±std deviation)
E. coli	**Infusion**	
ATCC 8739	5000	4.447 ± 0.834
	2500	4.405 ± 0.132
	1250	4.576 ± 0.028
	625	4.421 ± 0.279
	312	4.602 ± 0.100
	Decoction	
	5000	5.097 ± 0.087
	2500	4.922 ± 0.066
	Control	4.564 ± 0.260
P. aeruginosa	**Infusion**	
ATCC 9027	5000	5.051 ± 0.022
	2500	4.718 ± 0.003
	1250	4.550 ± 0.192
	Decoction	
	5000	5.085 ± 0.038
	%96 ethanol	
	312	4.500 ± 0.097
	Control	4.452 ± 0.156
K. aerogenes	**Infusion**	
ATCC 1348	2500	4.505 ± 0.129
	1250	4.800 ± 0.085
	625	4.905 ± 0.011
	312	4.947 ± 0.004
	Decoction	
	1250	4.540 ± 0.049
	Control	5.066 ± 0.049

incubated at 25°C under static conditions while being covered with gas-permeable membranes. Negative control wells without any extracts were present on each plate. Manual scoring was done for worm survival.

Effect of extracts on the colonization of urinary system pathogens using *C. elegans*

The nematodes were infected with urinary system pathogens and transferred on 96-well plates as described earlier. An equal volume of the extracts (50 µL) was also added to the plates. The nematodes were washed 3 times with M9 buffer containing 1 mM sodium azide. An average of 10 nematodes were transferred to a microcentrifuge tube, and the final volume was made up to 250 µL. Nematodes were lysed using a sterile pestle. Serial dilutions of the suspension were prepared and plated on Luria-Bertani agar plates to determine the bacterial counts (CFUs). The CFU values for each nematode were determined according to the formula (number of colonies x dilution factor x 50 µL nematode lysate)/number of nematodes, and logarithmic conversions of CFU/nematode values were performed (Table 1). The colonization of the pathogens in the medium without the extract was also calculated, and the data were given as controls in the table.

Student's t-test was used to determine for a possible statistically significant difference between the colonization values calculated from the nematodes in the wells to which the extracts were added and the colonization values calculated from the nematodes in the wells without the extract for each pathogen.

Results and discussion

Yields of plant extracts

Yields for extracts prepared from *Tripleurospermum callosum* were calculated as infusion (3.2%), decoction (3.7%) and alcohol extracts (3.0%).

Phytochemical analysis by LC-MS/MS

LC-MS/MS analysis was performed on infusion, decoction, and 96% ethanol extracts (Fig. S1) and 24 compounds were identified, six of which were determined as chlorogenic acids and quinic acid esters and the remaining as flavonoids (Table 2). Chlorogenic acid (3-Caffeoylquinic acid) was determined in the aqueous extracts. 5-Caffeoylquinic acid (Fig. S2) was found as the main compound in all extracts. However, caffeic acid was detected only in the aqueous extracts. 4,5-Dicaffeoylquinic acid (Fig. S3) was determined in all extracts, but in trace amounts in the ethanol extract. All extracts contained 1,5-dicaffeoylquinic acid (Fig.S4), which was one of the main compounds. The main flavonoid-type compounds were identified as quercetin glucoside (Fig. S5), quercetin acetylglucoside (Fig. S6), luteolin glucoside (Fig. S7), luteolin acetylglucoside (Fig. S8), apigenin glucoside (Fig. S9), apigenin acetylglucoside (Fig. S10), isorhamnetine (Fig. S11), and luteolin, respectively (Fig. S12). The mass spectra of the detected major compounds were listed in the Suplementary material.

Previous studies were generally on chemical analysis of *Tripleurospermum* sp. essential oils (Yaşar et al. 2005; Kilic and Bagci 2012). All *Tripleurospermum* compounds except isorhamnetin diglucoside, quercetin glucuronide, quercetin acetylglucoside and luteolin acetylglucoside were previously reported (Greger 1975; Sibul et al. 2020; Chen et al. 2022).

To the best of our knowledge, all *Tripleurospermum callosum* compounds reported in the present study were detected by LC-MS/MS for the first time. The relative concentration of the compounds analysed within the extracts was highest in the infusion, less in the decoction, and the least in the ethanol extract.

Table 2. LC/MS-MS analysis of *T. callosum* extracts.

No	Rt	$[M-H]^-$	Fragments	Identified as	Extract	Ref
1	3,7	499	353, 205, 191, 173	p-coumaroyl-caffeoylquinic acid	I, D,E	(Clifford et al. 2006)
2	4,4	391	373, 217, 191, 173, 155	Citric acid derivative	I, D	(Mena et al., 2012; Spínola et al. 2015)
3	8,6	353	191, 179,135	3-Cafeoylquinic acid	I, D	(Clifford et al. 2003)
4	12,1	353	191, 179,161	5-Caffeoylquinic acid*	I, D, E	(Clifford et al. 2003)
5	14,1	179	135	Caffeic acid	I,, D,	(Clifford et al. 2003)
6	15,3	337	191,173,163	5-p-Coumaroylquinic acid	I, D, E	(Clifford et al. 2003)
7	17,1	479	317, 287	Smilar to myrcetin glucoside	I, D,	(Abdel-Hameed et al., 2014; Tuominen et al. 2013)
8	17,9	463	301, 287, 151,	m/z 287 + glucuronide	I, D, E	
9	18,4	681	519, 477, 315, 191	Isorhamnetin diglucoside	I, D,	(Boukhris et al. 2016; Shang et al., 2006)
10	19,4	463	301, 151, 107	Quercetin glucoside*	I,D, E	(Abu-Reidah et al. 2013)
11	19,5	515	353, 335, 191, 179, 173,161	1,5-Dicafeoylquinic acid*	I,D, E	(Clifford et al. 2005)
12	19,9	477	301	Quercetin glucuronide	I	(Abu-Reidah et al. 2013)
13	20,7	447	285, 256, 151, 133	Luteolin-7-glucoside*	I, D, E	(Abu-Reidah et al. 2013)
14	21,2	609	301, 273, 179, 151	Rutin	I,D, E	(Barros et al. 2011)
15	21,4	463	300, 179, 151	Quercetin glucoside*	I, D, E	(Abu-Reidah et al. 2013)
16	21,8	515	353, 335, 191, 179, 173,161	4,5-Dicafeoylquinic acid*	I,D,E	(Clifford et al. 2005)
17	22,5	505	301, 179, 151	Quercetin acetylglucoside*	I, D, E	(Ruiz et al. 2015)
18	22,6	489	447, 429, 327, 285	Luteolin acetylglucoside	I, D, E	(Lin and Harnly 2010)
19	22,9	477	313, 300, 287, 283, 271	Isorhamnetin glucoside*	I, D, E	(Barros et al. 2013)
20	23,1	431	269,241, 149, 117	Apigenin glucoside*	I, D, E	(Lin and Harnly 2010)
21	23,8	489	447, 327, 284	Luteolin acetylglucoside*	I, D, E	(Lin and Harnly 2010)
22	24,9	473	413, 268	Apigenin acetylglucoside*	I, D,E	(Helmja et al. 2008)
23	25,6	315	299/300, 285, 271	Isorhamnetin*	I, D, E	(Simirgiotis et al. 2013)
24	27,5	301	179, 151	Quercetin	I,D,E	(Taamalli et al. 2012)
25	28,6	285	175, 151, 133	Luteolin*	I, D, E	(Abu-Reidah et al. 2013)

I: infusion, D: decoction, E: EtoH extract, *: Main compounds.

Minimum inhibitory concentrations (MIC, µg/mL)

In the present study, the extracts were tested against the urinary system pathogens *E. coli*, *S. aureus, P. aeruginosa*, *K. aerogenes*, and *C. albicans* standard strains for the first time. The MIC values of the extracts are listed comparatively in Table 3. The water extracts showed similar MIC values of >2500–1250 µg/mL, while the ethanol extract showed a MIC concentration of 312.5–1250 µg/mL. The ethanol extract was more effective with a MIC value of 625 µg/mL against *E. coli*, *S. aureus*, *K. aerogenes*, and 312.5 µg/mL against *C. albicans*, respectively. To the best of our knowledge, this is the first study on the antimicrobial effects of *T. callosum*.

Evaluation of plant extract on the life span of *C. elegans*

According to the results obtained with the infusion extract, no significant difference was found for the concentrations of 5000–1250 µg/mL, however a significant difference was observed for the concentrations of 625 µg/mL and 312 µg/mL ($p < 0.05$). The experimental results showed that 625 µg/mL and 312 µg/mL concentrations did not shorten the lifespan of the nematodes, according to the calculated TD_{50} values. The TD_{50} concentration values were 625 µg/mL and 312 µg/mL for 14.2 days, and 15.9 days, respectively. To compare the results, TD_{50} values for the control group were 12.8 days. Thus, all tested concentrations used in the study for the infusion extract were regarded as non-toxic for *C. elegans* (Figure 1A).

Similar results were obtained for the decoction extract. No significant difference was observed for the concentrations of 5000–312 µg/mL, however a significant difference was observed here also for the concentrations of 625 µg/mL ($p < 0.05$). TD_{50} calculated from the group treated with 625 µg/mL decoction extract was 12.3 days. Compared to the control group, the tested concentration was also classified as non-toxic. The decoction extract concentrations used in the study did not affect the life span of the nematodes ($p < 0.05$) (Figure 1b).

Interestingly, the 96% ethanol extract concentrations used were effective on *C. elegans* lifespan. According to the experimental results for the ethanol extract, a significant difference was observed for the 5000–312 µg/mL concentrations ($p < 0.05$). The tested 5000–625 µg/mL concentrations shortened the life span of the nematodes (Figure 1c). TD_{50} results calculated from the group with 312 µg/mL ethnaol extract were 14.5 days, compared to the control group regarded as non-toxic.

Both infusion and decoction extracts at a 5000 µg/mL concentration, which was above the *in vitro* MIC, were also non-toxic for tested *C. elegans*. The non-toxic concentration (312 µg/mL) determined for the 96% ethanol extract was equal to the *in vitro* MIC value obtained for *C. albicans*, while it was below the *in vitro* MIC determined for *E. coli*, *S. aureus*, *K. aerogenes* and *P. aeruginosa*, respectively.

Table 3. The minimum inhibitory concentration of *T. callosum* extracts (MIC, µg/mL).

samples	Microorganisms				
	E. coli ATCC 8739	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 9027	*K.aerogenes* ATCC 1348	*C.albicans* ATCC 10231
Infusion	**1250**	**1250**	**>2500**	**2500**	**>2500**
Decoction	**1250**	**1250**	**>2500**	**2500**	**>2500**
Ethanol (96%) extract	**625**	**625**	**1250**	**625**	**312.5**
Clarithromycin	**>64**	**4**	**2**	**4**	-
Moxifloxacin	**0.125 >**	**0.125 >**	**0.125 >**	**2**	-
Cefuroxime	**8**	**2**	**4**	**2**	-
Ampicillin	**1**	**2**	**8**	**16**	-
Terbinafine	-	-	-	-	**>64**
Fluconazole	-	-	-	-	**>64**

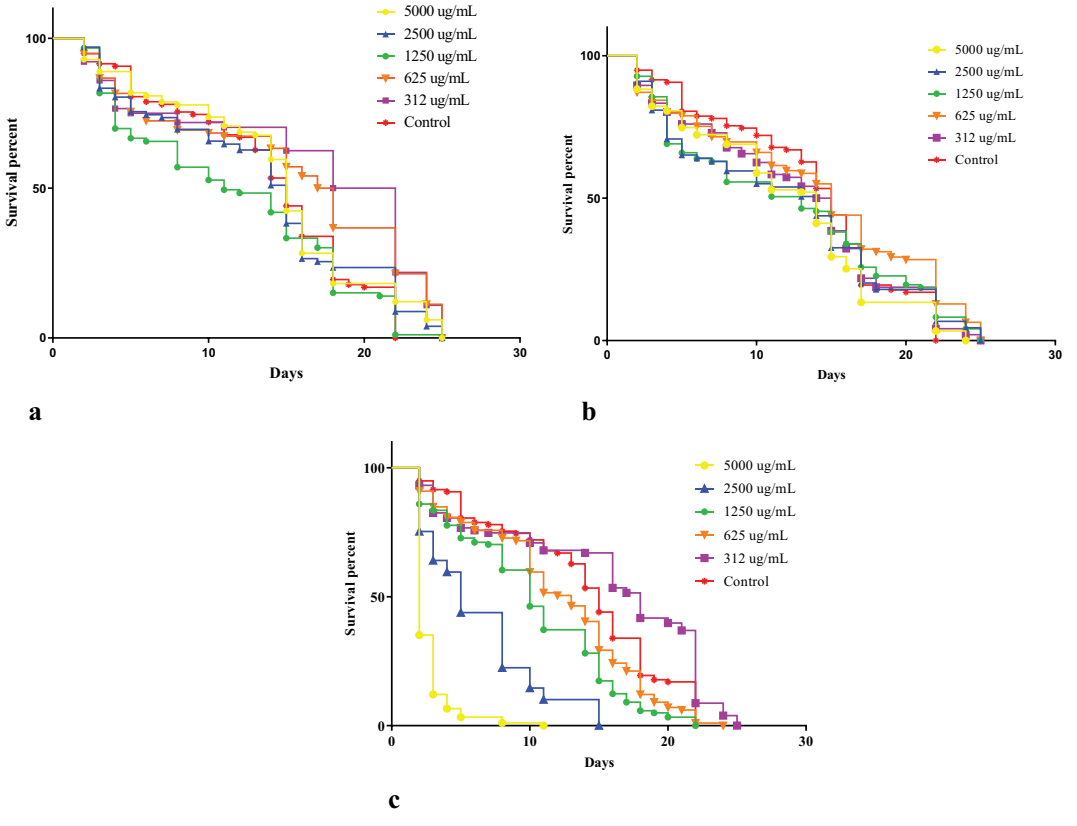

Figure 1. (a) Effect of infusion extract on life span of *C. elegans* (b) Effect of decoction extract of life span of *C. elegans* (c) Effect of alcohol extract of life span of *C. elegans*.

To the best of our knowledge, this is the first *in vivo* data on analysed and characterized *T. callosum* extracts. Information on the toxic concentrations of *T. callosum* extracts is important because it provides critical information about extracts for potential safe use.

As it is known, lifespan assays in *C. elegans* play an important role in toxicology studies. Some mammalian organs or tissues, like lungs, livers, kidneys, and blood transport systems, are absent in *C. elegans*. Therefore, compared to other mammalian models, it still has some limitations in its utilization as a model system. When compared to screenings utilizing mammalian models, the *C. elegans* assay has many benefits. The *C. elegans*-pathogen infection assay can help identify compounds that impact the pathogen's growth *in vitro*, pathogenicity *in vivo*, or even the host's immune system. The whole-animal *C. elegans* assays have many advantages over using an *in vivo* mammal approach, such as their small brood size, long reproductive cycles, cheap application costs and being free of ethical considerations.

In addition, toxicity studies show a correlation between toxic concentrations of heavy metals and organophosphates in *C. elegans*, and toxic concentrations both in mice and rodents (Williams and Dusenbery 1988; Cole et al. 2004).

Determination of the pathogenicity of the urinary system pathogens in *C. elegans*

To determine the pathogenicity of urinary system pathogens on *C. elegans*, TD_{50} values were calculated for nematodes infected with each pathogen used in the study. While evaluating the *in vivo* anti-infective effects of the extracts, the percentage of live nematodes for the day calculated

as the TD_{50} value was used. TD_{50} values and the day of evaluation for *P. aeruginosa*, *E. coli*, *K. aerogenes*, *S. aureus* and *C. albicans* were listed in Table 4.

As a result of pathogenicity trials in the *C. elegans* nematode model system, five strains including *E. coli, S. aureus, P. aeruginosa, K. aerogenes and C. albicans* were found to be pathogens in the *C. elegans* nematode model system.

Anti-infective effect of extracts using the *C. elegans* model

To determine the anti-infective effect of plant extracts in the *C. elegans* nematode model system, results were evaluated as positive if the number of nematodes in the wells with extract was >50% compared to the number of nematodes in the control wells without extract at the end of the experiment and as negative if it was ≤20% at the day of evaluation. The percentage values of alive nematodes infected with the pathogens on the day of the evaluation are listed in Table 5.

The results for *C. elegans* infected with *P. aeruginosa* showed that the concentrations of 5000–2500–1250 µg/mL for the infusion extract; 5000 µg/mL for the decoction extract, and 312 µg/mL for the 96% ethanol extract was *in vivo* anti-infective. For *C. elegans* infected with *E. coli*, concentrations of 5000– 2500-1250- 625, and 312 µg/mL for the infusion; and 5000 and 2500 µg/mL for the decoction extract showed *in vivo* anti-infective effects. The *C. elegans* infected with *K. aerogenes*, concentrations of 2500–1250 625, and 312 µg/mL for the infusion extract, and 1250 µg/mL for the decoction extract showed *in vivo* anti-infective effects. The results showed that infusion, decoction and 96% ethanol extracts did not show an *in vivo* anti-infective effect on the *C. elegans* nematode model infected with *S. aureus* and also *C. albicans*.

The widest range of *in vivo* anti-infective concentrations was observed in the infusion extract. For 96% ethanol extracts, the *in vivo* effect was determined only against *P. aeruginosa*. For the infusion extract, the *in vivo* effective doses against *P. aeruginosa*, *E. coli* and *K. aerogenes* were found to be equal to or below the *in vitro* MIC values. For the decoction extract, it was determined that the *in vivo* effective dose only against *K. aerogenes* was below the *in vitro* MIC value.

Contrary to conventional antibiotics, *in vivo* effective doses of various natural extracts and compounds can be at lower concentrations compared to *in vitro* effective concentrations (Moy et al. 2006). The effects of synthetic compounds and plant extracts against *Enterococcus faecalis* in *C. elegans* nematode model and showed that *in vivo* anti-infective effective concentrations were much lower than the *in vitro* MIC values (Moy et al. 2006). Also, similar results were obtained in the study of anti-infective natural extracts and compounds against *Staphylococcus aureus* in the *C. elegans* model (Kong et al. 2016).

Effect of extracts on the colonization of urinary system pathogens using *C. elegans*

To determine the effect of test extracts on the colonization of urinary system pathogens in *C. elegans*, colonization in the nematodes was calculated. The colonization of pathogens by which only the extracts have an *in-vivo* anti-infective effect on nematodes was determined and the effect of the extracts on this colonization was investigated. Concentrations of extracts were anti-infective for

Table 4. TD_{50} values and the day of evaluation for *in vivo* anti-infective effect of the extract.

Pathogens	TD_{50} (day)	The day of evaluation
P. aeruginosa ATCC 9027	3.5	4
E. coli ATCC 8739	4.4	5
K.aerogenes ATCC 1348	6	6
S. aureus ATCC 6538	5.3	6
C. albicans ATCC 10,231	5.5	6

Table 5. The percentage (%) of live nematodes infected on the day of the evaluation.

The percentage values of live nematodes at the day of evaluation (%)					
P. aeruginosa ATCC 9027					
	5000 µg/mL	2500 µg/mL	1250 µg/mL	625 µg/mL	312 µg/mL
Infusion	58*	66*	75*	38	41
Decoction	76*	44	46	45	48
Ethanol (96%)	-	-	-	-	70*
***E. coli* ATCC 8739**					
Infusion	72*	53*	52*	57*	53*
Decoction	71*	65*	50	35	50
Ethanol (96%)	-	-	-	-	29
***K.aerogenes* ATCC 1348**					
Infusion	30	56*	52*	54*	55*
Decoction	28	42	52*	38	38
Ethanol (96%)	-	-	-	-	39
***S. aureus* ATCC 6538**					
Infusion	39	40	39	25	44
Decoction	28	33	21	22	19
Ethanol (96%)	-	-	-	-	21
***C. albicans* ATCC 10231**					
Infusion	30	26	33	25	18
Decoction	23	17	35	19	17
Ethanol (96%)	-	-	-	-	23

* indicates *in vivo* anti-infective effects.

each pathogen. In addition, colonization in nematodes was calculated on the day of evaluation to determine the extracts' anti-infective effects.

No significant difference was observed between the colonization data (CFU/nematode) in the media with different concentrations of the extracts and the control group for each pathogen ($p < 0.05$). The results showed that the indicated concentrations of the extracts did not effect the colonization of the pathogens used in the study. The infusion extract showed the widest activity concentration range in the nematode model system. Considering these data, a Pearson correlation test was used to show the possible correlation between the survival percentage obtained at the concentrations of the infusion extract determined to have anti-infective effect in the nematode model system, and the colonization data in nematodes, where no correlation ($r = -0.3840$ for *E. coli*, $r = -0.9755$ for r = −0.4233 for *P. aeruginosa*, *K. aerogenes*) was observed. The Pearson correlation test was also used to analyze the potential correlation between the colonization data obtained and the TD_{50} values, calculated in the assay to determine the pathogenicity of the pathogens in *C. elegans*, resulting with no correlation ($r = 0.5618$). Similar results were found in a study with *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii*, which belong to the "ESKAPE" group of pathogens. No correlation was found between the colonization levels of the tested pathogens for 48 hours, and pathogenicity (Scott et al. 2020).

These results suggest that the urinary tract pathogens used in the present study colonized in the *C. elegans* nematode model system; however, pathogenicity was developed independently dependent on the amount of colonization.

Conclusion

Herbal medicines for human health are derived from plants that have been used for centuries. For this purpose, ethnobotanical wisdom may direct and provide rational medicinal application in light of appropriate experimental design. Overall, in the present study, *in vitro* and *in vivo* anti-infective effects of *Tripleurospermum callosum* were demonstrated for the first time supporting the ethnobotanical use. The selectively non-toxic anti-infective

effect concentrations of the extracts, and the pathogen strains that were effective were determined using the *C. elegans*. The present data demonstrated that the infusion extract was comparatively more effective, using the *in vivo* the *C. elegans* model. Still there is more molecular and mechanistic studies needed to prove the safety and efficacy of natural preparations.

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