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Original article

Phytochemical insights into *Thymus carmanicus* Jalas and its antileishmanial potential: A synergistic therapy

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Abstract

Objective: The aim of this study was to investigate phytochemical composition and antileishmanial activities of *Thymus carmanicus* different extracts individually and in combination with meglumine antimoniate (Glucantime®; MA) to introduce possible complementary drugs against *Leishmania major* using experimental methods.

Materials and methods: The aerial parts of *T. caramanicus* were extracted by maceration using a series of solvents, including petroleum ether, chloroform, ethyl acetate, 80% methanol, and water. Total phenolic and flavonoid content was determined using colorimetry and thymol content was determined by HPLC method. Experiments assessed *in vitro* antileishmanial activity and cytotoxicity.

Results: Although the combination of the petroleum ether extract with meglumine antimoniate exhibited the strongest raw antileishmanial activity against *L. major* promastigotes (IC_{50} = 236.5 µg/mL, $p<0.001$) and amastigotes (IC_{50} = 43.15 µg/mL, $p<0.001$), the combination index (CI) value of 1.23 indicated an antagonistic interaction. In contrast, the methanolic extract combined with MA proved to be the most effective candidate for combination therapy, as demonstrated by a CI of 0.92 (suggesting moderate synergism), a satisfactory selectivity index (SI) of 7.04, and a high amastigote mortality rate.

Conclusion: The methanolic extract with compounds like polyphenolics, flavonoids, and thymol, shows high anti-leishmanial activity and safety, making it a good option for combination therapy.

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Introduction

The World Health Organization recognizes leishmaniasis as a considerable public health concern, especially in areas where the disease is endemic. Initiatives are currently underway to develop strategies aimed at its management and prevention (Organization 2023). It is well-documented that zoonotic cutaneous leishmaniasis caused by *Leishmania major* exhibits a significant prevalence in the rural regions of middle east, accounting for approximately 70% of reported cases (Bamorovat et al. 2024). After over 100 years, yet treatment options for this disease remain scarce, largely because the limited available drugs are associated with side effects. Antileishmanial drugs such as miltefosine may cause vomiting and diarrhea, amphotericin B may lead to fever, generalized pain including muscle and joint pains and tachypnoea, and meglumine antimoniate may result in general malaise, difficulty in breathing, skin rash, and facial edema (Organization 2022). Many studies have shown antileishmanial activity of plant extracts or their chemical derivatives *in vitro* against promastigote and amastigote forms or *in vivo* in Leishmania-infected animals (da Veiga et al. 2020; Ghadimi et al. 2020; Sharifi et al. 2023; Sharifi et al. 2021). Moreover, the use of herbal medicine as combination therapy could help to solve problems in relation to parasitic resistance and relieve the side effects of the applied medicine.

Thymus is a large genus of the Lamiaceae family with 300–400 species worldwide, particularly in the Mediterranean region. *Thymus caramanicus* Jalas with local name "*Avishan e Kermani*" is an endemic species of Iran. While several *Thymus* species have been studied for their anti-leishmanial potential, largely focusing on widely distributed species such as *T. syriacus* (Battah 2025) or *T. vulgaris* (Nilforoushzadeh et al. 2008). The endemic Iranian species, *T. carmanicus* Jalas, possesses a distinct phytochemical signature. This species possesses a unique carvacrol/thymol ratio that distinguishes it from previously reported counterparts and implies a distinctive biological activity, yet its antileishmanial potential has not been systematically assessed. According to previous reports, carvacrol (42-80.7%), thymol (4.1-11.8%), parasimen (2.5-12.8%), gamma terpinene (3.7-3.7%) and borneol (3.8-3.1%) are the main compounds of the essential oils of the ecotypes, and carvacrol was the main compound in the investigated ecotypes in different parts of Iran (Makkizadeh Tafti et al. 2010). *T. caramanicus* can be used in the pharmaceutical industry due to its significant amount of essential oil and valuable phenolic compounds like thymol and carvacrol (Mazandarani 2015). In Iranian traditional medicine, the leaves of the plant are used in the treatment of skin disorders and rheumatism, and as an antibacterial agent (Zargari 1990). According to ethnobotanical research in the Rhine region of Kerman province, Iran, local people use boiled incense of the plant to treat sinusitis, colds, and respiratory ailments such as asthma as well as diarrhea (Rajaei and Mohamadi 2012). The plant has shown antimalaria (Mousavi 2023), antifungal (Khorasany 2016), and antimicrobial activity (Malekpourzadeh 2023). To the best of our knowledge, there is no scientific report on antileishmanial therapeutic effects of *T. carmanicus*. Therefore, for the first time, we investigated the anti-leishmanial activity of *T. carmanicus* various extracts and essential oil against promastigote and amastigote forms of *L. major* alone and in combination with MA.

Materials and Methods

Plant material

T. caramanicus was collected in July 2023 from Soutkooh mountain (3185 m) in Kerman, Iran. The plant species was identified by Dr. Mansour Mirtajadini, Shahid Bahonar university of Kerman and a voucher specimen (KF1553) was

deposited in the herbarium of Kerman university of medical science.

Extraction and preparation of herbal samples

The aerial parts of *T. caramanicus* were extracted by maceration using a series of solvents, including petroleum ether, chloroform, ethyl acetate, 80% methanol, and water. For this purpose, 250 g of dried plant was powdered, macerated in solvents for 24 hr, concentrated under vacuum, dried at 40°C, and stored at -20°C for future experiments.

The groups included aqua extract (AQ), chloroform extract (CHL), ethyl acetate extract (EA), essential oil (EO), petroleum ether (PE) and methanolic extract (MET). Serial concentrations ranging from 100 to 400 µM of various extracts were prepared for the promastigote assay, while concentrations of 25 to 400 µM were used for the amastigote assay. In the context of combination treatment, a formulation comprising identical concentrations from the respective groups alongside Glucantime® (MA) was utilized.

T. caramanicus essential oil (EO) preparation

Dried leaves and thin branches were grounded and passed through a 300-mesh sieve. About 100 g of the plant sample was soaked in water for 24 hr before distillation to increase the penetration of the solvent and hydro distillation was used for EO extraction. Samples with a ratio of 1 to 10 (equivalent to 1 g of dry matter in 10 ml distilled water) were poured into the clevenger apparatus and EO was collected within 3 hr. The EO was separated, dehydrated using anhydrous sodium bicarbonate and was stored at -20°C.

Phytochemical analysis

Quantification of total phenols and total flavonoids

Total phenol content was measured using the Folin-Ciocalteu method. To 1 mL of plant extract (500 µg/mL) or standard gallic acid, 1. 0 mL of Folin reagent and 1. 0 mL of saturated sodium carbonate solution (7. 5%) were added, with the total volume adjusted to 25 mL using de-ionized water. After 10 min of incubation, absorbance was recorded at 700 nm using a UV-Vis spectrophotometer (Agilent Cary 60 UV-Vis, USA). Data is expressed as milligrams of gallic acid equivalents per gram of plant extract. A calibration curve was made using different gallic acid concentrations from a 1000 µg/mL stock diluted to 10-100 µg/mL (Jamshidzadeh et al. 2017).

To find the total amount of flavonoids, thin layer chromatography (TLC) was used to identify the main flavonoids in the plant, with rutin being the main one. Adding 2% aluminum chloride to rutin showed a maximum wavelength of 415 nm. A calibration curve for rutin (31.28-500 µg/mL) was created, and the absorbance of the plant extract was measured at 415 nm using a UV-Vis spectrophotometer (Agilent Cary 60 UV-Vis, USA) after aluminum chloride addition to calculate the total flavonoids (Shraim et al. 2021).

Thymol content determination using HPLC

In the present work, thymol was analyzed in different extracts of *T. caramanicus* using HPLC method. Separation was carried out using HPLC system (Yang, South Korea) and a Waters® C18 column (250×4.6 mm, 4 µm) (USA). The plant extracts (100 µg/ml, 20 µl) were passed through a syringe filter (0.2 µm) and injected into HPLC. The data analysis was done using Clarity version 6.2.0.208 software. The mobile phases utilized in this study consisted of deionized water (A): methanol (B). A mixture comprising 40% of phase A and 60% of phase B was employed for a duration of 30 min. The wavelength designated for UV detection was established at 330 nm. For the purpose of constructing a calibration curve, a series of standard solutions of thymol within the

concentration range of 0.0312-10 µg/ml were utilized.

Gas chromatography-mass spectrometry

The chemical profile of the essential oil was determined using a Shimadzu QP 5000 gas chromatograph-mass spectrometer (GC-MS), equipped with a Chrom-pack HP-5 MS capillary column (30 m × 250 µm × 0.25 µm) and a Shimadzu QP 5050 mass selective detector operating in electron impact mode at 70 eV. Helium was used as the carrier gas at a flow rate of 1 mL/min. The column oven temperature program began at 50°C held for three minutes, then rose to 160°C at a rate of 3°C/min, and finally increased to 240°C at 10°C/min. The mass spectrometer transfer line and the injector were set at 220°C and 290°C, respectively. Compound identification was carried out by comparing their mass spectra and retention times with those of reference standards, data from the Wiley 2001 GC-MS spectral library, and previously reported scientific literature.

Parasite culture

In this study, Promastigotes of L. major strain MRHO/IR/75/ER were obtained from the Pasteur Institute of Iran, Tehran, Iran, and cultured in RPMI1640 medium enriched with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% (v/v) fetal calf serum, and incubated at 25°C.

Macrophage culture

The murine macrophage cell line (J774-A1, GCACC) was obtained from Pasteur Institute in Tehran, Iran. The cells were cultured in Gibco's Dulbecco's adjusted Eagle's Medium (DMEM) supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum (FBS), and maintained in a 5% CO_2 environment (Sharifi et al. 2023).

Anti-promastigote activity

About 100 mL of the promastigotes (10^6 cells per ml) from the logarithmic growth stage were placed into a 96-well plate. Then, 20 µl of essential oil and different extracts of *T. carmanicus* (100-400 µg/ml), or the combination concentrations of aqua extract (AQ), chloroform extract (CHL), ethyl acetate extract (EA), essential oil (EO), petroleum ether (PE) and methanolic extract (MET) alone and in combination with with MA (100+100, 2000+200, 300+300 and 400+400 µg/ml) were added to each well, followed by incubation for 72 hr at 25°C. Each well received 20 µl of MTT solution (Sigma Aldrich, USA) from stock with a 5 mg/ml concentration. It was incubated right away for three hours at 25°C. After using isopropanol alcohol to stop the reaction, the optical density (OD) absorbance was measured at 490nm (Mohamadi et al. 2023). The IC50 result was recorded for the probit test in SPSS software version 20.

Anti-amastigote activity

To evaluate the effect of the EO and different extracts of *T. carmanicus* (25–400 µg/ml), MA and their combinations against *L. major* amastigotes, murine J774 macrophages cell line (J774 A.1 ATCC®TIB-67™) at 1 × 10^5/ml were cultured in six-chamber slides (Lab-Tek, Nalge Nunc, NY, USA). They were kept at 37 ± 1 °C and 5% CO_2 for 12 hr. Then, the macrophages were infected with 1 × 10^6/ml of promastigotes post-annular forms and incubated for 24 hr (macrophage to parasite ratio 1:10). After incubation, the slides were fixed with methanol, stained with Giemsa, and examined under a light microscope to determine the number of intracellular amastigotes per macrophage. The infection index was calculated by counting infected macrophages and the number of amastigotes per cell (Tadtong 2026). Free parasites and old medium were replaced with fresh medium. Different concentrations of aqua extract (AQ), chloroform extract (CHL), ethyl acetate extract (EA), essential oil (EO), petroleum ether (PE) and methanolic extract (MET) alone and in combination with MA (25-400 µM) were added to macrophages, and after

72 hr, slides were prepared to count amastigotes (Bahraminejad et al. 2022).

Cytotoxicity activity

To assess the possible cytotoxic effects of the EO, various extracts of *T. carmanicus*, MA, and their combined application on J774 murine macrophages, cells were seeded at a density of 5×10^5 cells/mL and treated with varying concentrations (25–400 µM) of the test substances in 96-well plates. The macrophages were incubated for 72 hr at 37±1°C in a 5% CO_2 atmosphere. An untreated control group consisted of macrophages cultured without any test substances for the same duration, allowing evaluation of the cytotoxic activity of the different treatments, whether MA alone or in combination. After incubation, the same volume of MTT solution used in the anti-promastigote assay was added, and the plates were incubated for another 3 hr. OD was then measured similarly using an ELISA reader at 490 nm after adding dimethyl sulfoxide (DMSO) solution. The cytotoxic activity at the 50% level (CC_{50} value) was calculated using SPSS software and a probit test. Drug safety was assessed using the selectivity index (SI), calculated by the formula $SI = IC_{50} / CC_{50}$. (Salarkia et al. 2023).

Combination index (CI) and Isobologram analysis

The Isobologram Analysis was employed to quantify the interactions between the essential oil and various extracts of *T. carmanicus* with MA in a combinatorial context utilizing CompuSyn software (CompuSyn software, CompuSyn, Inc., Paramus, NJ, USA). The foundational principles and applications pertaining to this methodology have been extensively evaluated by Huang et al. (Huang et al. 2019). The Combination Index (CI) represents a quantitative assessment that elucidates the extent of synergistic and antagonistic interactions between two pharmacological agents with respect to a designated ultimate outcome. All experimental procedures were conducted in triplicate to ensure reliability and reproducibility of the results (Nooshadokht et al. 2022).

Statistical analysis

Statistical evaluations were performed using SPSS software version 22.00 (Chicago, Illinois, USA) and GraphPad Prism version 8.0 (California, USA). One-way analysis of variance (ANOVA) was used to assess statistical differences between different drug concentrations, while paired t-test was used to determine any significant differences between groups. The significance level was considered to be $p<0.05$.

Results

Extract yield

The yields increased with solvent polarity, with methanol giving the highest extraction efficiency. The percentage yields (w/w) of petroleum ether, dichloromethane, ethyl acetate, and methanol extracts were found to be 4.11%, 6.15%, 8.41% and 15% respectively.

Phytochemical analysis

Total phenolic, and flavonoids content

The quantification of total polyphenolic compounds was obtained based on the standard calibration curve of concentrations of gallic acid ($y = 0.0014 x + 0.0559$; $R^2 = 0.9890$). The content of polyphenolic compounds to be 147.76 ± 2.25 mg of gallic acid equivalent per g of dry extract. The quantification of flavonoid content was completed based on the standard calibration extinction curve of rutin concentrations ($y = 0.0028 x + 0.008$; $R^2 = 0.9931$). The flavonoid content expressed as rutin equivalents, was 9.46 ±0.79 mg rutin equivalent/g extract.

Thymol content in methanolic extract

A calibration curve for thymol was created using HPLC data, showing a linear

relationship with a correlation coefficient of 0.9980 over the concentration range of 0.0315-5 µg/ml. The regression equation was determined as Y= 6635.8 X1-3.7227. Limit of detection (LOD), and limit of quantitation (LOQ) were 0.000134 and 0.00416 µg/ml, respectively. Each gram of the methanolic extract yielded 286.2 mg of thymol (Figure 1).

EO chemical composition analyses

Chemical compositions of the EOs of aerial parts of *T. carmanicus* are presented in Table 1. GC–MS analyses showed that the main ingredients were thymol (73.39 %) followed by *para*-cymene (6.30%), and. γ-terpinene (6.12 %).

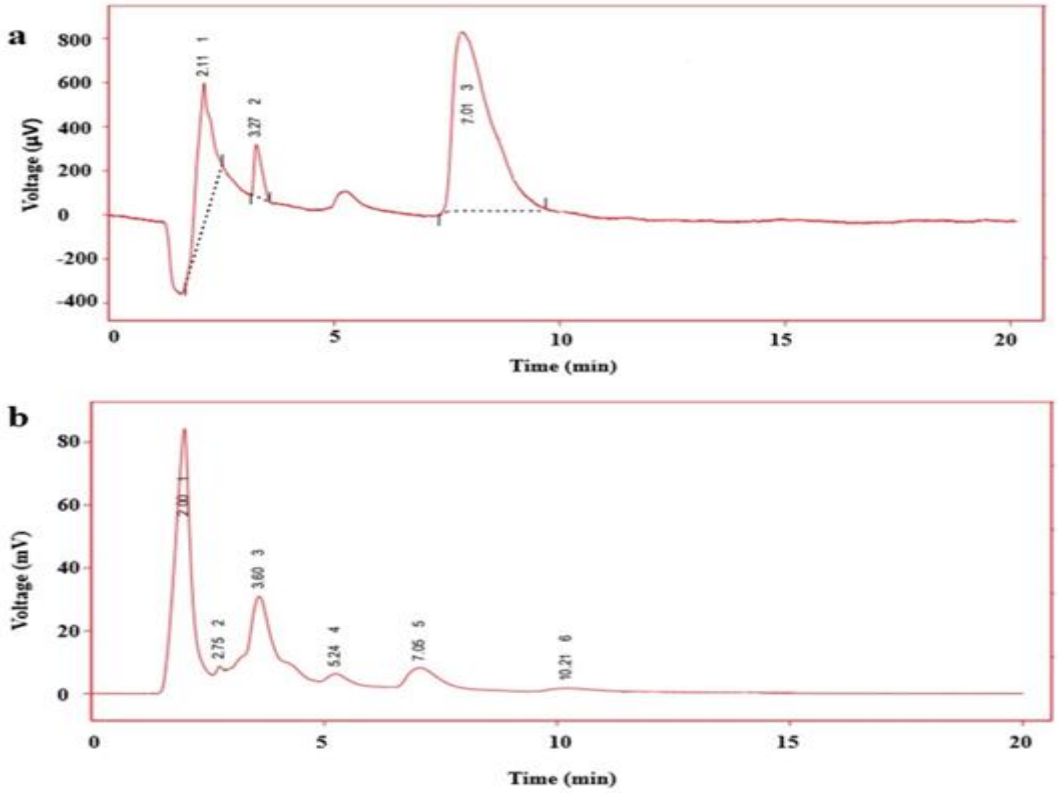

Figure 1. Typical chromatograms of thymol 5 µg/ml (as standard) , and plant sample 100 µg/ml (b). The retention time of 7.01 min is related to thymol.

Table 1. Chemical compositions of the EO of aerial parts of *T. carmanicus*

No	RT	%	Components	KI	Type
1	11.01	0.85	α-Thujene	928	MH
2	11.40	0.56	α-Pinene	936	MH
3	12.28	0.25	Camphene	953	MH
4	14.30	0.96	β-Cymene	993	MH
5	15.78	1.17	α-Terpinene	1022	MH
6	16.30	6.30	*Para*-Cymene	1032	MH
7	16.44	0.24	Limonene	1035	MH
8	16.63	0.32	Eucalyptol	1038	MO
9	17.99	6.12	γ-Terpinene	1065	MH
10	18.72	0.33	Trans-Sabinene hydrate	1079	MO
11	20.20	0.35	Linalool	1108	MO
12	24.06	1050	Borneol	1186	MO
13	24.40	1.05	Terpinen-4-ol	1193	MO
14	26.65	0.46	Thymol methyl ether	1240	MO
15	27.07	0.58	Carvacrol methyl ether	1249	MO
16	30.05	73.39	Thymol	1312	MO
17	30.28	4.95	Carvacrol	1318	MO
		99.35	Total Identified		
Monoterpene Hydrocarbons MH				Oxygenated Monoterpenes MO	

Effect of EO and different extracts, MA, or extracts-MA combination on promastigotes mortality

The IC50 data revealed that combining MA with all tested extracts produced significantly greater activity against promastigotes than the individual treatments alone ($p<0.001$).The IC_{50} value of MA was found to be 110.6 µg/ml**.** The petroleum ether extract combined with MA exhibited the highest potency (IC_{50}=236.5 µg/ml), followed by the combination of ethyl acetate extract (IC_{50}=242.1 µg/ml), chloroform extract (IC_{50}=247 µg/ml), EO (IC_{50}=257.2 µg/ml), methanolic extract (IC50=265.75 µg/ml), and ultimately aqueous extract (IC_{50}=273.5 µg/ml) with MA. Figure 2 shows that various extracts and MA alone effectively reduced the mean mortality rates of *L. major* promastigotes, with improved results when combined ($p<0.01$).

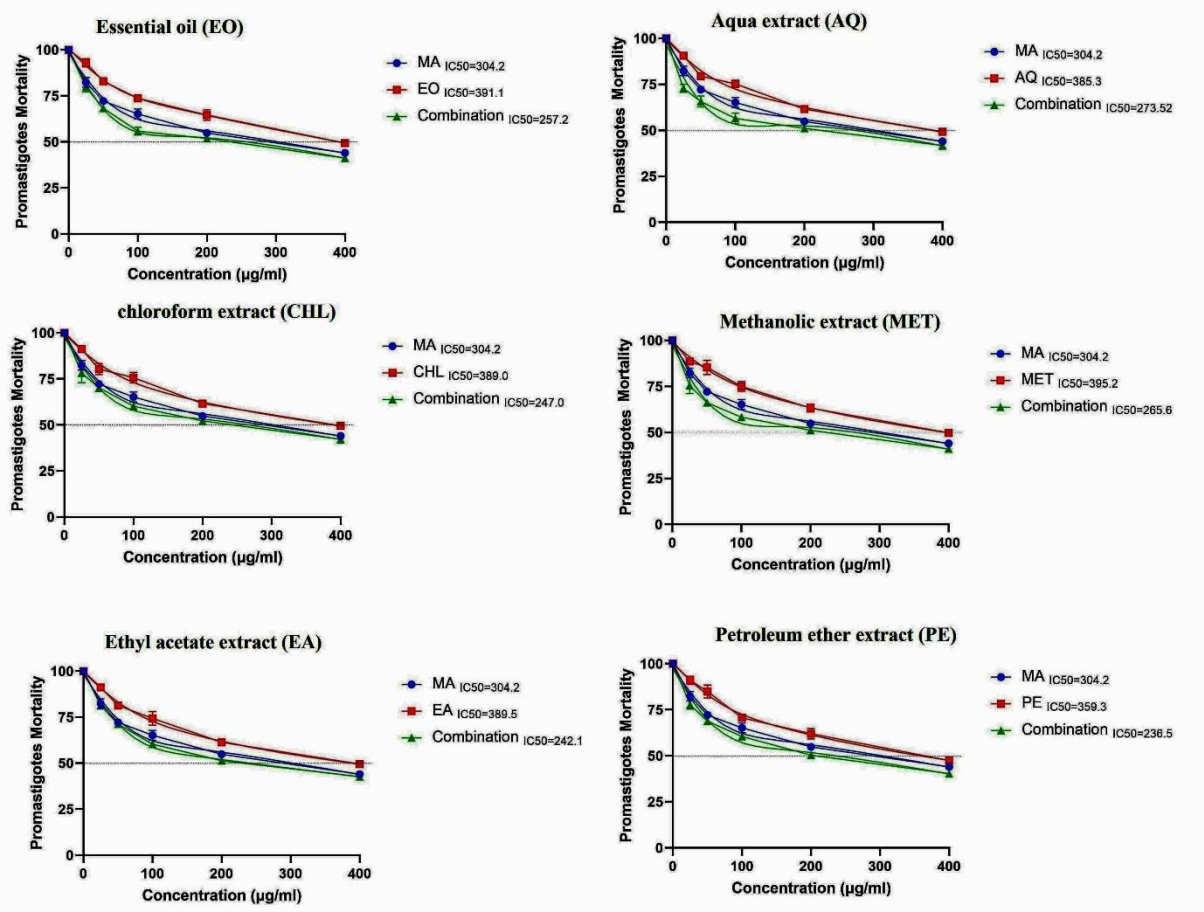

Figure 2. The mortality rate of *L. major* promastigotes at different concentrations of essential oil and different extracts of *T. carmanicus* alone, MA and their combination by MTT assay.

Effect of EO and different extracts of *T. carmanicus*, MA, or extract-MA combination on amastigotes mortality

The successful infection of macrophages by *L. major* amastigotes was confirmed by Giemsa staining, showing clear intracellular localization of parasites (Figure 3). The results demonstrated a significant variation in the indices of *L. major* intracellular amastigotes compared to the untreated control group ($p<0.001$). There was a marked decrease in the number of amastigotes at various concentrations ($p<0.001$) of the plant extracts when compared to the untreated control. According to the data, there was no statistically significant difference between the groups treated with different extracts

compared to the groups treated with MA in terms of reduction in amastigote burden. The highest efficacy, evidenced by the IC50 values, was observed when the petroleum ether extract and MA were used in combination (43.15 µg/ml), followed by the combined use of chloroform extract (51.72µg/ml), methanolic extract (66.75µg/ml), EO (70.7µg/ml), ethyl acetate extract (78.66µg/ml), and aqueous extract (79.44µg/ml) ($p<0.001$) with MA (Table 2).

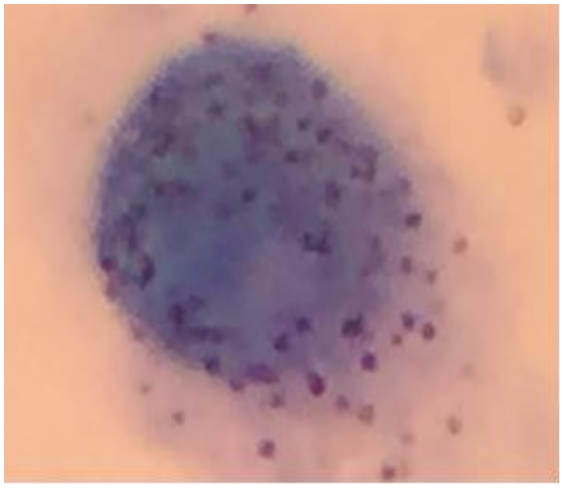

Figure 3. Giemsa-stained murine macrophages infected with *L. major* amastigotes.

Cytotoxicity activity

The CC_{50} values for various concentrations of plant extracts and essential oil (ranging from 100-400 µg/ml) were determined by analyzing the mean quantity of intracellular amastigotes present within murine cell lines. The cytotoxicity evaluation indicated that the percentage of macrophage viability was significantly increased in the EO alone and the combination group (EA+MA) in comparison to MA alone ($p<0.001$), indicating its efficacy against the parasites while imposing lesser toxicity on host cells (Table 2).

Selectivity index (SI)

Table 3 shows the IC_{50}, CC_{50}, and SI values for all the different extracts, MA, and their combination. Cytotoxicity tests showed no lethal effects, with a safety index value of at least 1. A higher SI value indicates a safer and more effective compound. So, in this study PE, CHL and MET in combination with MA showed the best result in terms of SI (SI=10.55, 9.05, and 7.04 respectively), and followed by EO, EA, and AQ combination with MA.

Table 2. The effect of different concentrations of essential oil and different extracts of *T. carmanicus* alone, MA and their combination on the mean number of intra-macrophage amastigotes.

Groups		UC	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml
MA[1]		48.0±2.1	44.2±1.8	39.1±2.0	27.3±1.3	12.2±1.6	0.0±0.0
EO[2]	Alone	48.0±2.1	46.3±3.8	42.0±3.3	29.0±4.1	14.0±3.5	2.0±1.1
	Combination	---	40.2±2.2	31.6±3.8	18.3±3.2	4.6±1.9	0.0±0.0
AQ[3]	Alone	48.0±2.1	45.4±4.2	43.1±4.8	32.3±4.1	16.4±4.2	2.9±1.9
	Combination	---	41.1±5.1	33.8±1.7	20.6±4.1	6.3±4.7	0.0±0.0
MET[4]	Alone	48.0±2.1	42.3±3.4	39.8±1.9	29.8±3.8	12.3±3.9	1.7±±1.8
	Combination	---	38.3±4.6	30.1±2.4	18.6±3.9	4.2±2.9	0.0±0.0
EA[5]	Alone	48.0±2.1	43.3±4.5	40.1±4.4	32.4±3.3	13.4±4.3	2.9±1.6
	Combination	---	40.1±3.0	33.8±2.1	20.6±3.5	6.4±2.1	0.0±0.0
CHL[6]	Alone	48.0±2.1	44.9±1.9	40.6±2.6	28.2±1.4	12.3±0.9	0.0±0.0
	Combination	---	33.2±2.6	28.6±1.5	12.4±2.8	0.0±0.0	0.0±0.0
PE[7]	Alone	48.0±2.1	43.7±2.9	39.6±2.6	27.8±3.0	11.8±2.3	0.0±0.0
	Combination	---	33.0±4.2	24.1±2.7	6.2±2.9	0.0±0.0	0.0±0.0

MA[1]: Meglumine antimoniate, EO[2]: Essential oil, AQ[3]: Aqueous extract, MET[4]: Methanolic extract, EA[5]: Ethyl acetate extract, CHL[6]: Chloroform extract and PE[7]: Petroleum ether extract, UC: Untreated group.

Table3. Evaluating the IC_{50}, CC_{50}, and the selectivity index (SI) of different concentrations of essential oil and different extracts of *T. carmanicus* alone, MA and their combination.

Groups	Amastigotes				Macrophage				Selective Index[1]	
	Alone		Combination		Alone		Combination		Alone	Combination
	IC_{50}[2](µg/ml)	p value Compare MA	IC_{50}	p value Compare MA	CC_{50}[3]	p value Compare MA	CC_{50}	p value Compare MA[4]		
MA[4]	110.6	---	---	---	532.2	---	---	---	4.81	--
EO[5]	117.8	<0.001	70.7	<0.001	583.3	<0.001	492.3	<0.001	4.95	6.96
AQ[6]	130.5	<0.001	79.44	<0.001	573.4	<0.001	486.3	<0.001	4.39	6.12
MET[7]	112.8	<0.001	66.75	<0.001	544.2	<0.001	469.7	<0.001	4.82	7.04
EA[8]	119.7	<0.001	78.66	<0.001	569.3	<0.001	501.3	<0.001	4.76	6.37
CHL[9]	115.5	<0.001	51.72	<0.001	572.3	<0.001	468.2	<0.001	4.95	9.05
PE[10]	111.7	<0.001	43.15	<0.001	571.3	<0.001	455.3	<0.001	5.11	10.55

Selectivity index[1]: (CC_{50}/IC_{50}). IC_{50}[2]: Drug concentration inhibited 50% of promastigotes and amastigotes growth. CC50[3]; Drug concentration that inhibited 50% of macrophage growth. MA[4]: Meglumine antimoniate. EO[5]: Essential oil, AQ[6]: Aqueous extract, MET[7]: Methanolic extract, EA[8]: Ethyl acetate extract, CHL[9]: Chloroform extract and PE[10]: Petroleum ether extract.

Combination index (CI) analysis

CI is a common index for drug interactions and is interpreted as follows: strong synergism <0.7, moderate to low synergism 0.7-0.9, almost additive synergism 0.9-1.1, low to moderate antagonism 1.1-1.45, antagonism 1.45-3.3, and strong to high antagonism >3.3. The (CI) value for the combination of EO+MA was determined to be 0.95, whereas the CI values for the combinations of AQ+MA, MET+MA, as well as EA+MA were measured at 0.92, which is indicative of synergistic interactions (CI<1). In contrast, the CI value for PE + MA, and CHL + MA was recorded at 1.23 and 1.14, thus classified as antagonistic properties indicating (CI > 1) and thus may indicate an antagonistic effect (Table 4 and Figure 4 and 5). These results show that the MET is the most valuable sample, both in terms of SI and CI index.

Table 4. Combination index (CI[1]) values for the combinations of *T. carmanicus* essential oil and various extracts with MA[2] against *L. major* amastigotes, calculated at the 50% inhibitory effect level (Fa = 0.5; equivalent to the IC_{50})

Drug/Combination	CI[1] value	Dose MA[2]	Dose Drug
MA		23.7919	
EO[3]			27.1591
MA+EO	0.95	12.0059	12.0059
MET[4]			22.6878
MA+MET	0.92	10.6964	10.6964
AQ[5]			25.30
MA+AQ	0.92	11.23	11.23
EA[6]			23.32
MA+EA	0.92	10.84	10.84
CHL[7]			25.03
MA+CHL	1.14	14.04	14.04
PE[8]			23.8530
MA+PE	1.23	14.6343	14.6343

CI[1]: Combination Index. MA[2]: Meglumine antimoniate. EO[3]: Essential oil, MET[4]: Methanolic extract, AQ[5]: Aqueous extract, EA[6]: Ethyl acetate extract, CHL[7]: Chloroform extract and PE[8]: Petroleum ether extract.

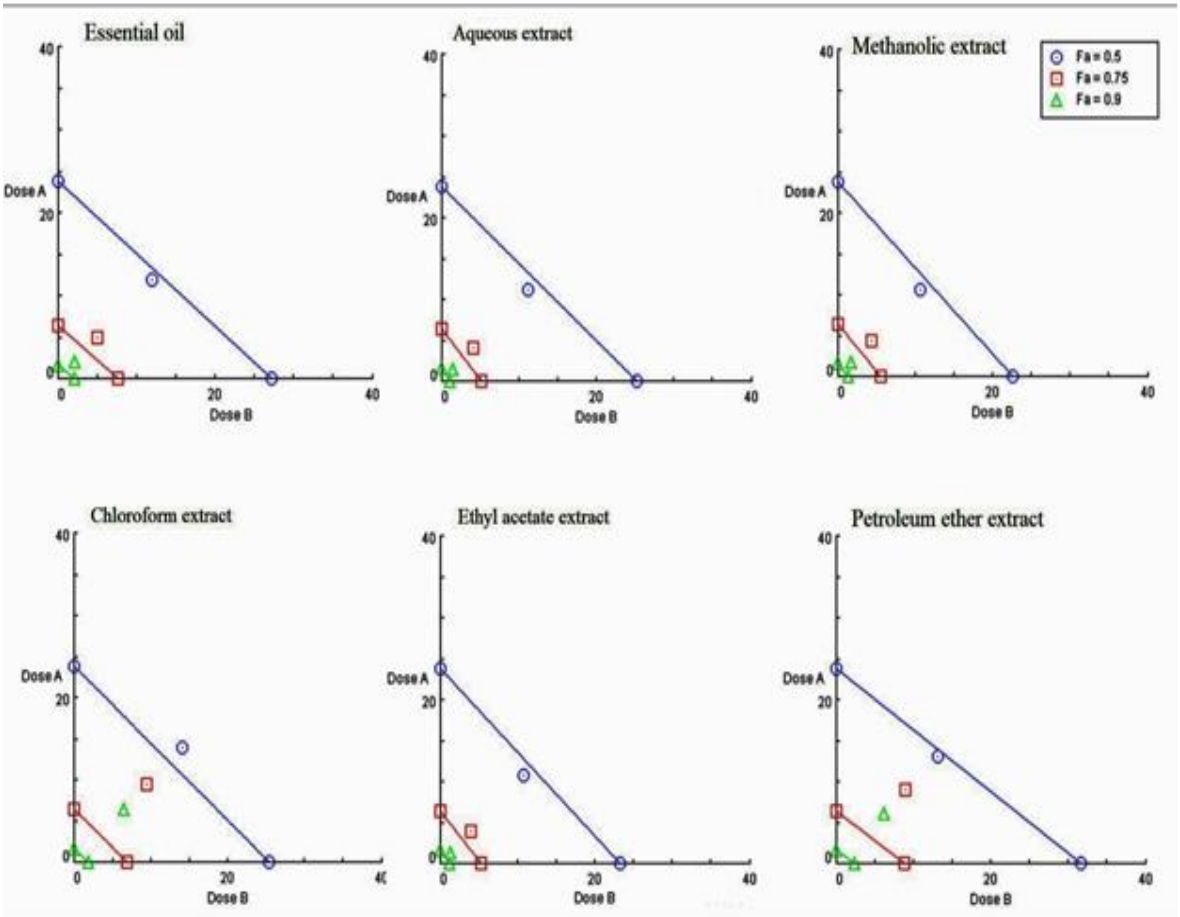

Figure 4. A combination index (CI) analysis was performed to evaluate the degree of synergy or antagonism between the essential oil, various extracts of *T. carmanicus*, and MA when combined against *L. major* amastigotes. fa = 0.5 = 50% of the parasites are inhibited (this corresponds to the IC50 value), fa = 0.75 = 75% of the parasites are inhibited, fa = 0.9 = 90% of the parasites are inhibited (close to the IC90).

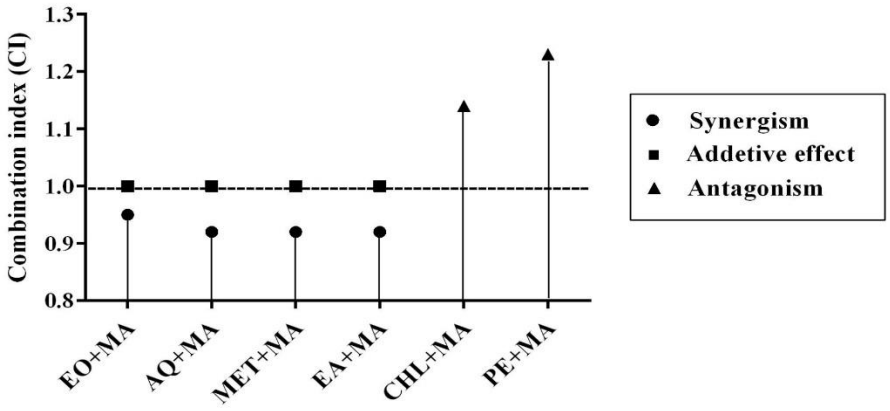

Figure 5. A combination index (CI) analysis was carried out to assess the synergistic or antagonistic effects of the combination treatments involving essential oil (EO), different *T. carmanicus* extracts, and MA on *L. major* amastigotes.

Discussion

In this study, we reported the phytochemical composition and antileishmanial activities of *T. carmanicus* different extracts individually and in combination with MA to introduce possible complementary drugs against *L. major* using experimental methods. The findings

of this study showed that petroleum ether extract had the greatest effect on the mortality of amastigotes and promastigotes. Although the combination of petroleum ether extract and MA had the greatest antileishmanial effect on *L. major* promastigote (IC50=236.5 µg/ml) and amastigote forms (IC50= 43.15), but the CI value for it was recorded at 1.23, which was classified as antagonistic properties. Therefore, the methanolic extract is considered the most effective for the combination treatment, considering CI 0.92, which indicates synergistic effect properties, acceptable SI 7.04, and high mortality of amastigotes. However, we fully acknowledge that this value is very close to the additive threshold (CI = 1), indicating only a mild or borderline synergistic effect.

Despite the petroleum ether extract of *T. carmanicus* demonstrating the highest individual activity (with IC_{50} values of 236.5 µg/mL against promastigotes and 43.15 µg/mL against amastigotes), its combination with MA resulted in an antagonistic interaction (CI = 1.23). Several biochemical explanations could account for this seemingly contradictory finding.

First, the non-polar components of the petroleum ether extract – which probably include sesquiterpenes, fatty acids, and waxes – may physically or chemically interact with the antimonial complex, thereby lowering its bioavailability or stability. Second, these lipophilic substances might compete for shared uptake routes, for instance endocytosis in macrophages or aquaglyceroporins in Leishmania, leading to reduced intracellular accumulation of MA. In contrast, the methanolic extract (CI = 0.92) produced genuine synergy with MA, presumably due to its polar phenolic constituents (such as thymol and rosmarinic acid) that complement MA mechanism without causing interference. These divergent outcomes highlight that not every "active" plant extract is suitable for combination therapy, and that antagonism can occur even with highly potent individual compounds. Future research should identify the specific petroleum ether compounds responsible for this antagonism through bioassay-guided fractionation, and examine their impact on antimonial uptake, efflux, and macrophage immune responses.

The content of thymol in methanolic extract was found 28.62%. According to the present research, PE and ME extracts had better antileishmanial effects than EO. So, it can be concluded thymol, the major compound of EO, does not seem to be responsible for antileishmanial activity. In this regard, it should be mentioned that according to a report, thymol derivatives like acetyl and benzoyl-thymol demonstrated high anti-leishmanial activity (de Morais et al. 2014). Molecular docking indicated strong binding of thymol to IFN-gamma (Interferon-gamma) and IL-12p40 (Interleukin-12p40) suggesting immunostimulatory potential (Mohamadi 2025). As a matter of fact, it is possible that the activity of extracts is due to the interaction between their constituents (Machado et al. 2014). There is evidence suggesting that crude plant extracts often exhibit greater biological activity than isolated compounds at equivalent doses (Rasoanaivo et al. 2011). This phenomenon indicates complex interactions in plant extract compounds that may contribute to their efficacy. Modern research suggests that activity loss upon fractionation- where an extract is separated into its components- indicates synergy. Minor compounds often work together to boost potency significantly. It has been demonstrated that this with *Vepris gossweileri*. A specific compound was 50 times more potent when combined with other minor molecules from the same extract than when tested alone (Langat 2022).

The current findings revealed a key pharmacological difference between the two extracts. The petroleum ether extract combined with MA showed a high combination index (CI) of 1.23, indicating that its components may either chemically

interfere with MA or compete for its uptake, thereby reducing MA effectiveness. In contrast, the methanolic extract combined with MA had a synergistic effect. Its compounds—such as polyphenols and thymol—likely operate through different mechanisms than MA, creating a complementary "pincer movement." This approach reduces the parasitic burden directly while MA clears any remaining infection, leading to a higher overall kill rate. The anti-leishmanial activity of *T. caramanicus*, especially its methanolic extract, appears to stem from a dual mode of action: directly attacking the parasite while also enhancing the host's immune response.

The methanolic extract of *T.carmanicus* has been found to contain various phenolic compounds and flavonoids which contribute to its antileishmanial activities. The anti-leishmaniasis activity of flavonoids such as quercetin and quercetin-derived compounds has been shown in various studies. Several targets of quercetin identified for the treatment of leishmaniasis include arginase and ribonucleotide reductase (da Silva et al. 2012). It has been shown that the flavonoid fistin inhibits the growth of promastigotes by inhibiting arginase activity and reducing intracellular glutathione levels and Nitric oxide (NO) production (Adinehbeigi et al. 2017). Rutin has been shown to be effective against *L. donovani* strains, promoting Th1 polarization and activating CD4+ and CD8+ T cells. Moreover, infected mice treated with rutin display up-regulation of NF-κB and Inducible Nitric Oxide Synthase (iNOS) genes (Chauhan et al. 2018). According to the studies some bioflavonoids like quercetin, gallic acid, and rutin are capable of suppressing essential enzymes like trypanothione reductase and trypanothione synthetase, which are important for leishmanial survival (Mehwish et al. 2021).

Thymus vulgaris ethanolic extract has shown a significant effect in reducing the size of the wound compared to MA (Nilforoushzadeh et al. 2008). The result of another study on *T. vulgaris* indicated that this plant is effective against *Trypanosoma cruzi* epimastigotes (Hikal et al. 2021). According to Ghadimi et al results, EOs of *Thymus hirtus* with IC50 of 0.25 µg/ml was found to be effective against *L. amazonensis, L. infantum and L. major* (Ghadimi et al. 2020).

Limitation: Although the methanolic extract exhibited a satisfactory selectivity index (SI) of 7.04, it falls short of the conventional threshold of >10, which is widely regarded as the gold standard for a safe drug candidate. This indicates that while the extract is relatively more toxic to the parasite than to mammalian cells, the therapeutic window is modest. Nevertheless, an SI value above 5 is often considered promising for further optimization, especially for natural products. Future studies could enhance the selectivity by isolating the most active synergistic compound(s) and encapsulating them in targeted drug delivery systems (e.g. liposomes, niosomes or nanoformulations) to reduce host cell cytotoxicity while maintaining antiamastigote efficacy.

Methanolic extract seems to be a promising candidate for combination therapy considering its high anti-leishmanial activity, synergistic effect and immunological properties. The methanolic extract, particularly when combined with MA referring to a specific active compound like polyphenolics, flavonoids, and thymol has shown leishmanicidal properties. However, more research is needed to isolate anti-leishmania compounds from plant extracts and assess the plant mechanism of action. *In vivo* studies (animal models) are necessary to confirm these findings before labeling it a good option for therapy.

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Conflicts of interest

The authors had no competing interests.

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Authors' Contributions

The authors confirm contribution to the paper as follows: study conception and design: Neda Mohamadi; antileishmanial activity data collection: Ehsan Salarkia and Simin Sharifi. Phytochemical analysis: Anis Ashrafzadeh, Aida Najafi, Sedigheh Afshar; analysis and interpretation of results: Fatemeh Sharifi and Neda Mohamadi, draft manuscript preparation: Fatemeh Sharifi and Neda Mohamadi.

Abbreviations

ANOVA – Analysis of Variance
AQ – Aqueous extract
CC_{50} – Half-maximal Cytotoxic Concentration
CD4+ – Cluster of Differentiation 4
CD8+ – Cluster of Differentiation 8
CHL – Chloroform extract
CI – Combination Index
DMEM – Dulbecco's Modified Eagle's Medium
DMSO – Dimethyl Sulfoxide
EA – Ethyl acetate extract
ELISA – Enzyme-Linked Immunosorbent Assay
EO – Essential oil
FBS – Fetal Bovine Serum
GC-MS – Gas Chromatography-Mass Spectrometry
HPLC – High-Performance Liquid Chromatography
IC_{50} – Half-maximal Inhibitory Concentration
IFN-γ (IFN-gamma) – Interferon-gamma
IL-12p40 – Interleukin-12p40
iNOS – Inducible Nitric Oxide Synthase
L. amazonensis – *Leishmania amazonensis*
L. donovani – *Leishmania donovani*
L. infantum – *Leishmania infantum*
L. major – *Leishmania major*
LOD – Limit of Detection
LOQ – Limit of Quantitation
MA – Meglumine Antimoniate (Glucantime®)
MET – Methanolic extract
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (assay)
NF-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells
NO – Nitric Oxide
OD – Optical Density
PE – Petroleum ether extract
RPMI – Roswell Park Memorial Institute (medium)
SI – Selectivity Index
SPSS – Statistical Package for the Social Sciences
T. carmanicus (*T. caramanicus*) – *Thymus carmanicus*
T. hirtus – *Thymus hirtus*
T. syriacus – *Thymus syriacus*
T. vulgaris – *Thymus vulgaris*
Th1 – T helper 1 cell
TLC – Thin Layer Chromatography
UV-Vis – Ultraviolet-Visible spectroscopy
WHO – World Health Organization

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