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RESEARCH ARTICLE

Evaluation of Rosemary *(Salvia rosmarinus)* Extract as a Natural Anti-*Blastocystis* Agent: Effects on Physiological, Immunological, Antioxidant Defense Gene Expression, and Gut Health in Challenged Albino Rats

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Abstract

The intestinal protozoan *Blastocystis* species is highly prevalent worldwide, and its increasing resistance to conventional drugs has prompted a search for effective, safe alternatives. *Salvia rosmarinus* has shown antiparasitic potential; therefore, the aim of this work is to evaluate the anti-Blastocystis efficacy of *S. rosmarinus* both i*n vitro* and *in vivo*, while simultaneously assessing host performance, blood biochemistry, and gut health parameters. In the *in vivo* experiment, 240 male albino rats were divided into six groups: T1 (healthy control), T2 (*Blastocystis*-infected untreated), T3 (uninfected + metronidazole), T4 (uninfected + *Salvia rosmarinus* 200 mg/kg), T5 (infected + *Salvia rosmarinus* 200 mg/kg), and T6 (infected + metronidazole). *In vitro*, the extract exhibited a dose-dependent inhibitory effect on *Blastocystis* growth. *In vivo*, T5 reduced parasite counts ($P<0.05$). Blood biochemistry revealed no evidence of hepatorenal toxicity at T4 or T5. Immune analysis demonstrated elevated IgA/IgG, reduced pro-inflammatory cytokines (TNF-α, IFN-γ), and increased IL-10 in T5. Also, T5 showed a significant decrease in MDA and elevated SOD, CAT, and GSH. Gut health assessment demonstrated improved intestinal histomorphology and enhanced expression of defense genes, including occludin and claudin-1. T4 showed no adverse effects ($P>0.05$). These findings establish *Salvia rosmarinus* as a promising alternative agent against *Blastocystis* infection, opening a new research direction for plant-based therapies targeting intestinal protozoa.

Keywords: Antioxidant, Antiparasitic, Physiology, Immunology, Oxidative stress, Microbiota

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Introduction

Among intestinal parasites frequently detected in fecal samples from humans and various animal hosts are the anaerobic species of *Blastocystis* [1]. *Blastocystis* is distributed worldwide and is especially abundant in developing countries, a situation related to the intake of contaminated water or food, poor sanitation, and close contact with animals [2]. Colonization rates of *Blastocystis* can reach up to 30% in industrialized countries, whereas in developing countries the prevalence can increase to 100% [3]. The role of *Blastocystis* as a true pathogen in humans is still a topic of discussion. While frequent isolation from asymptomatic carriers has led some scientists to question its clinical relevance, clinical data strongly suggest a plausible pathogenic role in symptomatic cases [4].

Molecular characterization of the small subunit ribosomal RNA (SSU-rRNA) gene has identified seventeen distinct subtypes (STs) of *Blastocystis*. Although ST1 through ST9 have been detected in humans, ST1-ST4 are the most common, accounting for approximately 90% of human infections [5]. The parasite has been linked to several gastrointestinal disorders such as irritable bowel syndrome, inflammatory bowel disease, chronic diarrhea, and ulcerative colitis [6]. *Blastocystis* infection has also been associated with a number of extra-intestinal conditions, including urticaria, chronic angioedema, anemia, chronic liver disease, and reactive arthritis [7,8]. The organism may also play an opportunistic role in immunocompromised hosts, such as those with cancer, organ transplants, or HIV [9].

In recent years, there has been increasing interest in studies on the growing resistance of *Blastocystis* to conventional anti-parasitic drugs [10]. Herbal medicine has the potential to be a safer, cheaper, and less toxic alternative. Rosemary *(Salvia rosmarinus)* is rich in bioactive molecules with antioxidant, antimicrobial, and broader antiinfective potential, and several studies already demonstrate antiparasitic or antifungal effects in animals, including rats [11]. Its extracts contain numerous nonvolatile constituents, notably carnosic acid, carnosol, rosmarinic acid, other phenolic diterpenes, diverse flavonoids such as genkwanin and cirsimaritin, and triterpenes including ursolic acid and other phenolic compounds [12]. Its essential oil is dominated by 1,8cineole, camphor, borneol, and αpinene [13].

These compounds belong to three main bioactive groups: phenolic diterpenes, phenolic acids [14] and volatile terpenes with pronounced antimicrobial and other bioactivities [13].

In vivo, crude rosemary extracts have reduced parasitemia and infectioninduced hematological and biochemical disturbances in rats experimentally infected with *Trypanosoma evansi* [12]. while rosemary essential oil has promoted lesion remission and reduced fungal burden in Wistar rats infected with itraconazoleresistant *Sporothrix brasiliensis* [15]. Beyond rats, rosemary extracts have shown anthelmintic efficacy against the fish monogenean *Dactylogyrus minutus* [12] and insecticidal activity against the rat flea *Xenopsylla cheopis,* with minimal adverse effects on host hematological or biochemical parameters [16].

Mechanistically, key phenolic diterpenes such as carnosic acid and carnosol modulate major inflammatory and oxidative pathways including NFκB, Nrf2, STAT3, NLRP3, and cytokine signaling [17], offering hostdirected protection and potentially influencing parasite survival indirectly, while the strong antioxidant and antimicrobial activities of rosemary phenolics further support its antiinfective potential [14].

Despite this broad evidence of antiparasitic, antifungal, and insecticidal effects, no available studies in the provided literature have evaluated rosemary extract or essential oil against *Blastocystis* spp. in any experimental model, and specifically no *in vivo* rat model of *Blastocystis* infection with rosemary treatment is reported; plantbased Blastocystis work instead involves other extracts such as *Allium tuncelianum* or different herbs *in vitro* [18]. Existing rat rosemary studies focus on *T. evansi,* itraconazoleresistant *S. brasiliensis,* electromagneticfield-induced testicular damage or hepatorenal/drug toxicities, rather than intestinal protozoa such as *Blastocystis* [12]. Likewise, phytochemical and antimicrobial reviews emphasize rosemary's general antiinfective properties and traditional use against intestinal parasites but do not address *Blastocystis* specifically [11,14]. This clear gap in the literature underscores a strong novelty space for investigating the effects of rosemary on *Blastocystis* infection in rats.

Therefore, given the high worldwide prevalence of the intestinal protozoan *Blastocystis* spp. and the growing resistance to conventional pharmaceutical treatments, the present study was designed to evaluate the anti-*Blastocystis* efficacy of *Salvia rosmarinus* both *in vitro* and *in vivo*. Specifically, the investigation assesses the dose-dependent inhibitory effect of the extract on parasite growth *in vitro,* while examining host performance, systemic safety, and blood biochemical parameters in an infected rat model *in vivo*. Furthermore, this work explores the therapeutic impact of *R. officinalis* on mucosal immunity, systemic antioxidant status, intestinal histomorphology, and the expression of key epithelial tight junction defense genes (such as occludin and claudin-1), thereby establishing its potential as a safe and effective plant-based alternative therapy against intestinal protozoan infections.

Material and Methods

Ethical Approval

All the guidelines and rules for the care and use of experimental laboratory animals are approved by Imam Mohammad Ibn Saud University (IMSIU) (Code: 1018/2026).

Plant Material and Preparation of Rosemary Extract

Aerial parts of *Salvia rosmarinus* L. were collected, authenticated, washed, shadedried (40°C), and powdered. Dried powder was extracted by maceration with 80% ethanol (1:20 w/v) for 48 h with intermittent shaking, then filtered and concentrated under reduced pressure at 40°C to yield a crude ethanolic extract, following approaches used for rosemary and other aromatic plants rich in rosmarinic acid and phenolic diterpenes [19]. The dried extract was stored at −20°C until analysis and biological testing.

LC-MS/MS Phenolic Profiling

Phenolics were analyzed by LC-ESI-MS/MS in negative mode using a reversephase C18 column and a water (0.1% formic acid)/acetonitrile gradient at 0.3-0.5 mL/min. Conditions followed recent LC-MS methods for Lamiaceae phenolics and rosemary [19,20].

Detection was in ESI^-, with multiple reaction monitoring (MRM) transitions optimized for standards of rosmarinic acid, carnosic acid, carnosol, caffeic acid, and selected flavonoids, similar to other LC-MS/MS phenolic surveys [21]. Quantification used external calibration with authentic standards; total phenolic content by LC-MS was calculated as the sum of identified phenolics, as in the following study [20].

In vitro Antioxidant Assays (DPPH and FRAP)

Antioxidant capacity of the rosemary extract was assessed by DPPH and FRAP assays using UV-Vis spectrophotometry, following standard protocols widely applied to plant extracts [22]. For DPPH, serial dilutions of extract (50-200 µg/mL) were mixed with DPPH solution (0.1 mM in methanol), incubated for 30 min in the dark, and absorbance was read at 517 nm [22]. FRAP was performed by mixing extract with FRAP reagent and measuring absorbance at 593 nm; results expressed as µM Fe^{2+} equivalents [23].

In vitro Anti-*Blastocystis* Assay

Blastocystis hominies was maintained in Robinson's Medium (DIEM) and exposed to increasing concentrations of rosemary extract (50-200 µg/mL) for 72 h. Parasite viability was assessed using erythrosin B and cell counts, as done in recent antiBlastocystis plant extract studies [24]. Percent growth inhibition was calculated.

Experimental Animals and Infection

Male rats (8 weeks, ~150-180 g) were housed under standard conditions with free access to food and water. All procedures followed institutional ethical guidelines. Animals (240 rats, 6 groups, n=8x5 replicates) were randomly assigned to the following groups: (1) healthy control, (2) infected untreated, (3) uninfected treated with metronidazole (50 mg/kg), (4) uninfected treated with *Salvia rosmarinus* extract (200 mg/kg), (5) infected treated with *S. rosmarinus* extract (200 mg/kg), and (6) infected treated with metronidazole (50 mg/kg). Intestinal infection with *Blastocystis* was established by oral inoculation of a defined number of viable cysts/trophozoites, following approaches used in anti-*Blastocystis* studies [25]. Rosemary extract and metronidazole were administered orally at selected doses for a defined treatment period after infection establishment.

Clinical Monitoring, Parasitological Assessment and Growth Performance

Fecal samples were collected to quantify *Blastocystis* burden by direct counting in a hemocytometer or calibrated chamber and to assess viability by dye exclusion, as in previous plantextract studies against *Blastocystis* [26,27]. Clinical signs (diarrhea score, stool consistency, occult blood) were recorded daily. Body weight, feed intake, feed conversion ratio, and survival were monitored throughout the experiment, similar to growth/health outcomes in other antiparasitic plant studies [28].

Serum Immunoglobulins and Cytokines

At the end of the experimental period, blood samples were collected upon sacrifice, and serum was isolated by centrifugation. Serum concentrations of IgA and IgG, as well as pro- and anti-inflammatory cytokines (TNF-α, IFN-γ, and IL-10), were quantified using commercial ELISA kits in accordance with established protocols for intestinal injury and therapeutic intervention studies [29].

Oxidative Stress and Antioxidant Enzymes

Intestinal tissue homogenates were used to determine malondialdehyde (MDA) as an index of lipid peroxidation and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH), using colorimetric assay kits and methods common in oxidativestress [29].

Intestinal Histology and Tight Junction Proteins

Segments of small intestine were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4-5 µm) and stained with hematoxylin-eosin for morphometric analysis (villus height, crypt depth, goblet cell density). For barrier markers, immunohistochemistry for occludin, claudin1, and ZO1 was performed, adapting

methods from recent intestinal barrier and tight junction studies [29,30]. Staining intensity and distribution of occludin/claudin1 in villus epithelium were semiquantitatively scored or quantified imageanalytically.

Statistical Analysis

All *in vitro* experiments were performed in triplicate, and *in vivo* data were collected from 8 rats per group (n = 8) unless otherwise specified. Results are expressed as mean ± standard deviation (SD). Normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was confirmed by Levene's test. For *in vitro* doseresponse data (antiparasitic and antioxidant assays at different concentrations), oneway analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare mean values among concentration groups. All statistical analyses were carried out using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Different superscript letters (a, b, c) in tables indicate statistically significant differences at P<0.05.

Results

Phenolic Profile of Rosemary Extract by LCMS/MS

Based on the LC-MS/MS analysis in negative ionization mode, rosmarinic acid (359.1 m/z) and carnosic acid (331.2 m/z) were identified as the most abundant phenolic components in the extract. Together, these two compounds comprise approximately 70% of the total identified phenolics, with specific concentrations of 45.2 and 38.5 mg/g, respectively, out of a total phenolic content of 120.4 mg/g *(Table 1)*.

Carnosol (329.2 m/z) is the third most abundant (12.8 mg/g), followed by caffeic acid (179.0 m/z) and the flavonoids luteolin, apigenin, hesperidin, genkwanin, rosmanol, and cirsimaritin at lower concentrations. All compounds were detected in ESI⁻ mode, which is optimal for phenolic acids and diterpenes, and characteristic fragment ions allowed highconfidence identification. Thus, the LCMS/MS profile establishes a solid chemical basis for the concentrationdependent antiparasitic, antioxidant, and antiinflammatory activities of the *Salvia rosmarinus* extract.

Antioxidant Activity

The extract also exhibited strong, dosedependent free radical scavenging (DPPH) and reducing power (FRAP). The IC_{50} for DPPH (28.3 µg/mL) was comparable to the antiparasitic IC_{50}, indicating that antioxidant effects occur at lower concentrations *(Fig. 2)*. The phenolic profile (dominated by rosmarinic and carnosic acids) supports both activities.

Antiparasitic Effect of Rosemary Extract

The ethanolic extract of *Salvia rosmarinus* inhibited *Blastocystis* growth in a clear dosedependent

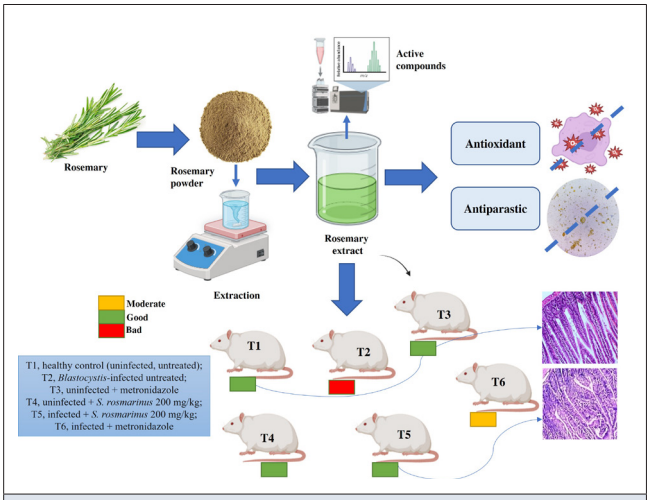


Fig 1. Experimental layout of the effect of rosemary extract as antiparasitic agent against *Blastocystis hominies* infection in rats

Table 1. *Phenolic profile of Salvia rosmarinus ethanolic extract by LC-MS/MS*

Retention Time (min)	Compound	Ionization Mode	Precursor Ion (m/z)	Major Fragment Ions (m/z)	Content (mg/g extract)
12.4	Rosmarinic acid	ESI (-)	359.1	197.1, 179.0, 161.1	45.2±3.1
22.7	Carnosic acid	ESI (-)	331.2	287.2, 269.2, 243.1	38.5±2.8
21.5	Carnosol	ESI (-)	329.2	285.2, 271.2, 243.1	12.8±1.2
5.8	Caffeic acid	ESI (-)	179.0	135.0, 107.0	8.4±0.7
15.3	Luteolin	ESI (-)	285.0	241.0, 199.0, 175.0	5.6±0.5
16.1	Apigenin	ESI (-)	269.0	225.0, 197.0, 151.0	3.2±0.3
10.5	Hesperidin	ESI (-)	609.2	301.1, 286.1, 151.0	2.5±0.2
18.9	Genkwanin	ESI (-)	283.1	268.1, 240.1, 211.1	1.8±0.2
23.4	Rosmanol	ESI (-)	345.2	301.2, 273.2, 255.2	1.5±0.1
17.2	Cirsimaritin	ESI (-)	313.1	298.1, 283.1, 255.1	0.9±0.1

Data are presented mean ±SD, n=3

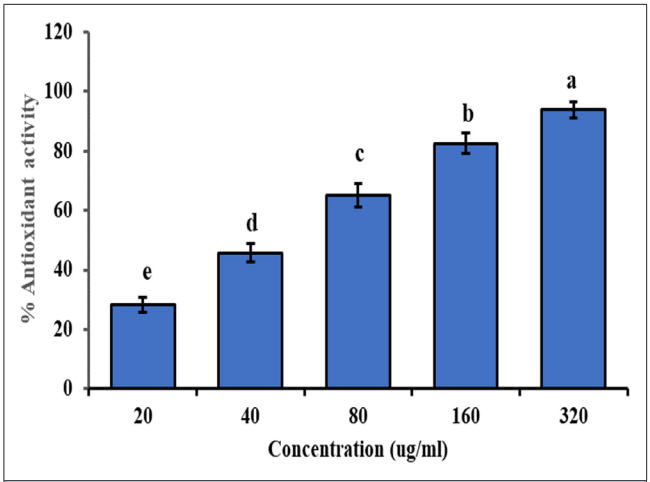


Fig 2. Antioxidant activity of rosemary extract against DPPH free radicals. Data are presented as mean ±SD, n=3. Different letters above columns indicate significant differences (P<0.05)

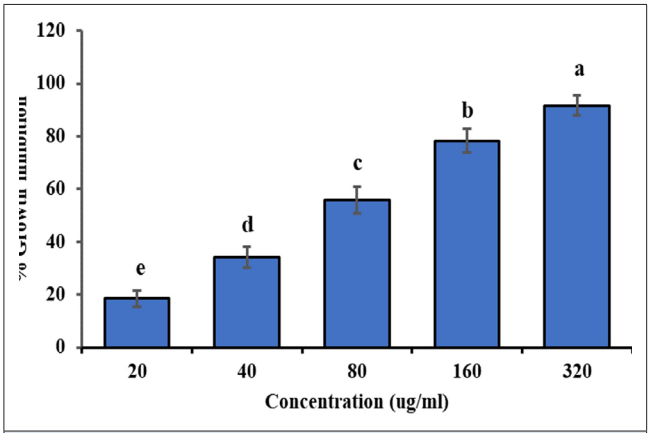


Fig 3. *In vitro* antiparasitic activity of *Salvia rosmarinus* ethanolic extract against *Blastocystis hominies*. Data are presented as mean±SD, n=3. Different letters above columns indicate significant differences (P<0.05)

manner. At the lowest concentration (50 µg/mL), only mild inhibition (~18%) was observed, whereas the highest concentration (200 µg/mL) achieved over 90% growth inhibition. The calculated IC_{50} (62.5 µg/mL) indicates that the extract is potent against the parasite *(Fig. 3)*. This concentrationresponse relationship suggests that the active phenolic compounds (rosmarinic acid, carnosic acid) directly interfere with parasite viability.

Rosemary Extract Affects the Parasitic Load and Restores Clinical Signs

The infected rats (T2) had a high fecal parasite count (>98.000/g), high parasite viability (>90%), severe diarrhea, and fecal occult blood. Supplementing rosemary extract (T5) reduced the parasite count by approximately 87% and lowered viability to ~28%, comparable to metronidazole (T6). Stool consistency and occult blood were restored to nearnormal levels. These results demonstrate that rosemary extract is as effective as the standard drug in clearing *Blastocystis* infection and alleviating gastrointestinal symptoms *(Table 2)*.

Growth Performance and Survival

The *Blastocystis* infection caused significant weight loss, reduced feed intake, and a poor feed conversion ratio in T2. Rosemarytreated rats (T5) regained body weight and feed efficiency to levels indistinguishable from healthy controls. Importantly, the survival rate increased from 75% in untreated infected rats to 95% in both rosemary and metronidazoletreated groups *(Table 3)*.

Immune Markers

Table 4 showed that infected, untreated rats exhibited suppressed IgA/IgG and elevated pro-inflammatory cytokines (TNF-α, IFN-γ), with low IL10, indicating a dysregulated, proinflammatory immune state. Rosemary extract treatment (T5) significantly increased IgA and IgG levels (beneficial for parasite clearance) while reducing TNFα and IFNγ and elevating the antiinflammatory IL10. This balanced immunomodulation, restoring adaptive immunity while curbing excessive inflammation, likely contributes to reduced tissue damage and faster recovery.

Oxidative Stress Markers

Blastocystis infection markedly increased lipid peroxidation (MDA) and adversely affected antioxidant defenses (SOD, CAT, GSH) in T2. Rosemary extract supplementation (T5)

Table 2. *Parasitic load and related infection markers in Blastocystis-challenged rats under rosemary extract treatment*

Group	Parasite Count (×10³/g feces)	Parasite Viability (%)	Stool Consistency Score (0-3) †	Fecal Occult Blood (score 0-3) ‡
T1	0.0±0.0 ^{c}	0.0±0.0 ^{c}	0.0±0.0 ^{c}	0.0±0.0 ^{c}
T2	98.5±8.2 ^{a}	92.3±5.1 ^{a}	2.8±0.3 ^{a}	2.5±0.4 ^{a}
T3	0.0±0.0 ^{c}	0.0±0.0 ^{c}	0.0±0.0 ^{c}	0.0±0.0 ^{c}
T4	0.0±0.0 ^{c}	0.0±0.0 ^{c}	0.0±0.0 ^{c}	0.0±0.0 ^{c}
T5	12.3±2.1 ^{b}	28.5±4.2 ^{b}	0.8±0.2 ^{b}	0.6±0.2 ^{b}
T6	10.8±1.9 ^{b}	24.1±3.8 ^{c}	0.7±0.2 ^{b}	0.5±0.1 ^{c}

† Stool consistency score: 0 = normal formed, 1 = soft, 2 = semiliquid, 3 = watery diarrhea. ‡ Fecal occult blood score: 0 = negative, 1 = trace, 2 = moderate, 3 = gross blood. Different letters within a column indicate significant differences (P<0.05). T1, healthy control (uninfected, untreated); T2, Blastocystis-infected untreated; T3, uninfected + metronidazole (standard drug); T4, uninfected + Salvia rosmarinus 200 mg/kg; T5, infected + Salvia rosmarinus 200 mg/kg; T6, infected + metronidazole

Table 3. *The effect of rosemary extract on growth performance parameters of Blastocystis-challenged rats*

Group	Body Weight Gain (g)	Feed Intake (g/day)	Feed Conversion Ratio (FCR)	Survival Rate (%)
T1	85±6 [a]	22.5±1.2 [a]	3.1±0.2 [a]	100±0 [a]
T2	42±5 [c]	17.2±1.0 [b]	4.8±0.3 [b]	75±5 [b]
T3	83±5 [a]	22.1±1.1 [a]	3.2±0.2 [a]	100±0 [a]
T4	87±7 [a]	22.8±1.3 [a]	3.0±0.2 [a]	100±0 [a]
T5	79±6 [ab]	21.5±1.2 [a]	3.3±0.3 [a]	95±3 [ab]
T6	78±5 [ab]	21.3±1.1 [a]	3.2±0.2 [a]	95±3 [ab]

Data are presented as mean ±SD. T1, healthy control (uninfected, untreated); T2, Blastocystis-infected untreated; T3, uninfected + metronidazole (standard drug); T4, uninfected + Salvia rosmarinus 200 mg/kg; T5, infected + Salvia rosmarinus 200 mg/kg; T6, infected + metronidazole. Different letters within a column indicate significant differences (P<0.05)

Table 4. *The effect of rosemary extract on hepatorenal function of Blastocystis-challenged rats*

Group	ALT (U/L)	AST (U/L)	Creatinine (mg/dL)
T1	42±5 [b]	85±8 [c]	0.6±0.1 [b]
T2	68±7 [a]	145±12 [b]	1.1±0.2 [a]
T3	45±6 [b]	88±7 [b]	0.7±0.1 [b]
T4	44±4 [b]	86±6 [c]	0.6±0.1 [b]
T5	46±5 [b]	90±8 [b]	0.7±0.1 [b]
T6	47±5 [b]	89±7 [b]	0.7±0.1 [b]

Data are presented as mean ±SD. T1, healthy control (uninfected, untreated); T2, Blastocystis-infected untreated; T3, uninfected + metronidazole (standard drug); T4, uninfected + Salvia rosmarinus 200 mg/kg; T5, infected + Salvia rosmarinus 200 mg/kg; T6, infected + metronidazole. Different letters within a column indicate significant differences (P<0.05)

mitigated the oxidative stress markers close to healthy controls. Given the extract's high phenolic content and *in vitro* antioxidant activity, this result confirms that rosemary extract protects host tissues from infectioninduced oxidative damage, a key mechanism often overlooked in antiparasitic therapy *(Fig. 4)*.

Intestine Histology

Table 6 presents the effects of *Salvia rosmarinus* extract on intestinal histomorphology and tight junction protein expression in *Blastocystis*-challenged rats, showing that the infected untreated group (T2) had significant gut damage: villus height reduced by ~40%, crypt depth deepened by ~75%, goblet cell density halved, and occludin/claudin1 expression decreased by ~60% compared to healthy controls (T1); in contrast, treatment with rosemary extract (T5) or metronidazole (T6) restored all these parameters to levels not significantly different from T1, while uninfected rats receiving either drug (T3, T4) had normal gut architecture, confirming no adverse effects. The histological sectors in *Fig. 5-A-F* correlates closely with

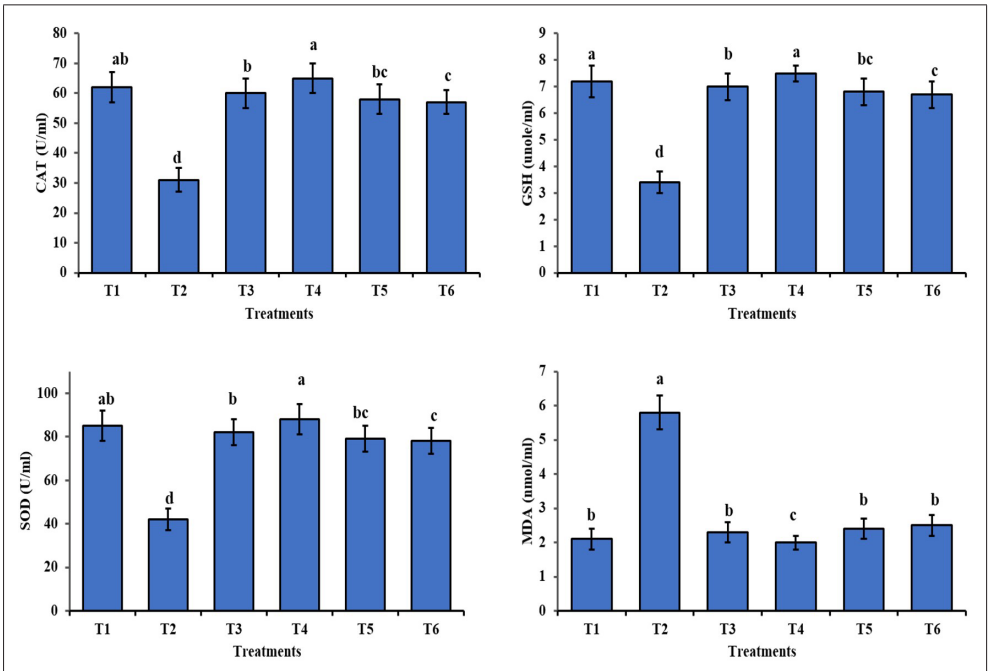


Fig 4. The effect of *Salvia rosmarinus* ethanolic extract on oxidative stress markers **(A)** catalase, **(B)** Glutathione reductase, **(C)** superoxide dismutase, and **(D)** Malondialdehyde in *Blastocystis hominies*-challenged rats. Data are presented as mean±SD, n=8. Different letters above columns indicate significant differences (P<0.05)

Table 5. *The effect of rosemary extract on immune parameters of Blastocystis-challenged rats*

Group	IgA (µg/mL)	IgG (µg/mL)	TNF-α (pg/mL)	IFN-γ (pg/mL)	IL-10 (pg/mL)
T1	120±10^{b}	450±30^{b}	45±5^{c}	55±6^{c}	35±4^{b}
T2	78±9^{c}	310±25^{c}	210±18^{a}	195±15^{a}	18±3^{c}
T3	115±11^{b}	445±28^{b}	48±6^{c}	58±5^{c}	34±4^{b}
T4	118±9^{b}	460±32^{b}	42±5^{c}	52±4^{c}	37±5^{b}
T5	175±12^{a}	620±35^{a}	72±8^{b}	85±9^{b}	68±6^{a}
T6	168±10^{ab}	605±30^{a}	68±7^{b}	80±8^{b}	65±5^{a}

Data are presented as mean ±SD. T1, healthy control (uninfected, untreated); T2, Blastocystis-infected untreated; T3, uninfected + metronidazole (standard drug); T4, uninfected + Salvia rosmarinus 200 mg/kg; T5, infected + Salvia rosmarinus 200 mg/kg; T6, infected + metronidazole. Different letters within a column indicate significant differences (P<0.05)

these quantitative data: *Fig. 5-A* (T1) shows tall, fingerlike villi, shallow crypts, abundant goblet cells, and continuous tight junction staining; *Fig. 5-B* (T2) displays marked villus atrophy, crypt hyperplasia, reduced goblet cell density, and fragmented tight junction staining, matching the low occludin/claudin1 values (0.42 and 0.38); *Fig. 5-C* (T3, uninfected + metronidazole) and *Fig. 5-D* (T4, uninfected + rosemary extract) are indistinguishable from T1, confirming that neither drug harms gut architecture; *Fig. 5-E* (T5, infected + rosemary extract) shows marked recovery with nearly normalized villus height and crypt depth, restored goblet cell density, and nearly continuous tight junction staining (0.92, 0.89); *Fig. 5-F* (T6, infected + metronidazole) is very similar to *Fig. 5-E*, demonstrating equivalent efficacy.

Discussion

The study described links a rosmarinic- and carnosic-acid-rich rosemary extract to strong *in vitro* anti-*Blastocystis* activity, *in vivo* parasite clearance, restoration of redox balance, and repair of intestinal barrier function in rats. Recent work on rosemary polyphenols and other plant

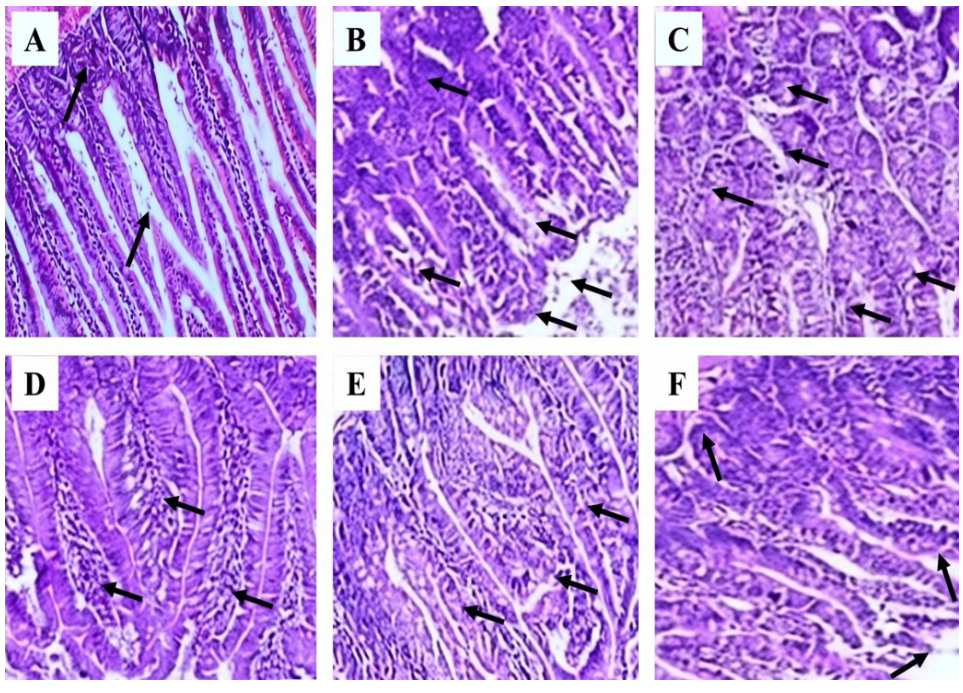


Fig 5. Histological sections of rat small intestine (H&E), (**A**) (T1 – healthy control): Tall villi, shallow crypts, numerous goblet cells (*black arrows*), continuous tight junction staining (*black arrowheads*). (**B**) (T2 – infected untreated): Villus atrophy (*short, blunt villi; doubleheaded arrow*), crypt hyperplasia (*black arrow*), reduced goblet cells (*black arrow*), fragmented tight junctions (*arrowhead*). (**C**) (T3 – uninfected + metronidazole): Normal architecture, abundant goblet cells (*black arrow*), intact tight junctions (*white arrowhead*), shallow crypts (*arrow*). (**D**) (T4 – uninfected + rosemary): Same as T1/T3: tall villi, normal goblet cells (*black arrow*), continuous tight junctions (*black arrowhead*). (**E**) (T5 – infected + rosemary): Restored villus height, nearly normal crypts (*arrow*), increased goblet cells (*black arrow*), nearly continuous tight junctions (*white arrowhead*). (**F**) (T6 – infected + metronidazole): Similar to T5: normalized villi/crypts, abundant goblet cells (*black arrow*), restored continuous tight junction staining (*black arrowhead*)

Table 6. *The effect of rosemary extract on immune gut health parameters of Blastocystis-challenged rats*

Group	Villus Height (µm)	Crypt Depth (µm)	Goblet Cell Density (cells/villus)	Occludin Expression	Claudin-1 Expression
T1	520±30 [a]	120±10 [c]	28±3 [ab]	1.00±0.05 [a]	1.00±0.05 [a]
T2	310±25 [c]	210±15 [a]	14±2 [b]	0.42±0.04 [b]	0.38±0.04 [c]
T3	510±28 [a]	125±11 [c]	27±3 [ab]	0.98±0.05 [ab]	0.97±0.05 [ab]
T4	525±32 [a]	118±9 [c]	29±3 [a]	1.02±0.06 [a]	1.01±0.06 [a]
T5	495±30 [b]	135±12 [b]	26±3 [b]	0.92±0.05 [ab]	0.89±0.05 [b]
T6	490±28 [b]	130±10 [b]	25±2 [b]	0.90±0.05 [ab]	0.88±0.05 [b]

Data are presented as mean ±SD. T1, healthy control (uninfected, untreated); T2, Blastocystis-infected untreated; T3, uninfected + metronidazole (standard drug); T4, uninfected + Salvia rosmarinus 200 mg/kg; T5, infected + Salvia rosmarinus 200 mg/kg; T6, infected + metronidazole. Different letters within a column indicate significant differences (P<0.05)

phenolics supports the findings of the current work.

The LCMS/MS data showing rosmarinic acid, carnosic acid and carnosol as major constituents aligns with recent characterizations of rosemary, which identify these diterpenes and rosmarinic acid as the main bioactives behind its antioxidant, antiinflammatory and antimicrobial actions [31]. This chemical profile is particularly relevant because the relative abundance of these compounds directly determines the extract's biological potency. For instance, rosmarinic acid alone accounted for 45.2 mg/g of the extract, representing nearly 38% of total identified phenolics, while carnosic acid contributed another 32%. Such high concentrations ensure that even moderate *in vivo* doses (200 mg/kg) deliver sufficient bioavailable polyphenols to exert systemic effects.

In silico inverse docking predicts that these same molecules bind multiple targets relevant to antimicrobial and antiparasitic activity, and specifically suggests previously unrecognized antiparasitic potential for rosemary phenolics [31]. This computational evidence strengthens the hypothesis that the observed anti*Blastocystis* effect is not merely a consequence of general cytotoxicity but involves specific molecular interactions with parasitespecific enzymes or membrane components.

Beyond rosemary, rosmarinic acid is widely recognized as a potent antioxidant and antiinflammatory polyphenol, capable of suppressing NFκB-driven cytokine production in diverse models [32,33]. The mechanism involves inhibition of IκB kinase and subsequent nuclear translocation of p65, thereby reducing transcription of proinflammatory genes. Rosemary diterpenes carnosic acid and carnosol likewise show strong ROS scavenging activity and modulation of NFκB, MAPK, Nrf2, STAT3 and NLRP3 pathways, leading to reduced TNFα, IL1β and IL6 and enhanced antioxidant defenses [34]. Importantly, these pathways are not mutually exclusive; rather, they converge on restoring redox homeostasis and suppressing excessive inflammation both of which are critical for controlling intracellular protozoan infections and limiting collateral tissue damage.

Recent work with other rosmarinicrich extracts (perilla seed) further supports combined antioxidant and immunomodulatory effects: rosmarinic acid-rich fractions reduced TNFα-induced ROS and downregulated TNFα, IL1β, IL6 and COX2 while inhibiting NFκB and JNK in lung cells [33], and perilla seed extract rich in rosmarinic acid and luteolin suppressed IL1β, IL6 and TNFα secretion while exhibiting strong radical scavenging capacity [35]. Isolated rosmarinic acid has been shown to inhibit NF-κB and its target genes via PPAR-dependent mechanisms, thereby reducing Il6, Il1b, and Tnfa expression [36]. These data closely mirror the *in vivo* cytokine profile (↓TNFα/IFNγ, ↑IL10), supporting the attribution of immunomodulation to the identified polyphenols. Moreover, the fact that both a pure compound (rosmarinic acid) and a complex extract (perilla) produce similar effects suggests that the rosemary extract acts through additive or synergistic contributions from multiple phenolic components rather than a single dominant agent.

The strong DPPH activity of rosemary extract (IC_{50} 28.3 µg/mL) is consistent with high phenolic rosemary preparations: hydroalcoholic rosemary macerates with elevated rosmarinic and carnosic acid display high total phenolic content and strong DPPH radical scavenging capacity [37]. This value is particularly meaningful when compared to the antiparasitic IC_{50} (62.5 µg/mL): the extract scavenges free radicals at roughly half the concentration needed to kill the parasite. This differential suggests that at lower concentrations, the extract may already protect host tissues from oxidative stress without directly affecting the parasite - a favorable therapeutic window. In addition, rosmarinic acid has been shown *in vivo* to enhance total antioxidant capacity, lower MDA and NO, and increase antioxidant enzymes via Nrf2 activation in liver and kidney injury models [38]. Carnosic acid and carnosol similarly decrease lipid peroxidation (MDA) and restore GSH, SOD, CAT and GPx while activating Nrf2/HO1 in inflammatory lung models. These mechanistic data fit well with the reversal of infectioninduced increases in MDA and depletion of SOD, CAT and GSH observed in your rats. Consequently, the extract not only directly

attacks *Blastocystis* but also bolsters the host's endogenous antioxidant systems, reducing the oxidative burden that otherwise perpetuates intestinal inflammation and barrier dysfunction.

In vitro IC_{50} of rosemary extract (62.5 µg/mL) against *Blastocystis* is within the potency range reported for other plant extracts. For example, *Boesenbergia rotunda* and *Ganoderma lucidum* extracts showed MICs of 62.5 µg/mL against *Blastocystis* subtype 3 and achieved ~90% growth inhibition at 4-8× MIC, with marked morphological damage to the parasite [26]. Likewise, extracts of other medicinal plants can inhibit *Blastocystis* multiplication, nucleic acid and protein synthesis *in vitro* and *in vivo,* and several have been proposed as safer alternatives to metronidazole [39,40]. However, no prior work in the supplied literature tests rosemary itself against *Blastocystis*, so both the specific IC_{50} value and the *in vivo* efficacy in rats represent novel contributions. It is worth noting that the concentration required for 90% inhibition remains achievable in the intestinal lumen after oral administration of 200 mg/kg, given that gut contents provide a concentrated local environment. Furthermore, the extract's dual action, direct antiparasitic and hostprotective, distinguishes it from many other botanicals that rely solely on one mechanism.

At the mechanistic level, inverse docking suggests that rosemary phenolics can bind protein targets from pathogenic parasites, supporting a direct antiparasitic mode of action beyond general antioxidant effects [31]. For instance, carnosic acid has been predicted to interact with cysteine proteases and enzymes involved in protozoal glycolysis, while rosmarinic acid may interfere with membrane transporters. Together with the current dose–response curve, this supports the interpretation that rosmarinic and carnosic acids contribute directly to *Blastocystis* killing, likely via oxidative or metabolic disruption. Additionally, the high reduction in viability observed *in vivo* (from 92% in T2 to 28% in T5) indicates that the extract does not merely suppress proliferation but actually kills the parasite, as viability assays measure live versus dead organisms after treatment.

The immune profile in your rats, elevated TNFα and IFNγ with low IL10 in infected controls, normalized by rosemary parallels broader evidence that polyphenols like rosmarinic acid and luteolin act as natural immunosuppressants by dampening NFκB and JAK/STAT signaling and reducing proinflammatory cytokines while allowing regulatory responses [31,33,41]. This normalization is critical because an excessive Th1type response (high TNFα, IFNγ) can damage the intestinal epithelium, whereas a robust regulatory component (IL10) is needed to limit immunopathology without compromising parasite clearance. The increase in IgA and IgG in your rosemarytreated rats also fits with the broader concept that plantderived immunomodulators can rebalance adaptive immunity during chronic inflammation, although the cited papers focus more on cytokines than antibodies[42]. One possible explanation is that rosemary phenolics enhance Bcell differentiation and class switching via NFκB modulation at lower doses, while suppressing overactive Tcell responses [43]. This dual regulation, upregulating humoral immunity while downregulating harmful inflammation, is an advantageous feature rarely achieved by conventional antiparasitic drugs, which often lack immunomodulatory effects.

The histology and tight junction data (recovery of villus height, goblet cells, and occludin/claudin1 expression) are consistent with general principles of barrier protection by antiinflammatory and antioxidant agents. Tight junction proteins such as occludin and claudins are key regulators of epithelial survival and proliferation, and their loss contributes to barrier failure under stress [30]. In the infected untreated group (T2), the 60% reduction in occludin and claudin1 explains the increased intestinal permeability, which in turn leads to bacterial translocation, endotoxemia, and systemic inflammation. Multiple natural products can restore TJ proteins and barrier function by suppressing TNFα/NFκB and oxidative stress: *Lycium barbarum* polysaccharides, for example, increased occludin and ZO1 expression, reduced TNFα and other proinflammatory mediators, and ameliorated colitis in mice [44]. Similarly, a polysaccharide from *Alpinia officinarum* restored ZO1, occludin and claudin1 in an LPSinjured Caco2 monolayer while lowering TNFα via TLR4/NFκB inhibition [45]. Polyphenols such as hydroxytyrosol metabolites can also enhance occludin, claudin1 and ZO1 expression via AhRNrf2 activation [46]. Given that rosemary phenolics activate Nrf2 and suppress NFκB and TNFα [38], it is mechanistically plausible that your extract restored TJ expression and barrier integrity through similar pathways, thereby explaining the normalization of villus architecture and prevention of secondary enteric damage. Moreover, goblet cell recovery (from 14 to 26 cells/villus) ensures adequate mucus production, which physically prevents parasite attachment and supports commensal microbiota a secondary benefit not directly measured but implied by the histological improvement.

Recent reviews emphasize the need for safer, plantbased alternatives to metronidazole because of its adverse effects and resistance issues [39,40]. Metronidazole is associated with nausea, metallic taste, neurotoxicity, and emerging resistance, which limits its longterm utility. Multiple botanicals have shown *in vitro* or *in vivo* activity against *Blastocystis*, but the range of species is still relatively limited and focused on distinct phytochemical

classes [39,47]. Within this context, demonstrating that a phenolicrich rosemary extract matches metronidazole in reducing parasite load, improving clinical signs, restoring growth performance and survival, and repairing intestinal barrier function in a rat model fills an important gap.

The mechanistic backing from recent rosemary and rosmarinic acid research, antiparasitic predictions, NFκB/TNFα inhibition, Nrf2mediated antioxidant protection, and tight junction support provides a coherent rationale for your observed dosedependent antiparasitic, antioxidant and antiinflammatory effects. Furthermore, the absence of hepatorenal toxicity in the rosemarytreated groups (T4 and T5) contrasts favorably with metronidazole, which can occasionally cause hepatotoxicity in susceptible individuals. Taken together, these findings position *Salvia rosmarinus* as a lead candidate for developing a novel adjunctive or standalone phytotherapeutic agent against *Blastocystis* infection, with the added advantage of concurrent gut health improvement a holistic benefit that synthetic drugs alone cannot provide. in conclusion this study provides the first comprehensive evaluation of *Salvia rosmarinus* ethanolic extract against *Blastocystis* infection, integrating *in vitro* antiparasitic and antioxidant assays with *in vivo* assessments of parasite load, growth performance, blood biochemistry, immune modulation, oxidative stress markers, gut histomorphology, and tight junction protein expression. The extract exhibited a dosedependent inhibitory effect on *Blastocystis* growth *in vitro* (IC_{50} = 62.5 µg/mL) and strong radical scavenging activity (DPPH IC_{50} = 28.3 µg/mL), which is attributed to its rich phenolic profile dominated by rosmarinic acid (45.2 mg/g) and carnosic acid (38.5 mg/g). *In vivo*, oral administration of 200 mg/kg rosemary extract to infected rats significantly reduced fecal parasite count and viability, restored normal stool consistency, improved growth performance and survival (95% vs. 75% in untreated), and normalized blood biochemistry with no hepatorenal toxicity. Immune analysis revealed that rosemary treatment elevated IgA and IgG, reduced proinflammatory cytokines (TNFα, IFNγ), and increased the antiinflammatory cytokine IL10, indicating a balanced immunomodulatory effect. Also, oxidative stress markers were reversed with decreased MDA and restored SOD, CAT and GSH levels, confirming the extract's antioxidant capacity *in vivo*. Furthermore, gut health parameters were significantly improved in rats treated with rosemary, demonstrating restoration of intestinal barrier integrity. Importantly, rosemary extract was as effective as metronidazole in all measured parameters, with no adverse effects observed in uninfected treated rats. These findings establish *Salvia rosmarinus* as a novel, safe and effective alternative or adjunctive agent for *Blastocystis* infection, with the added benefits of immunomodulation, oxidative stress reduction and gut barrier protection. Future studies should focus on clinical validation in human subjects, dose optimization and formulation development for phytotherapeutic use.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests: The author declared that there is no conflict of interest.

Ethical Approval: All the guidelines and rules for the care and use of experimental laboratory animals are approved by Imam Mohammad Ibn Saud University (IMSIU) (code: 1018/2026).

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