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VERIFYING THERAPEUTIC POTENCY OF SHILAJIT AGAINST CRYPTOSPORIDIOSIS: AN EXPERIMENTAL STUDY

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Abstract

In immunocompromised hosts, diarrheal disease is primarily caused by *Cryptosporidium* spp. infect-ion. Up- to-date, there is neither vaccine nor safe or efficient treatment. Shilajit is one the medicinal plants. This study evaluated Shilajit anti-cryptosporidiosis alone or with Nitazoxanide® (NTZ) in immunocompromised Albino mice. The study comprised fifty mice divided into five gr-oups of ten mice each. The oocysts shedding were assessed parasitologically by stained fecal smears, histologically in liver and small intestine, and immunologically by evaluation of IFN-γ and IL-10 levels.

The results showed that shilajit improved the liver and small intestine significantly by oocysts reduction in feces and small intestine. Mice treated with both shilajit and NTZ showed the best improvement. IFN-γ increased and IL-10 decreased ($P< 0.001$) in all treated mice.

Key words: *Cryptosporidium parvum,* Shilajit, Nitazoxanide, Immunocompromised mice

Introduction

One of the worldwide prevalent protozoa parasites that induce gastrointestinal troubles in animals and humans is *Cryptosporidium parvum* (Khan and Witola, 2023). About 1.7 billion cases and 525,000 deaths annually were attributed to diarrhea as the second most common cause of mortality in young children (Hartman *et al,* 2023). Many agents, such as viruses, protozoa, and bacteria, or even allergy to some drugs can cause diarrhea (Shrestha *et al,* 2022). Cryptosporidiosis is a water- and food-borne zoonotic disease caused by *C. hominis* and *C. parvum* transmitted by fecal-oral & respiratory routes as well as animal contact; oocysts resist disinfectants including chlorine (Helmy and Hafez, 2022). Immunocompetent hosts suffer from acute, self-limited gastroenteritis infections, but immunocompromised and neonatal animals may develop chronic fatal diarrhea (Gerace *et al,* 2019). Immunocompromised patients and young children suffered from risky cryptosporidiosis due to lack of an effective treatment (Gilbert *et al,* 2023).

Nitazoxanide® is the only proven anti-cryptosporidiosis approved by FDA (Caravedo and White, 2023).

Generally, Shilajit, or *Asphaltum punjabianum*, is one of the medicinal plants (Yaqoob *et al,* 2023). It grows naturally in various rocky mountainous regions, mainly the Himalayas, Altai, Caucasus, and others (Elahi *et al,* 2024). Its black and yellow form have been used in fortified food products, with available structural protein for skin, eyes, bones, ligaments, tendons, and muscles (Neltner *et al,* 2022). Due to its benefits, Shilajit has been utilized as food, a supplement, or as part of traditional medicine (Patel *et al,* 2023). Shilajit is composed of 60-80% organic components and 20-40% minerals (Zhernov *et al,* 2021) Shilajit showed that it has great potential for treating various diseases and proved to have anti-ulcerogenic, antioxidant, cognitive and memory enhancer, anti-diabetic, anxiolytic, antiallergic, immunomodulator, anti-inflammatory, analgesic, antimicrobial, antiviral, and anti-fungal activities (Abylaeva and Kaya, 2023).

This present study aimed to evaluate, may be for the first time, the therapeutic effects of Shilajit in treating *Cryptosporidium parvum* immunocompromised mice versus the Nitazoxanide® as the golden standard drug.

Material and Methods

Animals and experiment design: Fifty laboratory bred male Albino Swiss mice aged 3-4 weeks and weighed 20-25g were purchased from Theodor Bilharz Research Institute provided. Mice were divided into five groups of ten mice each. They were housed in plastic cages in conditioned room and fed a regular diet of 24% protein, 4% fat, and roughly 4-5% fiber. Special water bottles were used to offer water, and fresh bedding was always available in addition to routine cage cleanings.

Ethical Committee: Following TBRI (Federal Wide Assurance FWA00010609), approval all mice experiments on were conducted using internationally valid guidelines.

Immunosuppression: Mice were given Dexamethasone 0.25mg/g/day oral doses for 14 consecutive days to induce immunity (Abdou *et al,* 2013).

Cryptosporidium oocysts from naturally infected diarrheal calves were collected and genetic identity as *C. parvum* (El-Wakil *et al,* 2024). After stool sample collection, the oocysts were purified (Arrowood and Donaldson, 1996). Potassium dichromate solution (2.5%) with purified oocysts was maintained at 4°C until needed. The infection was given using the oral-gastric gavage method. About 10^4 oocysts in PBS (200µl) were given to each mouse for at zero day post-infection (Ghasemkhani *et al,* 2021).

Drug preparation: 1- Shilajit powder extract used was supplied by Sunfood Natural Spirit Trading, and given orally as 250 mg/kg/day for five consecutive days started on 7^{th} day post-infection (Ghaaazi *et al,* 2018). 2- Nitazoxanide 100mg/kg/ day were given for five consecutive days started on 7^{th} day post-infection (Li *et al,* 2003).

Parasitological drugs' assessment**:** On 7^{th} day post-infection (PI), feces of mice were collected and modified Ziehl-Neelsen stained smears microscopically examined to prove presence of oocysts (Garcia, 2007). On 12^{th} PI, all mice were sacrificed and oocysts/gram feces were counted. The following equation was used (Penido *et al,* 1994).

Reduction % = 100(C- E)/C; where C was control mice, and E was experimental mice

Histopathological examination: Mice sacrificed on 12^{th} day PI liver and small intestine were histopathologically examined in TBRI pathology department after being preserved in 10% buffered formalin solution, and processed for paraffin sectioning and hematoxylin and eosin staining (Culling, 2013).

Immunological assessment of IFN-γ & IL-10 levels: Blood samples were collected and centrifuged for 15 minutes at 3000 rpm to separate serum, aliquot and kept at -20°C until needed. IFN-γ & IL-10 levels in sera were measured by double-sandwich ELISA (Bioneovan Co; Ltd, Beijing, China), following the manufacturer's instructions. Wavelengths of 450 & 630nm filters assessed the optical density. Standard curve and assay ranges of 3-200pg/ml were used.

Statistical analysis: Data were computerized and analyzed by using SPSS version 28. Means ± standard deviation (SD) was displayed. Paired sample T-test was used for two group comparison, and ANOVA tested more than two groups. $P > 0.05$ was considered non-significant, $P < 0.05$ significant, and $P < 0.001$ highly significant.

Results

Oocyst: GIII, GIV, and GV showed significant reduction in oocysts count in stool and small intestinal contents compared to positive control ($P < 0.001$). GV given combined nitazoxanide and shilajit extract showed reduction of 94.39% in stool and 88.92% in small intestine, respectively, as compared to mice received either Nitazoxanide (NTZ) or Shilajit extract (74.81%, & 67.47%) in stool and (72.72%, & 65.11%) in small intestine.

Histopathological examination: Small intestine of normal control (GI) showed normal structures with a typical villi structure. Posi-

tive control (GII) showed distorted villi and an edematous core associated with many intraepithelial inflammatory cells. Mice in GIII, GIV, & GV after treated with single and/or combined NTZ, and shilajit extract showed improvement as scattered mononuclear cells, mild infiltration, & regular villi.

Liver showed typical architecture as negative control, but localized lobular and portal inflammatory cellular infiltration in positive one. GIII & GIV showed mild inflammatory cellular infiltrations. GV improved typical hepatic architecture with combined drugs.

IFN-γ highly increased in positive control (92.6±0.88) as to negative one (15.0±0.75). IFN-γ levels were reduced in GIII, GIV, & GV compared to positive mice (P< 0.0001). Least IFN-γ levels in GV given combined treatment (56.7±0.77). GIV shilajit treated (69.8±0.89) showed lower IFN-γ levels than GIII treated NTZ (78.9±0.68) mice with a high significant difference (P< 0.0001).

IL-10: A highly significant decrease in IL-10 level in GII (32.5±0.95) compared to GI (56.82±0.87). IL-10 levels were reduced in GIII, GIV, & GV compared to GII (P< 0.0001). IL-10 levels were highly reduced in GV given combined NTZ and shilajit extract (51.1±0.66). GIV mice given shilajit extract (47.2±0.74) showed higher IL-10 levels than GIII treated with (NTZ) (39.2 ± 0.82) with a highly significant difference (P< 0.0001)

Details were shown in tables (1, 2, 3, & 4) and figures (1, 2, & 3).

Table 1: Oocysts shedding and reduction % in fecal of different groups.

Groups	Oocysts shedding in stool	Reduction %
GI	0	--
GII	319.12±28.7	--
GIII	80.4±18.7	74.81%
GIV	103.8±16.5	67.47%
GV	17.9±6.8	94.39%
P1=0.0000**; *P2, P3, P4* < 0.0001**; *P5*= 0.000**; *P6*= 0.008*		

Table 2: Oocysts shedding and reduction% in small intestines of different groups.

Groups	Oocysts shedding in small intestine	Reduction %
GI	0	--
GII	38.12±2.4	--
GIII	10.4±1.7	72.72%
GIV	13.3±1.2	65.11%
GV	4.2±0.8	88.92%
P1=0.0000**; *P2, P3, P4* < 0.0001**; *P5*= 0.000**; *P6*= 0.0003**		

P<0.001** a high significant difference, *P*<0.05 * significant difference, *P*1 between all groups, *P*2 GII vs. GIII, *P*3 GII vs. GIV, *P*4 GII vs. GV, *P*5 between (GIII, GIV, GV), & *P*6 GIII vs. GIV

Table 3 IFN-γ expression in all groups

Groups	IFN-γ
GI	15.0 ± 0.75
GII	92.6 ± 0.88
GIII	78.9 ± 0.68
GIV	69.8 ± 0.89
GV	56.7 ± 0.77
P1=0.0000**; *P2, P3, P4, P5* < 0.0001**; *P6*= 0.0000**; *P7* < 0.0001**	

Table 4: IL-10 expression in all groups

Groups	IL 10
GI	56.82 ± 0.87
GII	32.5 ± 0.95
GIII	39.2 ± 0.82
GIV	47.2 ± 0.74
GV	51.1 ± 0.66
P1=0.0000**; *P2, P3, P4, P5* < 0.0001**; *P6*= 0.0000**; *P7* < 0.0001**	

P<0.001** highly significant difference; *P*<0.05 * significant difference, *P*1 between all groups, *P*2 GI vs. GII, *P3* GII vs. GIII, *P*4 GII vs. GIV. *P*5 GII vs. GV, *P*6 between all treated GIII, GIV, GV, & *P*7 GIII vs. GIV

Discussion

Many phytochemical (alkaloids) plant extracts are now used in treatment microorganisms infectious diseases (Abouel-Nour *et al,*

2016). The first recorded expedition for obtaining living plants was that of the famous Queen Hatshepsut to the Punt land, Northeastern Coast of Africa, to fetch the fabulous biblical resins frankincense or Boswellia and myrrh or Commiphora (Janick, 2007). In traditional folk medicine, shilajit, a herbomineral natural substance made from lichen and plant deposits, is one of the most widely utilized forms with a variety of medicinal advantages (Kangari *et al,* 2022).

In the present study, shilajit powder extracts alone or combined to treat *C. parvum* infections, showed significant reduction in oocysts number as compared to positive control ($P < 0.001$). The mice treated with combined shilajit extract and NTZ showed the highest oocysts reduction rate (94.39%) in the small intestine and (88.92%) in the feces. However, mice treated with shilajit extract alone showed the least reduction oocysts in feces (67.47%) and small intestine (65.11%). Mice treated with the reference drug, NTZ alone, showed a moderate oocyst reduction in small intestine (72.72%) and in feces (74.81%). This agreed with El-Wakil *et al*. (2022), who successfully used *Camellia sinensis* (green tea) in treating experimentally infected cryptosporidiosis mice. This also agreed with Esmat *et al.* (2022), who rep- orted that combined treatment with low-dose clofazimine and half-dose NTZ significantly improved the enterocyte cellular structures without cryptosporidium oocysts. Namazi and Razavi (2024) reported that medicinal plants contain bioactive constituents such as polyphenols, flavonoids, anthocyanins, coumarins, tannins, and alkaloids, with mode of action and responsible for anti-cryptosporidial properties.

In the present study, shilajit extract, either alone or combined with NTZ significantly improve the small intestinal changes, such as scattered mononuclear cells, mild infiltration, regular villi, and more or less improved liver architecture in combined treated mice.

In the present study, immunocompromised infected mice showed increase in inflammatory IFN-γ and decrease in anti-inflammatory IL-10 as compared to normal control ($P<0.0001$). IFN-γ levels were down-regulated and IL-10 levels were up-regulated in combined treated mice with a highly significant difference ($P<0.0001$). The group showed best improvement. Undoubtedly, crucial cytokine IFN-γ produced in the early infection stages with intracellular pathogens, triggers multiple processes in infected intestinal epithelial cells (IEC) to eliminate invasive pathogens as *Cryptosporidium* species (Pardy *et al,* 2024). Innate lymphoid cells (ILC) produce IFN-γ necessary to manage cryptosporidiosis in a mouse model (Gilbert *et al,* 2023). Also, IL-10 has a significant role in regulating mucosal immunity, with an attempt to restrain immune system, and restored the intestinal epithelium post cryptosporidiosis treatment (Shah *et al,* 2016).

Conclusion

Combined nitazoxanide and shilajit extract proved to be a promising therapeutic agent for *C. parvum*. This combination protected the liver and intestine by a significant IFN-γ decreasing and IL-10 increasing versus negative control. The antiparasitic activity was marked in the shilajit powder extract. More studies on shilajit extract as a supplemental treatment for other parasitosis are ongoing and will be published elsewhere in due time.

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Explanation of figures

Fig. 1: Flow chart of animal groups.

Fig. 2: Intestine A: Negative control showed no *Cryptosporidium* oocysts (yellow arrow) and few scattered mononuclear cells (red arrow) (x200); B: positive control showed many oocysts (yellow arrow) and distorted villi with oedematous core (blue arrow) associated with many intraepithelial inflammatory cells (red arrow) (x200); C: Infected and treated with NTZ, showed no oocysts (yellow arrow) and few scattered mononuclear cells (red arrow) (x200); D: Infected and treated with shilajit powder extract showed no oocysts (yellow arrow) and mild mononuclear cells infiltration (red arrow) (x200); E: infected and treated with combination of NTZ and shilajit extract showed no oocysts with regular villi (yellow arrow) and few scattered mononuclear cells (red arrow) (x100).

Fig. 3: Liver A: Negative control mice showed typical hepatic architecture (x200); B: Positive control showed focal lobular (yellow arrow) and portal (red arrow) inflammatory cellular infiltration (x200), C: Infected and treated with NTZ mice showed portal (red arrow) inflammatory cellular infiltration (x400), D: Infected and treated with shilajit extract showed mild portal (red arrow) inflammatory cellular infiltration (x200); E: Infected treated with both NTZ and shilajit extract showed nearly typical hepatic architecture (x200).