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Research Article
In Vitro and *In Vivo* Anti-blastocystis Efficacy of Olive Leaf Extract and Bee Pollen Compound

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Abstract

Background: *Blastocystis* is the most common enteric protozoa in humans and animals worldwide. The hazardous side effects and potential development of drug resistance to the standard drug, metronidazole (MTZ), necessitate the search for safer and more efficient alternative therapeutics. **Objective:** The present study was designed to investigate the *in vitro* activity and *in vivo* efficacy of two natural medicinal compounds, Olive Leaf Extract (OLE) and Bee Pollen Compound (BPC) against subtype 3 *Blastocystis* isolated from symptomatic patients. **Materials and Methods:** Cultured parasites were challenged with a graded concentration of OLE (500, 1000 μg mL^{-1}), BPC (500, 1000 μg mL^{-1}) and MTZ (150, 250 μg mL^{-1}) to assess its *in vitro* growth by counting the number of viable cells after one and two h of incubation and morphological ultrastructural changes were evaluated using Transmission Electron Microscope (TEM). The anti-*Blastocystis* effects of OLE and BPC were determined in 40 laboratory bred Swiss albino mice inoculated with *Blastocystis* isolates using parasitological, histopathological and immunohistochemical methods. **Results:** *Blastocystis* isolated from symptomatic patients were subtype 3. The OLE and BPC showed statistically significant ($p<0.05$) *in vitro* growth inhibition of *Blastocystis* in a concentration dependent manner and a statistically significant ($p<0.05$) *in vivo* reduction of the number of *Blastocystis* in stool samples and intestinal contents of infected treated mice higher than MTN effects with apoptotic-like death and Programmed Cell Death (PCD) in *Blastocystis*. The BPC normalized villous architecture and increased local IgA secretory villous cells. **Conclusion:** These findings highlighted the potential therapeutic effects of OLE and BPC as potent safe natural alternatives against blastocystosis. The BPC was superior to OLE in ameliorating the severity of tissue pathology and had an immunostimulatory effect on intestinal cells.

Key words: *Blastocystis*, bee pollen compound, olive leaf extract, metronidazole, genotyping, ultrastructure

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Blastocystis is a unicellular, anaerobic, eukaryotic, extracellular and luminal parasite that lives in the intestinal tract of different hosts including humans. It has a worldwide distribution and it is one of the most frequently identified parasites in human fecal samples in the developing countries in the tropics and subtropics[1]. Its prevalence might exceed 50% in developing countries[2] and reached about 20% in developed countries[3]. Its prevalence in Egypt reached to 33%[4]. Human infection is related to poor personal hygiene, absence of sanitation, exposure to animals and consumption of contaminated food or water. *Blastocystis* transmission is proposed to be by the fecal-oral route[5]. Blastocystosis can be asymptomatic or cause gastrointestinal symptoms[6,7]. *Blastocystis* might act as an opportunistic pathogen in immunocompromised patients[8]. Symptomatic irritable bowel syndrome and inflammatory bowel disease patients also had increased rates of *Blastocystis* spp. infection[9].

Blastocystis has 4 main forms which have been described in stools and/or *in-vitro* cultures: vacuolar, granular, amoeboid and cyst forms[10]. The extensive genetic diversity of *Blastocystis* spp. has been demonstrated using different molecular assays[11,12].

The diagnosis of blastocystosis is currently based on microscopic detection of the protozoa in direct smears carried out before or after cultivation of the fecal sample or molecular identification of the parasite DNA[13]. The pathogenicity and treatment of *Blastocystis* infections remain controversial. However, it is generally acceptable that treatment of *Blastocystis* infection is needed when debilitating symptoms are present with presence of several *Blastocystis* cysts in stool specimens and with absence of other clear cause of symptoms[14].

Although MTZ is the standard therapy for *Blastocystis* infections, there are several reports of treatment failure, indicating the presence of drug-resistant isolates[15]. Furthermore, MTZ has many side effects and drawbacks, including: headache, vertigo, nausea and a metallic taste in the mouth with pancreatitis, central nervous system toxicity at higher doses and reversible neutropenia[16]. It is also mutagenic in bacteria and carcinogenic in mice and rats when given at high doses over long periods of time[17]. All these reasons motivated the search for alternative therapies of *Blastocystis* infection, particularly, the study of natural active agents.

Olives and olive oil are considered the cornerstone of the Mediterranean diet and are well-known for their overall health benefits[18]. Propolis, royal jelly and bee pollen are recognized as valuable therapeutic components and as healthy foods. Several manuscripts discovered in ancient Egypt, China and Greece described the active healing properties of bee products. Many of them are widely used in medicinal purposes for the treatment of bacterial and viral infections, to stimulate the immunity, for treatment of poorly healing wounds, in a variety of tumor diseases, in gastro-intestinal infections, to promote potency and fertility. Bee products have comparable healing properties as established drugs but without side effects[19].

The aim of the present study was to evaluate the *in vitro* and *in-vivo* therapeutic efficacy of two natural medicinal agents; OLE and BPC on subtype *3 Blastocystis* in comparison with MTZ, the standard therapy for *Blastocystis* infection.

MATERIAL AND METHODS

Blastocystis isolates

Stool sample Collection: Fresh stool samples were obtained from 20 symptomatic patients complaining of intestinal symptoms including diarrhoea, abdominal cramps and bloating attending Theodor Bilharz Research Institute (TBRI) outpatient clinic.

Stool sample processing: Stool specimens were microscopically examined immediately after collection by a wet smear preparation in saline and staining with Lugol's iodine solution before and after formalin/ethyl acetate concentration and faecal smears stained with modified trichrome and modified Ziehl-Neelsen acid-fast staining[20] to exclude the presence of other parasites and to identify cases of *Blastocystis* infection. Portion of each *Blastocytis* positive stool sample was cultured on the same day for *in vitro* assays and another portion was kept at -20°C for molecular assay and genotyping.

***Blastocystis* molecular characterization:** Only three out of the 20 samples were proven to be infected with *Blastocystis* and were included in this study. At the Lab of Molecular Medical Parasitology (LMMP), Department of Medical Parasitology, Kasr Al-Ainy Faculty of Medicine, Cairo University, *Blastocystis* isolates were genotyped. Genomic DNA from the positive stool samples was extracted using Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001), according to manufacturer's instructions.

As recommended by Stensvold[21], the extracted DNA was amplified by PCR targeting specific SSU rDNA using the primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3`) and BhRDr

(5'-GAGCTTTTTAACTGCAACAACG-3') to amplify the ~600 bp fragment[22] for confirmation of the presence of *Blastocystis*. The reaction mixture and condition were done in a total volume of 25 µL according to Scicluna *et al.*[22], then seven standardized subtype-specific Sequence-Tagged-Site (STS) primers were used (Table 1) in a PCR reaction mixture and condition according to Yoshikawa *et al.*[23]. The amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining.

***Blastocystis in vitro* culturing:** Positive *Blastocystis* stool samples were cultivated in 3 mL of Jones' medium without rice starch and supplemented with 10% horse serum[24], 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin[11].

Blastocystis culture was incubated at 37°C with 5% CO_2 and monitored daily for 72 h by light microscopy. Culture was regarded as negative when it is failed to detect *Blastocystis* after 72 h[25]. Cultures were considered suitable for drug assessment when *Blastocystis* number mL^{-1} exceeded 1×10^3 vacuolar forms.

Drugs and drug preparation

MTN preparation: MTN (Flagyl) was manufactured and purchased from Sanofi-aventis Egypt s. a. e., under licence of Sanofi-aventis/France. It was used as a reference antiprotozoal drug[26]. Tablet was grinded and dissolved in sterile distilled water to prepare the stock solution of 1 mg mL^{-1}, then stored in a dark bottle at 4°C. Final concentrations of MTZ were adjusted to 150 and 250 µg mL^{-1}.

Preparation of natural products

***Olea europaea* (OLE):** About 430 mg per capsule, containing 20% oleuropein; purchased from Nature's way, USA and Queen's Magic BPC: distributed by Eden Pond Labs, USA; each capsule containing propolis (1000 mg), royal jelly (1500 mg) and bee pollen (750 mg). The powder of each capsule from each product was dissolved in sterile distilled water to get a final stock solution of 1 mg mL^{-1}. Final concentrations were adjusted to 500 and 1000 µg mL^{-1}.

In vitro anti-*Blastocystis* activity of OLE, BPC and MTN

***In vitro* experiment:** Parasite inoculums of size 50×10^4 parasites mL^{-1} from cultures in logarithmic growth phase were introduced into each set of culture tubes containing Jones medium at the different concentrations of OLE, BPC or MTN to study parasite growth. Non-treated control cultures of the parasites and negative control (culture media only) were subjected to the same procedure used for the other tested cultures. Culture tubes were used in triplicate for every concentration of each OLE, BPC, MTN and non-treated cultures. Parasites were challenged with a graded concentration of OLE (500, 1000 µg mL^{-1}), BPC (500, 1000 µg mL^{-1}) and MTN (150, 250 µg mL^{-1}). The tested cultures were incubated in humidified CO_2 at 37°C for examination after 1 and 2 h.

The treatment period of at least 1 h was used in previous standardized studies and had shown that this time period is necessary to induce the cytotoxic response in these parasites[27].

Assessment of *in vitro* anti-*blastocystis* activity of OLE, BPC and MTN

Light microscopy for the *in vitro* study: The activity of the tested drugs against *Blastocystis* infection was evaluated by counting the number of viable cells after 1 and 2 h using eosin-brilliant cresyl blue dye which stained viable cells with green color and nonviable cells with red color[28]. Enumeration

Table 1: *Blastocystis* subtype-specific Sequence-Tagged-Site (STS) primers

		Primers	
Primer pairs	Target organisms	Types	Sequences (5' –3')
SB83	*Blastocystis* sp. ST1	F	GAAGGACTCTCTGACGATGA
		R	GTCCAAATGAAAGGCAGC
SB340	*Blastocystis* sp. ST2	F	TGTTCTTGTGTCTTCTCAGCTC
		R	TTCTTTCACACTCCCGTCAT
SB227	*Blastocystis* sp. ST3	F	TAGGATTTGGTGTTTGGAGA
		R	TTAGAAGTGAAGGAGATGGAAG
SB337	*Blastocystis* sp. ST4	F	GTCTTTCCCTGTCTATTCTGCA
		R	AATTCGGTCTGCTTCTTCTG
SB336	*Blastocystis* sp. ST5	F	GTGGGTAGAGGAAGGAAAACA
		R	AGAACAAGTCGATGAAGTGAGAT
SB332	*Blastocystis* sp. ST6	F	GCATCCAGACTACTATCAACATT
		R	CCATTTTCAGACAACCACTTA
SB155	*Blastocystis* sp. ST7	F	ATCAGCCTACAATCTCCTC
		R	ATCGCCACTTCTCCAAT

*ST: Subtype, F: Forward, R: Reverse

was done in duplication and only intact cells were counted by using a Neubauer cell counting chamber under a Zeiss light microscope (Oberkochen, Germany). The percentage of inhibition of *Blastocystis* multiplication in the treated cultures in relation to non-treated culture was calculated according to the Eq. 1:

$$\text{Growth inhibition}(\%) = \frac{a-b}{a} \times 100 \quad (1)$$

where, "a" is the mean number of parasites in control cultures and "b" is the mean number of parasites in treated cultures.

TEM for the *in vitro* study: For detection of ultrastructural changes, 1 mL of cultured sediment was taken from untreated culture (control groups) and treated cultures with, 250 μg mL^{-1} of MTZ, 500 μg mL^{-1} of OLE and 500 μg mL^{-1} of BPC after 2 h of incubation, then washed twice in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose and fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h, then washed twice in buffer; followed by fixation in 2% osmium tetra oxide (OsO_4) and in 1% potassium ferrocyanide solution for 1 h then washed twice in 0.5 M sodium cacodylate buffer. The cells were processed[29] and tested by JEOL Ltd (JEM-1400 TEM, Japan). These drugs concentrations were selected for TEM according to the least dose of OLE, BPC and MTZ causing significant growth inhibition ($p<0.05$) in the *in vitro* study.

In vivo anti-*Blastocystis* activity of OLE, BPC and MTN

In vivo experiment

Experimental animals: A batch of 40 laboratory bred Swiss albino mice of CD-I strain 3-5 weeks old, weighing 20–25 g were used in this study. Mice were kept in well-ventilated cages of the animal house in the Parasitology Department, TBRI. Throughout the study, the animals were kept on a standard diet containing 24% protein, 4% fat and about 4-5% fiber and water *ad libitum*. They were kept at room temperature (28±2°C) and away from direct sunlight. The cages were cleaned twice a week to ensure good sanitary condition until required for use. The mice were allowed to adapt to the laboratory environment for 1 week before the experiment and their stools were examined microscopically daily to exclude the presence of parasites.

The OLE and BPC were used in a dose equivalent to the daily recommended dose to maintain good health. The doses were calculated by extrapolation of human therapeutic doses to animal doses according to the table of Paget and Barnes[30]. The used drugs were given 3 weeks after establishment of infection for seven consecutive days.

Experimental design: The Swiss albino mice used were divided into 5 groups each of which consisted of 8 mice as follows: normal control (uninfected, untreated), infected control (infected, untreated), infected treated with MTN, infected treated with OLE and infected treated with BPC.

Animal infection: Mice were orally infected by intragastric inoculation of 10^4 vacuolar forms of *B. hominis* in 4 days old axenic culture per mouse using oesophageal tube.

Three weeks after infection of the mice, faecal pellets were collected and subjected to parasitological examination to detect *Blastocystis* and to ensure that all mice had been infected.

Scarification: Scarification was done after cessation of treatment by using over dose of ether. Intestinal sections were dissected from individual mice, fixed in 10% formalin, embedded in paraffin for histological examination of 4 mm sections stained with haematoxylin and eosin and for immunohistochemical examination.

Assessment of *in vivo anti-Blastocystis* activity of OLE, BPC and MTN

Parasitological studies: Quantitative estimation of the intensity of infection in stool samples of *Blastocystis* infected mice after administration of drugs was determined by microscopic counting of *Blastocystis* per gram stool. Also, the number of vegetative forms (trophozoites) in intestinal contents was counted in five successive high power fields per animal and then the average was calculated.

Histopathological studies: Intestinal sections from scarified mice were excised, opened longitudinally and embedded in paraffin wax. Transverse sections of paraffin blocks were cut by microtome and mounted on glass slides. Thickness of 5 μm of the deparaffinized sections were performed by dipping slides in 100% xylene and descending grades (100, 95, 80 and 70%) of ethanol for rehydration. Finally, sections were stained with Ehrlich's hematoxylin and counter stained with eosin[31]. Five slides/mouse and 3 sections/slide in each group were prepared and examined to detect the histopathological changes occurred due to *Blastocystis* infection and to assess the cure rates and the degree of healing of intestinal mucosa after drug administration.

Immunohistochemical studies: Immunohistochemical staining was performed on 4 μm, formalin-fixed,

paraffin-embedded intestinal sections using IgA antibody at 1:50 dilution (DAKO, Carpinteria, CA). Antigen retrieval was performed in all cases by steam heating the slides in a 1 mmol L^{-1} solution of EDTA (pH 8.0) for 30 min. After blocking of endogenous biotin, staining was performed using an automated immunostainer followed by detection by using a streptavidin-biotin detection system (DAKO). Positive and negative control sections were used for each assay.

The IgA expression was evaluated according to H score in an attempt to describe the extent of immunohistochemical staining which appeared brown in color. The H score is considered a qualitative scale for the number and intensity of staining, ranging from zero for no staining (scanty), 1+ for mild and 2+ for moderate[32].

Statistical analysis: Data were reported as Mean counts±Standard deviation. Statistical analyses were done using computerized statistical software program SPSS 19.0. The independent sample t-test was used to assess the statistical significance of the mean difference between the two study groups. Statistical significance was defined as $p<0.05$.

Ethical consideration: The protocol of this study was approved by scientific research ethics committee of TBRI. Patients included in the study were informed verbally about the purpose of the study and the collection of stool samples was performed after obtaining their consent. The animal experiments were performed in accordance with the TBRI committee for laboratory animals research guidelines.

RESULTS

Amplification and genotyping of *Blastocystis* isolates: Genotypic assessment of the isolated *Blastocystis* samples revealed the subtype 3 with 526 bp band amplified with SB227 primer (Fig. 1, 2).

***In vitro* study:** Effect of different concentrations of OLE, BPC and MTN on the growth of *Blastocystis* vacuolar form after 1 and 2 h is illustrated in Table 2.

***In vivo* study:** Quantitative assessment of the intensity of *Blastocystis* infection in stool samples and intestinal contents of infected mice after administration of drugs was performed by the conventional diagnostic techniques to count number of *Blastocystis* per gram stool and results are illustrated in Table 3.

TEM results: TEM micrographs (Fig. 3) showed that untreated cultures had classical *Blastocystis* morphology (Fig. 3a). MTN-treated cultures showed morphological changes in the form of prominent Mitochondrion-Like Organelle (MLO) at the end of the peripheral cytoplasm with vacuolar compression

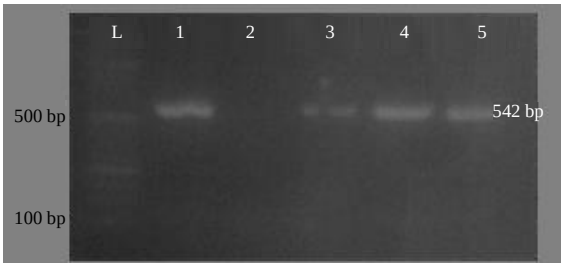

Fig. 1: DNA analysis of a *Blastocystis* samples
Lane L: 50 bp DNA ladder. Lane 1: Positive control, Lane 2: Negative control, Lane 3-5: Isolated samples ~600 bp band amplified with SSU rDNA primer pair

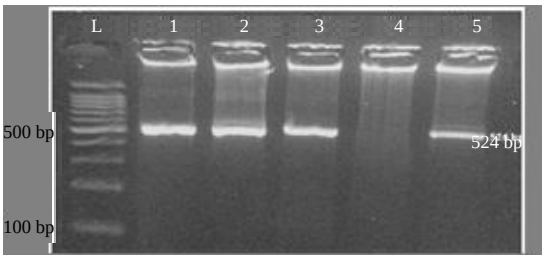

Fig. 2: Sub-typing DNA analysis of a *Blastocystis* samples
Lane L: 100 bp DNA ladder, Lane 1-3: Isolated samples with 526 bp band amplified with SB227 primer (subtype 3), Lane 4: Negative control, Lane 5: positive control

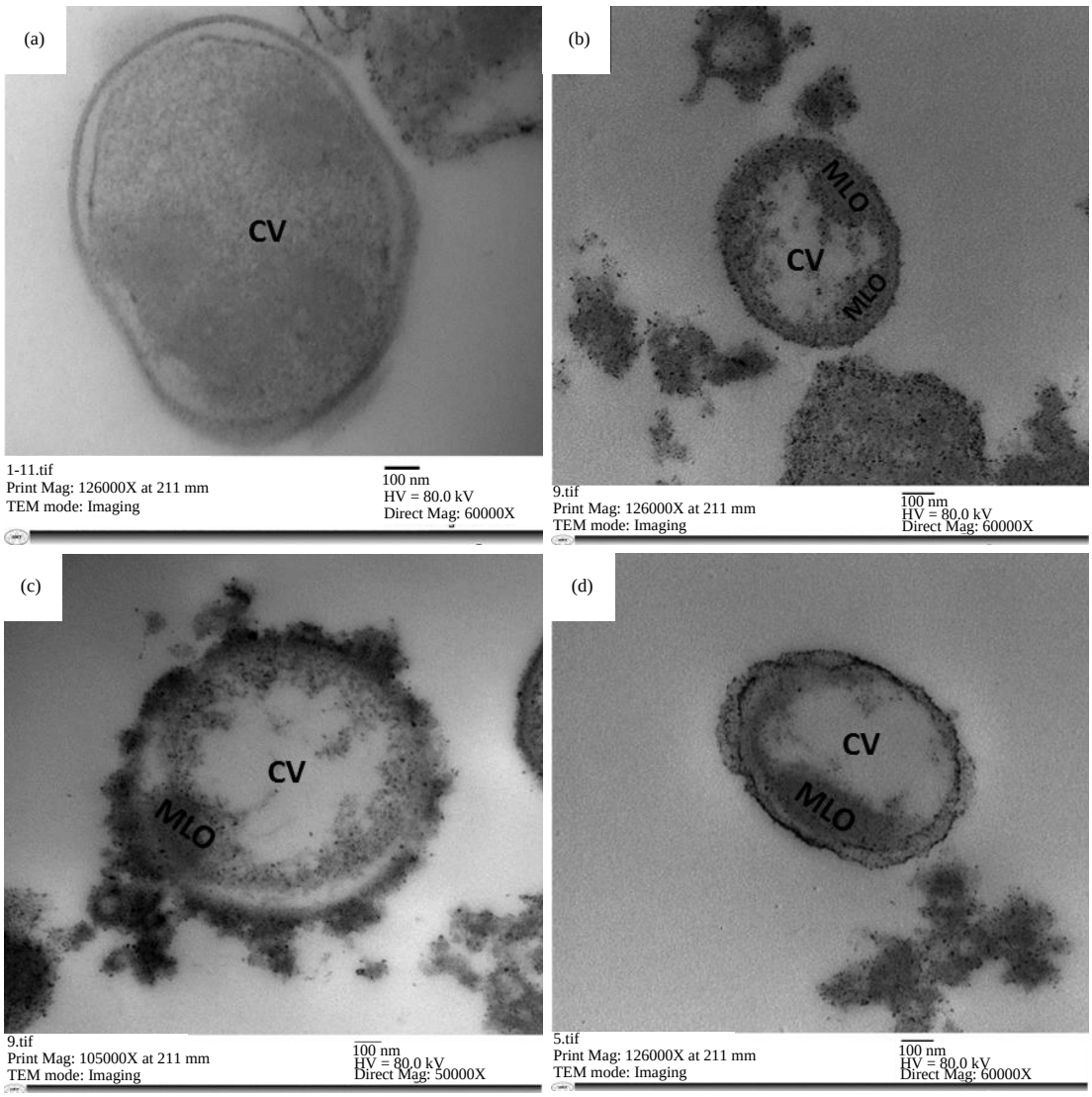

Fig. 3(a-d): TEM photographs of (a) Untreated cultures showing healthy *Blastocystis* cell, (b) MTN, (c) OLE and (d) BPC-treated cultures showing prominent MLO with vacuolar compression and less electron dense Central Vacuole (CV)

Table 2: Effect of MTN, BPC and OLE on the *in vitro* growth of *Blastocystis* after different incubation periods

Drugs/concentration (μg mL^{-1})	Cells mL^{-1} (Mean±SD) (1 h)	Cells mL^{-1} (Mean±SD) (2 h)
Control	$54.67\times10^4\pm2.52$	$54.67\times10^4\pm2.52$
MTN 150	$48.00\times10^4\pm10.54$ (12.20%)	$15.33\times10^4\pm2.31$* (71.96%)
MTN 250	$21.33\times10^4\pm2.08$* (60.98%)	$8.67\times10^4\pm1.53$* (84.41%)
BPC 500	$16.33\times10^4\pm2.08$* (70.13%)	$3.67\times10^4\pm1.53$* (93.29%)
BPC 1000	$6.67\times10^4\pm2.52$* (87.80%)	$2.33\times10^4\pm0.58$* (95.74%)
OLE 500	$27.33\times10^4\pm4.04$* (50%)	$6.67\times10^4\pm3.79$* (87.80%)
OLE 1000	$9.33\times10^4\pm3.51$* (82.93%)	$4.33\times10^4\pm0.58$*(92.10%)

Values are expressed as Mean±SD and Growth Inhibition (%) from control, *Statistically significant compared to infection control group ($p<0.05$), BPC: Bee Pollen Compound, OLE: Olive Leaf Extract

and a less electron dense central body with presence of electron dense intracellular bodies (Fig. 3b). The OLE-treated cultures showed prominent MLO at one end of the peripheral cytoplasm with a less electron dense central body and the presence of membrane-bound electron-dense particles (apoptotic bodies) in the central vacuole which were released

Table 3: Effect of MTN, BPC and OLE on *Blastocystis* number in intestinal contents and stool of infected mice 4 weeks post-infection

Experimental groups	Vegetative forms (trophozoites) in intestinal contents/field (Mean±SD)	Cysts g^{-1} stool (Mean±DM)
Control	31.00±7.94	211.67±10.41
MTN	15.33±3.06* (50.55%)	126.00±13.89* (40.47%)
BPC	9.33±1.53* (69.90%)	73.33±20.82* (65.36%)
OLE	11.67±1.53* (62.35%)	105.33±2.52* (50.24%)

Values are expressed as Mean±SD and Reduction (%) from control, *Statistically significant compared to infection control group ($p<0.05$), BPC: Bee Pollen Compound, OLE: Olive Leaf Extract

into the extracellular space with loss of membrane integrity and presence of electron dense intracellular bodies (Fig. 3c). The BPC-treated cultures showed prominent MLO at one end of the peripheral cytoplasm with vacuolar compression and a less electron dense central body (Fig. 3d).

Histopathological results: Histopathological examination of intestinal sections in infected non-treated mice revealed profound histopathological changes in the morphology of intestinal mucosa in the form of shortening and broadening of villi, loss of villous architecture, crypt hyperplasia, decrease the ratio of villous height and crypt length, mucosal ulceration and infiltration of inflammatory cells (Fig. 4a, b).

Histopathological examination of intestinal sections in treated groups with MTN or OLE revealed partial improvement in the histopathological changes, with persistent moderate villous atrophy and moderate inflammatory reactions (Fig. 4c, d). In contrast, treatment group with BPC revealed marked improvement in the form of returning of the villous like pattern, villous-crypt ratio with presence of few scattered inflammatory cells and focal broad villi (Fig. 4e).

Immunohistochemical results: Immunohistochemical staining of intestinal sections in infected non-treated mice revealed few IgA secretory cells which appeared as brown color in the lamina propria cells of the core of villi (Fig. 5a), while immunohistochemical staining of intestinal sections in treated groups with MTN or OLE revealed occasional IgA secretory cells (Fig. 5b, c), however, with BPC treatment there was a significant increase ($p<0.05$) in the number group IgA secretory cells (Fig. 5d).

DISCUSSION

In the present study, genotypic assessment of the isolated positive *Blastocystis* samples revealed infection with subtype 3 *Blastocystis*. This is agreed with several epidemiological studies which reported that subtype 3 is the most predominant subtype[3,33-35]. These studies demonstrated the importance of subtype 3 in terms of its prevalence, pathogenic implications and treatment.

Both OLE and BPC showed a statistically significant ($p<0.05$) growth inhibition of *Blastocystis* in a concentration dependent manner higher than MTZ effects.

The BPC showed the highest percentage of reduction in the number of *Blastocystis* in stool samples and intestinal contents of infected treated mice followed by OLE with a statistical significant difference ($p<0.05$) in comparison with the control group.

Mokhtar *et al.*[36] stated that ST1 and ST3 *Blastocystis* were susceptible to MTZ, at two concentrations (50 and 100 μg mL^{-1}) without growth after 24 h, while the concentration of 10 μg mL^{-1} showed a significant reduction ($p<0.05$) in the parasite counts for all incubation periods (24, 48, 72 h) with a cytocidal efficacy after 72 h.

Similarly, Yakoob *et al.*[37] reported a sensitivity of all *Blastocystis* isolates to MTZ in comparison to healthy controls at concentrations of 0.01 and 0.1 mg mL^{-1}, these isolates were mostly ST3 and coinfection of ST3 and ST1, however in the same study isolates from IBS patients showed variable efficacy of MTZ and were mostly of ST1. Also, Girish *et al.*[38] demonstrated high growth inhibition of MTZ (0.1 and 1 mg mL^{-1}) against ST1, ST3 and ST5. Moreover, many clinical studies reported effective parasite clearance with MTZ[39,40]. This controversy about MTZ efficacy may be due to intra-subtype differences in the susceptibility mechanisms of the drug, possibly this may be attributed to the presence of different alleles in each subtype[41].

Olive trees and leaves are considered low-cost sources which are rich in phenolic compounds with recognized antioxidant and anti-inflammatory properties[42]. Oleuropein, which is the main component of olives and olive leaves, have several biological properties: antitumor, antiviral, antibacterial and antiparasitic activity[43-49]. In the year 2000, oleuropein was pretended in a U.S. patent (Fredrickson, Num: 6,117,844) as having powerful antiviral activity.

Oleuropein demonstrated antiparasitic activity against *Toxoplasma gondii*, it showed proliferation inhibition of Madin-Darby bovine kidney cells infected with *Toxoplasma gondii* tachyzoites (*in vitro*) and decreased the parasite expansion (55.4%) in the peritoneal cavity of *Toxoplasma*-infected mice[48]. Also, oleuropein revealed

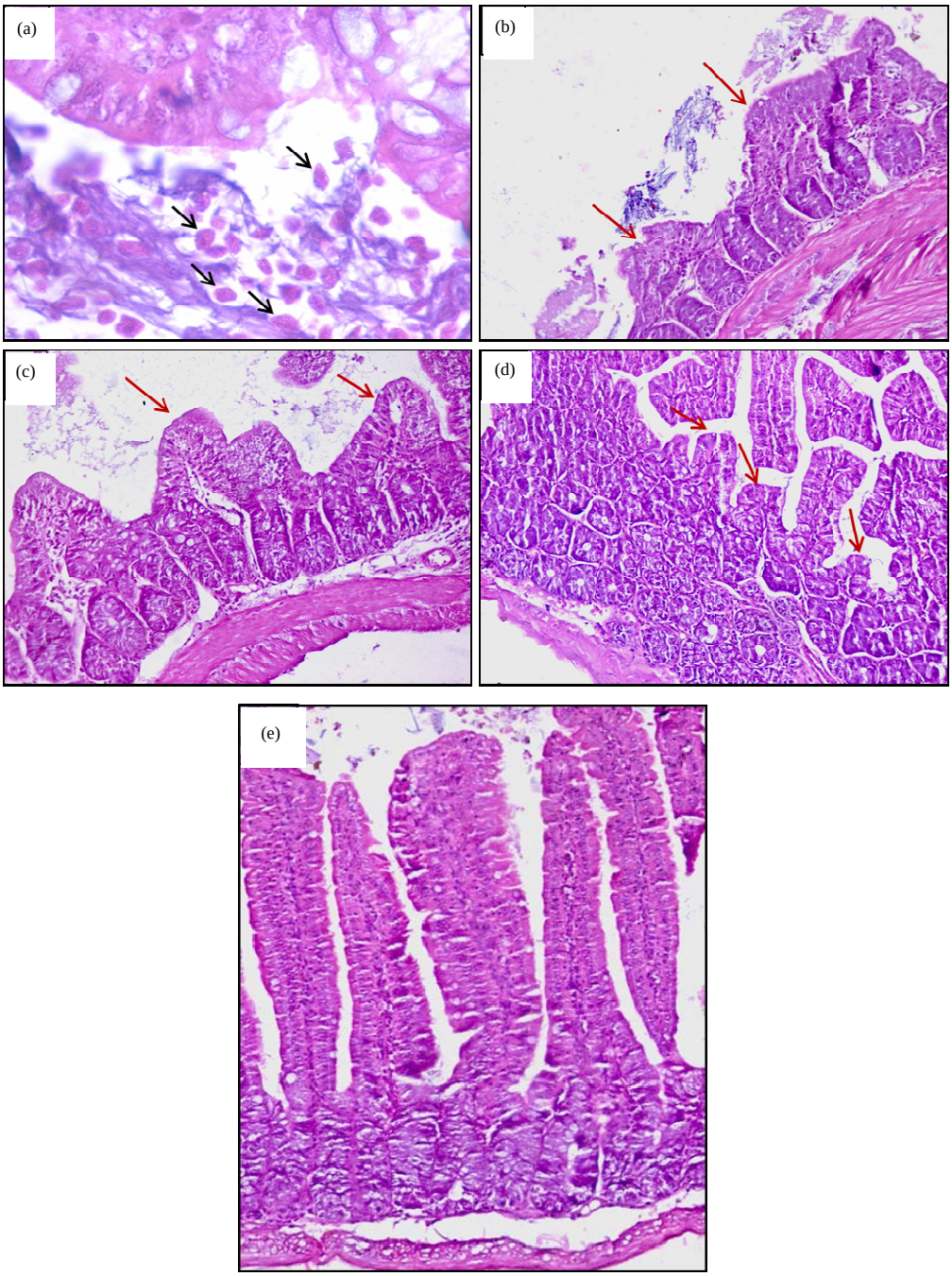

Fig. 4(a-e): Histopathological examinations of intestinal sections from (a, b) Infected untreated control group showing several *Blastocystis* granular forms (black arrows) (H&EX1000), severe villous atrophy (red arrows), (c) MTN, (d) Olive Leaf Extract (OLE) treated infected groups showing moderate villous atrophy (red arrows) and (e) BPC treated infected group showing normal villous architecture (H&EX200)

anti-parasitic activity against *Leishmania infantum*, *Leishmania donovani* and *Leishmania major*[50]. Similarly, Sifaoui *et al.*[51] stated that flavonoids present in five different Tunisian olive extracts, had leishmanicidal activities against the four *Leishmania* species, an antioxidant efficacy and a protective role against membrane lipoperoxidative damages. Propolis showed potent antimicrobial properties, it has been used as a chemotherapeutic agent since ancient times and it is considered as an anti-inflammatory drug and wound healing agent. Propolis is one of the most powerful natural

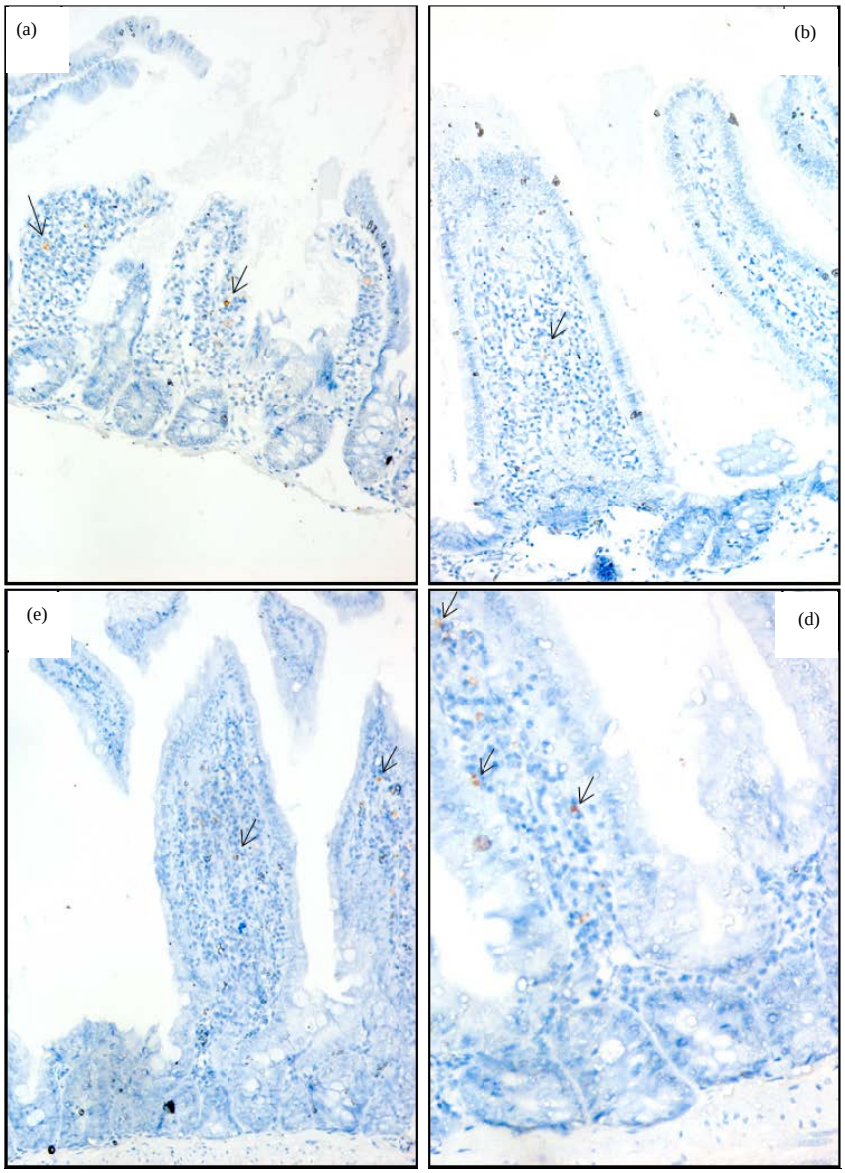

Fig. 5(a-d): Immunohistochemical examinations of intestinal sections from (a) Infected untreated control group showing few IgA secretory brownish cells, (b) MTN, (c) OLE treated infected groups showing scanty IgA secretory brownish cells and (d) BPC treated infected group showing mild focal increase of IgA secretory brownish cells (ImmunohistochemistryX200)

antibiotics with a wide spectrum efficacy. Its therapeutic applications did not stimulate germ resistance and did not damage useful microflora[52].

Several studies stated the anti-parasitic activities of propolis against cultured *Blastocystis* ST3 and ST1[37], *Leishmania infantum* and *Leishmania tropica* strains[53], *Giardia lamblia*, *Trichomonas vaginalis*, *Toxoplasma gondii*, *Leishmania donovani* and *Trypanosoma cruzi*[54,55].

Similarly, AlGabbani *et al.*[56] demonstrated that methanolic extract of Saudi propolis had antimalarial and antioxidant properties and provided protection against spleen tissue destruction in *Plasmodium chabaudi* infected mice. In addition, royal jelly and pollen acted as a potent antibiotic, they caused inhibition of all types of bacteria, both Gram-positive and Gram-negative and microbes. They were beneficial for a wide range of health conditions[57,58].

In the present study the OLE and BPC caused apoptotic-like death and PCD in treated cultured *Blastocystis* as was evidenced by ultrastructural morphological changes.

The Programmed Cell Death (PCD) pronounces a biochemical and pathological process of cell removal that theatres a significant role in upholding tissue homeostasis[59,60].

Mishra *et al.*[61] and Mishra[62] had provided detailed reviews on the biotechnological approach of apoptosis in living organisms and some of the relative features of PCD in plants and animals.

Similarly, Nasirudeen *et al.*[63] reported apoptosis-like features and PCD in growing cultures of MTN treated axenic *B. hominis*. Raman *et al.*[64] revealed a raise in the number of MLO in symptomatic *Blastocystis* subtype 3 treated with MTN.

The presence of MLOs' implicated that these treated parasites need greater energy for the purposes of internal reorganization in response to the drug effects[64] which revealed that there was an increase in the MLO number in symptomatic *Blastocystis* subtype 3 due to MTN treatment.

In addition to its anti-*Blastocystis* activity, BPC in the present study reduced *Blastocystis* induced intestinal tissue pathology, as evidenced by the return of the villous like pattern, villous-crypt ratio and few scattered inflammatory cells present. Also, BPC improved host defense *Blastocystis* infection as evidenced by increased IgA secretory levels in intestinal mucosa. These IgA secretory cells play a central and predominant role in the host defense against *Blastocystis* infection. Compared to BPC, both MTZ-treated and OLE-treated mice showed only partial improvement in villous atrophy with moderate inflammation, as well as the presence of few local secretory IgA in the immunohistochemical stained intestinal sections.

Likewise, Dimov *et al.*[65] reported that bee products have a non-specific immunostimulant efficacy and Scheller *et al.*[66] found that propolis ethanolic extract stimulates antibody production by mice spleen cells.

Wang *et al.*[67] reported that polyphenol-rich propolis extracts strengthen the function of intestinal barrier which is attributed to activation of AMP-activated protein kinase and extracellular signal–regulated kinases signaling pathways, which is highlighted the potential application of propolis for human gut health.

CONCLUSION

It is concluded that OLE and BPC had potential therapeutic effect and could be used as safe natural therapeutic alternatives against *Blastocystis* infections. In addition to its anti-*Blastocystis* activity, BPC has a restorative effect on the host tissue as it normalized the villous architecture and increased the local IgA secretory villous cells.

SIGNIFICANCE STATEMENT

This study highlighted the possible therapeutic effects of olive leaf extract and bee pollen compound that can be beneficial and potent safe natural alternatives for blastocystosis. This study may open fresh avenues for development of an alternative therapy in order to augment or even replace some of the standard chemotherapeutic agents currently employed in the treatment of blastocystosis.

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