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IN VITRO ANTI-PROTOZOAL ACTIVITY OF PROPOLIS EXTRACT AND CYSTEINE PROTEASES INHIBITOR (PHENYL VINYL SULFONE) ON *BLASTOCYSTIS* SPECIES

By

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Abstract

Blastocystis is one of the commonest enteric protozoan parasites worldwide. Despite its controversial clinical significance, frequent association with symptoms has necessitated treatment of cases with persistent symptoms. For long time, metronidzole (MTZ) was considered as a basic drug for blastocystosis treatment, however reports of treatment failure as well as its well-known side effects has promoted the search for more safe and effective alternatives. *In vitro* antiprotozoal activity of ethanolic extract of Egyptian propolis and a cysteine protease inhibitor, phenyl vinyl sulfone (PVS) on *Blastocystis spp.* was assessed through challenging with graded concentrations of propolis extract (125, 250, 500 & 1000µg/ml) and PVS (100, 200 and 300 µg/ml) compared to MTZ (10, 50 and 100 µg/ml) and viable parasites were counted after 24, 48 and 72 hr. of incubation. Molecular subtyping of *Blastocystis spp.* was done using subtype specific sequence-tagged site (STS) primers. Propolis extract inhibited the growth of *Blastocystis spp.* in both of the detected subtypes (ST1 and ST3), which was especially observed in cultures exposed to 500 & 1000 µg/ml through all incubation periods with the later concentration producing comparable results to MTZ. While PVS showed significant parasite count reduction on ST3 isolates, especially with the highest concentration, however the effect on ST1 isolate was nonsignificant. These findings highlight the potential antiprotozoal activity of propolis extract as a potent natural alternative for MTZ in treatment of blastocystosis.

Key words: *Blastocystis* spp., *in vitro*, propolis, phenyl vinyl sulfone.

Introduction

Blastocystis species (*spp.*) are single-celled anaerobic parasites that inhabit the lower intestinal tract of man and many animals (Yoshikawa *et al,* 2011). It was first described in1912as a harmless yeast and later as a protozoan parasite (Arisu, 2002). This emerging parasite has a worldwide distribution especially in underdeveloped nations, probably due to poor sanitation, exposure to animals and consumption of contaminated food or water (Stensvold *et al,* 2009) and considered as the most common parasite that can be detected in human stool samples, where its prevalence has shown a dramatic increase in recent years (Souppart *et al*, 2010). The infection rates ranged from 10-70% in different countries with high rates in adults than in children (Wong, 2008; El Safady *et al,* 2014). In Egypt, *Blastocystis* spp. was the most common identified parasite (33.3%) among the patients complaining of GIT symptoms (Rayan *et al,* 2007).

One of the greatest controversies surrounding *Blastocystis* was its equivocal association with bowel disease, where it is frequently found not only in GIT symptomatic patients but also in asymptomatic individuals (Tan, 2008b). However, numerous epidemiological, molecular, immunological and animal studies have helped clarify the pathogenic potential of *Blastocystis*, with evidence strongly suggesting its pathogenic potential (Tan, 2008a). Genetic analysis of *Blastocystis spp.* revealed presence of up to 17 subtypes (ST), at least 9 of which have been identified in humans where ST1-ST4 account for about 90% of human infections (Alfellani *et al,* 2013; Stensvold, 2013). Intestinal manifestations of symptomatic blastocystosis can be variable, ranging from mild diarrheal illness or acute gastroenteritis

with profuse watery diarrhea to chronic diarrhea (Gupta and Parsi, 2006; Nassir *et al*, 2004; Shah *et al*, 2012). Other nonspecific symptoms may be included as anorexia, nausea, vomiting, abdominal pain and flatulence (Jones *et al*, 2009). Moreover, the association of *Blastocystis spp.* with irritable bowel syndrome (IBS) and allergic skin disorders has been reported by several studies (Doğruman-AI *et al*, 2010; Yakoob *et al*, 2010; Hameed *et al*, 2011). *Blastocystis*-associated symptoms are generally self-limiting and may last from 1 to 14 days, however, some infections may persist for months or years if left untreated (Ustün and Turgay, 2005; Tan, 2008b).

Despite the fact that MTZ is the most frequently prescribed drug for treatment of blastocystosis (Dinleyici *et al,* 2011), yet it was reported to have widely varying rates of efficacy ranging from 0-100% and often associated with treatment failure as a possible result of drug resistance (Stensvold *et al*, 2010; Dunn *et al,* 2012). In addition to its undesirable side effects, MTZ has shown to induce a potential risk of carcinogenic, embryongenic and teratognic effects (Calzada *et al,* 2007). Hence, there is an urgent need to develop safe and effective alternative antiprotozoal agents to overcome these drawbacks.

Several studies have been conducted to find an effective natural therapy for blastocystosis to avoid exposure to potentially toxic drugs, including screening of plant extracts and plant-derived compounds (Vital and Rivera, 2009; Yakoob *et al*, 2011; El-Deeb *et al*, 2012). Propolis is a resinous substance collected by honeybees from plant exudates and used in beehives to seal crakes and as a building insulation material (Greenaway *et al,* 1990). For a long time, it has been widely used in folk medicine (Bankova, 2005). Recently, it has gained popularity as a food supplement (Monzote *et al,* 2012). Broad spectrum therapeutic activities of propolis have been described by numerous studies; including anti-inflammatory, antimicrobial, antioxidant (Bankova *et al,* 2000; Wang *et al,* 2013), antitumor (Popolo *et al,* 2009), antiulcer, anti-HIV (Ito *et al,* 2001) and antihelminthic activities (Hegazi *et al,* 2007). Major antiprotozoal activities of the propolis were also reported, including antigiardial, antileishmanial, antiplasmodial and antitrypanosomal activities (Abdel-Fattah and Nada, 2007; Monzote *et al,* 2012). However, no reports have investigated its effect on *Blastocystis spp.* until now. The chemical characteristics of propolis have been related to variations in geographical location, source plants and bee species. Generally, the propolis is composed of 50% plant resins, 30% wax, 10% essential and aromatic oils, 5% pollens and 5% other organic substances (Huang *et al,* 2014). Numerous studies have concluded that the therapeutic effects might result of synergistic action of its complex constituents (Sforcin *et al,* 2005; Bueno-Silva *et al,* 2013).

Cysteine proteases (CP) of protozoan parasites are enzymes that have been involved in a number of important biological functions including host cell invasion, immune evasion, pathogenesis and virulence (Sajid and McKerrow, 2002). These enzymes have emerged as promising targets for antiparasitic drug development (Atkinson *et al,* 2009). Non-specific cysteine protease inhibitors (CPIs) such as PVS have shown promising results in treatment of experimental *Schistosoma mansoni* infection (Abaza *et al,* 2013). Also, the PVS showed activities against *Trypanosoma cruzi* (Engel, 1998), *Plasmodium falciparum* (Pandey *et al,* 2006) and *Toxoplasma gondii* (Larson, 2009).

The present study aimed to investigate the *in vitro* effects of ethanolic extract of Egyptian propolis and a cysteine protease inhibitor, PVS at different concentrations on the growth of *Blastocystis* parasite.

Patients, Materials and Methods

Stool samples collection and examination: Fecal samples were collected from acutely GIT symptomatic patients with no history of antibiotic administration, attending the Gas-

troenterology Outpatient Clinic, The General Hospital, Ismailia, Egypt. Collected samples were examined by wet mount, iodine-stained smear, formalin-ethyl acetate concentration techniques (Garcia, 2007). Samples positive for *Blastocystis* spp. were further subjected to trichrome and acid-fast Trichrome staining to exclude common pathogenic parasites (Garcia, 2007). In addition, stool culture using *Salmonella–Shigella* and MacConkey agar were performed to exclude common pathogenic bacteria causing GIT symptoms (Cheesbrough *et al,* 2006). Only samples positive for *Blastocystis* without co-infection with other pathogenic parasites or bacteria were included in the study.

In vitro cultivation of *Blastocystis* parasite: Three *Blastocystis*-positive samples (isolate I-III) were cultivated in duplicate tubes into Jones' medium without rice starch, supplemented with 10% horse serum, 100 UI/ml penicillin, and 100µg/ml streptomycin at 37ºC (Leelayoova *et al,* 2002). Repeated subcultures were done every 3–4 days in fresh medium for about a month (Girish *et al,* 2015) to ensure sterile culture with minimum fecal debris.

Molecular subtyping of *Blastocystis* isolates: *Blastocystis* DNA was extracted from positive stool cultures with a DNA extraction kit, according to manufacturer's directions (QIAamp; Qiagen Inc., Hilden, Germany).

Polymerase chain reaction using seven pairs of STS primers namely; SB83 (351 bp) for ST1, SB155 (650 bp) for ST2, SB227 (526 bp) for ST3, SB332 (338 bp) for ST4, SB340 (704 bp) for ST5, SB336 (317 bp) for ST6, and SB337 (487 bp) for ST7 (Yoshikawa *et al,* 2004) were used to identify *Blastocystis spp.* subtypes according to the method described by Tan *et al.* (2008).

Propolis extract: Crude brown Egyptian propolis was supplied from an Egyptian honeybee keeper from bees feeding on buds of Egyptian clover or Berseem (*Trifolium alexandrinum*) as the source plant. Propolis 30% ethanolic extract was prepared. Briefly, 30gm of propolis was cut into small pieces and the volume was completed to 100 ml with 70% ethanol, with occasional stirring at room temperature. Stirring was repeated twice a week but otherwise it was left in a dark place. After a week, the extract was filtered using Wattman paper number 1 producing a clear dark brown filtrate free of particles. The solvent was evaporated at 40ºC under reduced pressure to obtain the dry extract which was weighed to calculate the final concentration (Miorin *et al,* 2003). The extract was dissolved in 70% ethanol at 10mg/ml and stored at 4ºC. The following concentrations of propolis were used for *in vitro* assessment of its effect on *Blastocystis* spp.; 125, 250, 500 and 1000µg/ml.

Preparation of PVS and MTZ concentrations: Cysteine protease inhibitor, phenyl vinyl sulfone (Mu-phe-Hph-ch2F; Sigma-Aldrich, Germany) serial dilutions were prepared as 100, 200 and 300 µg/ml of the compound in sterile distilled water. The metronidazole (Flagyl) was used as a reference antiprotozoal drug (Sawangjaroen and Sawangjaroen, 2005). The stock solution of 1mg/ml was prepared and stored in a dark bottle at 4°C. Final concentrations of MTZ were adjusted to 10, 50 &100µg/ml (Yakoob *et al,* 2011).

In vitro experimental design: From each *Blastocystis* isolate an inoculum size of $2x10^5$ parasites/ml from cultures in logarithmic growth phase was prepared by counting the number of live parasites using a Neubauer cell counting chamber after staining with 0.4% Trypan blue solution as an indicator of viability. After calculation of the inoculum volume required from each isolate and the corresponding quantities of propolis extract, PVS and MTZ (according to corresponding concentrations), the amount of the added Jones' medium was calculated to obtain a final volume of 1 ml (Grabensteiner *et al,* 2008). Non-treated control (NTC) and 70% ethanol-treated control (ETC) cultures were prepared by the same procedure. Parasites were challenged with a graded concen-

tration of propolis extract (125, 250, 500 &1000µg/ml), PVS (100, 200 & 300µg/ml) and MTZ (10, 50 & 100µg/ml) and viable parasites were counted after 24, 48 & 72 hr. All experiments were performed in triplicate.

Effect of propolis extract and PVS on the *in vitro* growth of *Blastocystis spp.*: The effect of concentrations of propolis extract, PVS & MTZ on each *Blastocystis* isolate was determined by detecting the cytocidal and cytostatic effects to all the viable (unstained) forms of *Blastocystis* parasite as assessed by Trypan blue solution (0.4%). The cytocidal effect was determined as the lowest concentration in which no viable parasites were seen and no growth was detected 72hr later when 100µl of the samples with no parasites found were cultured into fresh Jones' medium (Vdovenko and Williams, 2000). While, the cytostatic effect (percentage of growth inhibition) was defined as the reduction of the number of *Blastocystis* parasite compared to the number of NTC, and was calculated according to the following equation (Ahmed *et al,* 2015):

Growth inhibition% = $\frac{a-b}{a}$ x100 where,

a= mean number of *Blastocystis* parasite in NTC, b= mean number in treated cultures.

Fifty parasites were randomly selected from each culture tube (treated & untreated) for size measurement at 48 h after their initial inoculation into Jones' medium and the percentages of granular forms were calculated (Tan *et al,* 2008).

Statistical analysis: Data were reported as mean counts ± standard deviation. Statistical analyses were done using statistical software program SPSS 16.0. The independent sample t test was used to assess the statistical significance of the difference between two study group means. Statistical significance was defined as *P* values <0.05 and highly significant as *P* <0.001.

Ethical considerations: An informed written consent was taken from all patients after explaining the purpose of the study. All procedures were conducted according to the ethical standard approved by the Institutional Human Ethics Committee, Faculty of Medicine, Suez Canal University, Egypt.

Results

Molecular subtyping of the three *Blastocystis* isolates revealed that 2 isolates (isolate I and III) belong to subtype (ST) 3 and one isolate (isolate II) belong to ST1 (Fig. 1). *In vitro* effect of propolis extract and PVS on the mean counts of *Blastocystis* parasite after 24, 48 and 72 hr were given (Tab. 1). Ethanol-treated cultures didn't show significant difference on the mean counts of the parasite compared to NTC at the same time of incubation. All isolates were susceptible to MTZ, with two concentrations (50 & 100µg/ml) showed no growth after 24 hr., while the concentration of 10 µg/ml showed highly significant reduction in the parasite counts in all incubation periods (24, 48, 72 hr) with a cytocidal effect after 72 hr. Propolis extract inhibited the growth of *Blastocystis* parasites of the three isolates and the level of inhibition varied according to the extract concentration and incubation times. The highest reduction of parasite growth was observed in cultures exposed to 500 & 1000 µg/ml of propolis, in all incubation periods (24, 48, 72 hr) with the later concentration producing comparable results to MTZ (10 µg/ml) with a cytocidal effect after 72 hr. As regards to PVS, it showed a significant cytostatic effect on both isolate I & III which also varied according to the concentration and incubation times with the highest concentration (300µg/ml) showing the highest reduction of parasite counts. However, isolate II didn't show significant reduction of the parasite counts at all the used concentrations of PVS (100, 200, 300 µg/ml) and through all the incubation periods (24, 48, 72 hr).

Propolis exerted a notable effect on *Blastocystis* morphology which was detected at higher concentrations (500 &1000µg/ml), where the size rang of the vacuolar and granular forms was significantly smaller than that of the NTC (*P* <0.001) and cultures

challenged with other concentrations of propolis or PVS (Figs. 2 & 3). The cultures treated with MTZ (10µg/ml) also showed reduced size range. All concentrations of propolis extract, as well as PVS (200 & 300µg/ml), showed increased percentages of granular forms counted at 48 hr. compared to NTC which showed shriveled appearance and was especially abundant at concentration of 1000 µg/ml (52%) replacing the typical vacuolar forms (Figs. 2 &4).

Table 1 Mean counts (x10^4) and percentage of growth inhibition of *Blastocystis* parasites in culture challenged with graded concentrations of propolis extract and PVS.

Treated / Non treated cultures (tested concentration)	Time of incubation					
	24 hr.		48 hr.		72 hr.	
	Mean ± SD	% Growth inhibition	Mean ± SD	% Growth inhibition	Mean ± SD	% Growth inhibition
Isolate I						
NTC	35± 10.8	00.0	117.6±8.7	00.0	197.3±8.3	00.0
ETC	34.6±5	1.1	107.3±12.8	8.7	188.6±16.6	4.7
MTZ (10 µg/ml)**	6.6±1.1	81.1	1.3±0.5	98.8	0	100
PE (125 µg/ml)	34.3±7.3	2	117.3±3	0.8	55±6.2*	72.1
PE (250 µg/ml)	22.6±7.7	35.4	50±8*	57.4	33.3±4.1*	83.1
PE (500 µg/ml)**	7.6±1.5	78.2	10.6±2.3	90.9	2.3±1.1	98.8
PE (1000 µg/ml)**	4.6±1.5	86.8	4.3±1.1	96.3	0	100
PVS (100 µg/ml)	15.3±1.1*	56.2	70.3±16.5*	40.2	182.6±2.3	7.4
PVS (200 µg/ml)	7.3±0.5**	79.1	36.3±4*	69.1	66.6±13.2*	66.2
PVS (300 µg/ml)**	6.3±0.5	82	9.3±2.3	92	2.3±3.2	98.8
Isolate II						
NTC	57.3±9	00.0	119.3 ±6	00.0	207.3± 1.1	00.0
ETC	48.6±6.3	15.1	108.6 ±7.5	8.9	194±8.7	6.4
MTZ (10 µg/ml)**	5.0±1	91.2	3.3±1.1	97.2	0	100
PE (125 µg/ml)	48.3±4.9	15.7	104.6±7.0	12.3	113.3±15.2*	45.3
PE (250 µg/ml)**	17.6±0.5	69.2	33.6±3.2	71.8	23.3±1.1	88.7
PE (500 µg/ml)**	14.3±1.1	75.0	2.0±0.0	98.3	2.0±0	99
PE (1000 µg/ml)**	7.3±1.5	87.2	1.6±0.5	98.6	0	100
PVS (100 µg/ml)	53±2.6	7.5	109.3±8.3	8.3	196±14.4	5.4
PVS (200 µg/ml)	49.3±1.1	13.9	114.6±11	3.9	194.6±9.4	6.12
PVS (300 µg/ml)	46.6±1.1	18.6	101.3±2.3	15	189.3±14	8.6
Isolate III						
NTC	45±3.6	00.0	159.3±7.5	00.0	227.3±16.1	00.0
ETC	36±4	20.0	133.3±7.5	16.3	218.6±1.1	3.7
MTZ (10 µg/ml)**	5.6±0.5	87.5	2.3±1.1	98.5	0	100
PE (125 µg/ml)	44.6±7.5	0.8	143.6±2	9.8	72.3±3.7**	68.1
PE (250 µg/ml)	24±8	46.6	110.3±3.7*	30.7	42.3±7.5**	81.3
PE (500 µg/ml)**	9.3±0.5	79.3	4.3±0.5	79.3	2.0±0	99.1
PE (1000 µg/ml)**	4.6±2.3	89.7	1.6±0.5	98.9	0	100
PVS (100 µg/ml)	23.6±1.5*	47.5	123.3±5	22.5	152±14.7*	33.0
PVS (200 µg/ml)*	23.6±1.5	47.5	99.3±16.2	37.6	116.3±10.5	48.8
PVS (300 µg/ml)	17.6±2.5*	60.8	43.6±3.5**	72.6	72.6±9**	68

* P <0.05 =significant difference in comparison to NTC at same time of incubation, ** P <0.001= highly significant difference in comparison to NTC at the same time of incubation, PE, propolis extract

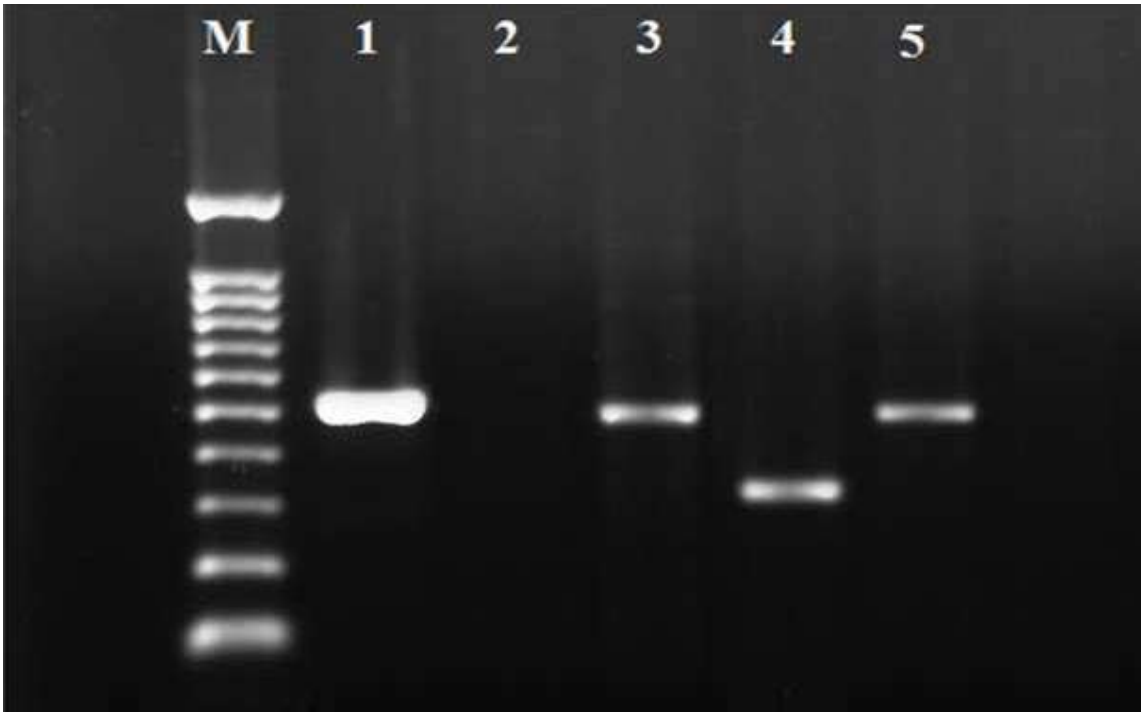

Fig. 1: PCR products of *Blastocystis spp.* on 1.5% agarose gel stained with ethidium bromide showing; M, molecular weight marker (100 bp), lane 1 a positive control for ST3 at 526 bp, lane 2 negative control, lanes 3 and 5 represent isolates I and III (ST3) and lane 4 represents isolate II (ST1 at 351 bp).

Discussion

Blastocystis spp. is considered as one of the commonest enteric protozoan parasites infecting humans worldwide. Despite its controversial clinical significance, accumulating reports suggest its pathogenic potential (Tan, 2008b). There is a consensus that therapy should be limited to persistently symptomatic patients after screening for alternative etiologies (Stensvold *et al,* 2010; Dinleyici *et al,* 2011). Several antiprotozoal drugs have been used to treat blastocystosis, but with variable rates of efficacy (Mirza *et al,* 2011; Robert *et al,* 2015). Among which MTZ stands as the first-line treatment, however development of drug resistance and its unpleasant adverse effects complicates its use suggesting that current treatment guidelines should be revised (Stensvold *et al,* 2010; Robert *et al,* 2015). Thus, there is a need to search for new treatment alternatives to target *Blastocystis*. In the present study, we have evaluated the *in vitro* antiprotozoal activity of ethanolic extract of Egyptian propolis and a cysteine protease inhibitor, PVS against *Blastocystis spp.* which, to the present best knowledge, was not assessed before by any reports.

The present isolates showed infection with ST1 & ST3. These subtypes were reported as the mostly detected subtypes worldwide and also in Egypt, with ST3 being the most prevalent (Souppart *et al,* 2009 and 2010; Abaza *et al,* 2014). Various reports have proven that the viable cell count method, applied in this study, was a reliable method to determine the activity of plant extracts against *Blastocystis* spp. (Yakoob *et al,* 2011; El-Deeb *et al,* 2012; Girish *et al,* 2015). In the present study, no MTZ resistant strains were detected. Similarly, Yakoob *et al.* (2011) reported sensitivity of all *Blastocystis* isolates from healthy controls to MTZ at concentrations of 0.01 and 0.1 mg/ml, these isolates were mostly ST3 and coinfection of ST3 and ST1, however in

the same study isolate from IBS patients showed variable efficacy to MTZ and were mostly of ST1. Another recent study demonstrated high growth inhibition of MTZ (0.1 and 1 mg/ml) against ST1, ST3 and ST5 (Girish *et al,* 2015). Moreover, several clinical studies reported effective parasite clearance with MTZ (Nigro *et al,* 2003; Aguilar and Lucía, 2010). This controversy about MTZ efficacy may be attributed to intrasubtype differences in mechanisms of susceptibility to the drug, possibly due to presence of different alleles of each subtype (Roberts *et al,* 2015).

The use of ethanol (70%) as a solvent for propolis extract had no significant effect on the growth of *Blastocystis* parasites in all isolates throughout the experiment. Similarly, the use of 95% ethanol demonstrated no activity on *Blastocystis* spp. as reported by Vital and Rivera (2009). In the present study, high concentration of propolis extract (1000 µg/ml) exhibited potent inhibitory effect on the *in vitro* growth of *Blastocystis spp*. regardless of ST difference with complete eradication of the parasite after 72 h which were comparable to results obtained by the reference drug (MTZ) at 10 µg/ml concentration. Reports highlighted the activity of propolis extract against intracellular and extracellular protozoan parasites; Fidalgo *et al.* (2011) evaluated the effect of eight Cuban propolis samples from different geographical locations where all samples showed inhibitory effect on *L. amazonenzis* while only five samples decreased the viability of *T. vaginalis* trophozoites. Also, Monzote *et al.* (2012) demonstrated major antiprotozoan activity against *T. brucei*, *T. cruzi*, *P. falciparum* and *L. infantum* using 20 different samples of red, brown and yellow Cuban propolis (YCP). They suggested that several bioactive compounds, with significant association with the YCP, could be responsible for the antimicrobial activities. Another study specified acetyl triterpenes as possible responsible constituents for this antimicrobial activity in YCP (Marquéz *et al,* 2010). The chemical composition of a raw Egyptian propolis sample from Dakahlia Governorate was investigated by Hegazi *et al.* (2004), where 65 compounds were recognized among which some new triterpenoids including lupeol and alpha-amyrin and two new caffeate esters were identified. Moreover, the Egyptian propolis showed highly significant antigiardial activity over the MTZ after 6 days of experimental mice infection (Abdel-Fattah and Nada, 2007). Propolis extract at high concentrations (8-16 mg/ml) also demonstrated potent amoebicidal effect on both trophozoites and cysts of *Acanthamoeba castellanii* (Topalkara *et al,* 2007).

In the present study, the effect of PVS was evident on ST3 isolates with significant reduction of cell counts especially with the highest concentration (300µg/ml), while it showed minimal effect on ST1 isolate. *Blastocystis* cysteine proteases were reported to degrade human secretory immunoglobulin A and induced up-regulation of interleukin 8, resulting in tissue damage and GIT disturbances, suggesting that proteases are potential virulence factors and contribute to parasite survival *in vivo* (Puthia *et al*, 2008). Recently, PVS was shown to inhibit the growth of *C. parvum* in mammalian cell line where high percentage of mice survived the infection compared to infected controls, moreover, it exhibited no toxicity *in vitro* or *in vivo* (Ndao *et al,* 2013).

On the other hand, two cysteine protease inhibitors (iodoacetamide & E-64) were investigated as potential chemotherapeutic agents against *Blastocystis spp.* and proved high susceptibility against all studied *Blastocystis* isolates (ST-1, 3, 4 & 7) *in vitro* (Mirza *et al,* 2011; Al-Mohammed *et al,* 2013). The variable effect of the PVS noted in this study could be attributed to subtype-dependent variability in susceptibility of *Blastocystis spp.* to PVS. This was previously suggested by Mirza *et al*. (2011), they examined four *Blastocystis* isolates with the ST4 and ST7 infection, and demonstrated

that the subtype-dependent variation in the susceptibility to the six different antiprotozoal drugs including MTZ. Indeed, large number of samples of the individual subtype infection was required to clearly demonstrate such variation.

In the present study, light microscopic observations of the morphology of treated *Blastocystis* parasites revealed significant reduction in the size range of vacuolar and granular forms exposed to high doses of propolis extract (500 and 1000µg/ml), moreover size reduction was also noted with cultures treated with MTZ (10 µg/ml). When keeping with the present findings, *G. lambia* trophozoites exposed to propolis extract exhibited changes of the pear-shaped appearance of cells with reduction of flagellar beating frequency (Freitas *et al,* 2006).

Furthermore, the MTZ treated *Blastocystis* cells have shown cell shrinkage with compaction of organelles in the cytoplasm using light microscopy. Such features were considered the important morphological evidence of apoptosis (Nasirudeen *et al,* 2004), which could be attributed to the loss of cytoplasmic fluids and denaturation of proteins in the apoptotic cells (Huppertz *et al,* 1999). The morphological changes observed here suggested that the propolis extract could have similar effects on *Blastocystis spp.* to that obtained by MTZ which has been suggested to activate the apoptotic machinery (Nasirudeen *et al,* 2004). Another light microscopic observation in this study was the abundance of granular forms with shriveled appearance at 48 h incubation especially in cultures treated with high doses of propolis extract and PVS. Similar finding was observed on *in vitro* treatment of *Blastocystis spp.* ST3 with *Ferula asafoetida* (oleo-gum-resin) using both powder and oil forms with high concentrations (20 and 50 mg/ml, respectively), where the viable vacuolar forms were replaced by granular forms which lost viability overtime and eventually disintegrated (El-Deeb *et al*, 2012).

The previous study has proposed that the granular forms may represent the sequential stages of the cell degeneration (Vdovenko, 2000) which could explain the present study outcome data.

Conclusion

Both the propolis extract and PVS showed potent inhibitory effect on *Blastocystis spp*. However, unlike PVS, propolis extract has shown complete eradication of the parasite and uniform effect across the studied subtypes and this was comparable to MTZ (reference drug). Further studies are needed to evaluate the effect of propolis on larger number of isolates and on other subtypes especially showing MTZ resistance and to evaluate its effect *in vivo*.

In addition, there is a need to study propolis from different geographical locations in Egypt with identification of its bioactive principles to achieve standardization before being introduced to clinical practice. Ongoing studies using other subtypes & MTZ resistant isolates with further evaluation of its effect *in vivo* will be published in due time.

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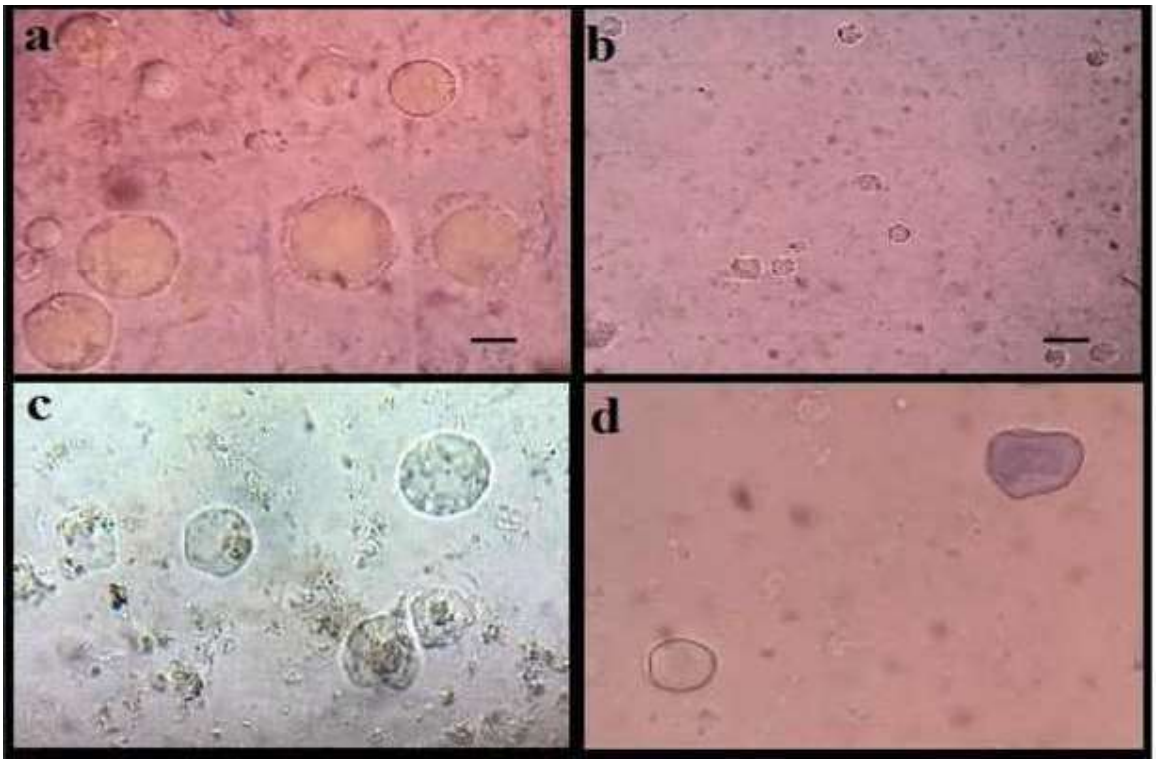
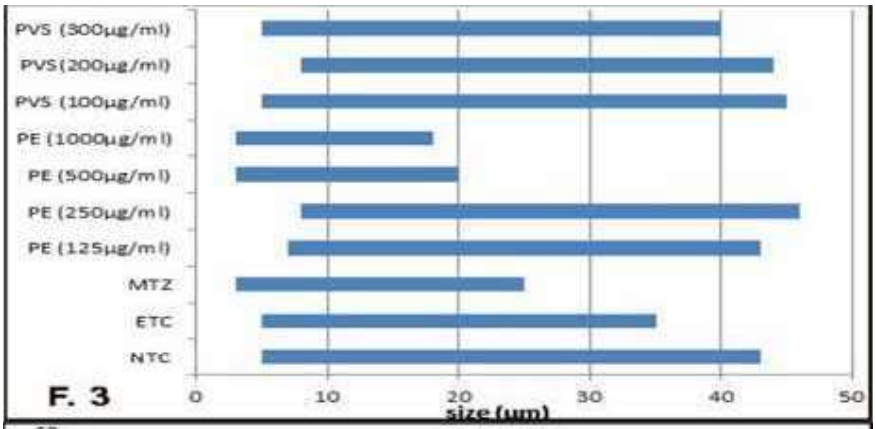
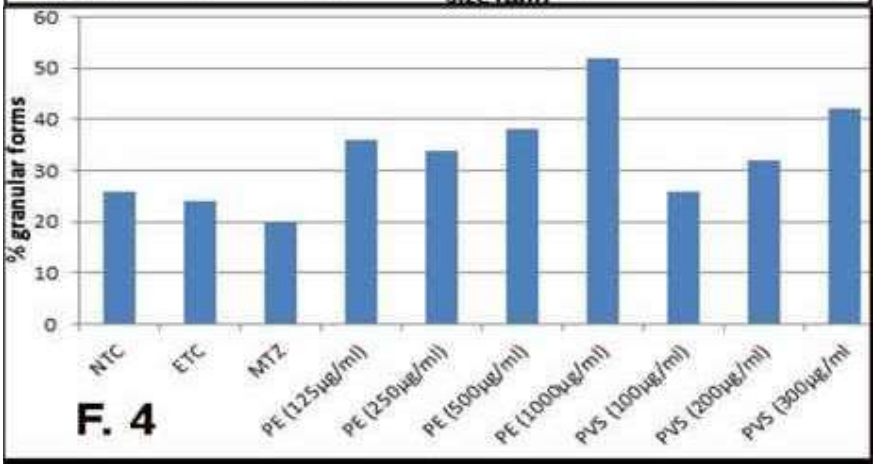
Explanations of figures

Fig. 2: *Blastocystis* parasite showing (a) vacuolar forms with large size in NTC, compared to (b) vacuolar forms with small size (x400) in 48 hr cultures treated with propolis extract (1000µg/ml), bars = 10 µm (c) abundance of granular forms (x1000) in 48 hr cultures treated with propolis extract (1000 µg/ml), (d) viable cell (unstained) and unviable cell (stained with Trypan blue), x400.
Fig. 3: Size ranges of *Blastocystis* parasites in treated cultures in comparison to non-treated control at 48hr
Fig. 4: Percentage of granular forms in treated cultures in comparison to non-treated control at 48hr,


a
b
c
d


PVS (300µg/ml)
PVS (200µg/ml)
PVS (100µg/ml)
PE (1000µg/ml)
PE (500µg/ml)
PE (250µg/ml)
PE (125µg/ml)
MTZ
ETC
NTC
0
10
20
30
40
50
size (um)

F. 3


% granular forms
60
50
40
30
20
10
0
NTC
ETC
MTZ
PE (125µg/ml)
PE (250µg/ml)
PE (500µg/ml)
PE (1000µg/ml)
PVS (100µg/ml)
PVS (200µg/ml)
PVS (300µg/ml

F. 4