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Original article

In vitro susceptibility of human *Blastocystis* subtypes to simeprevir

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ABSTRACT

Introduction and aim: *Blastocystis* is a common enteric parasite, having a worldwide distribution. Many antimicrobial agents are effective against it, yet side effects and drug resistance have been reported. Thus, ongoing trials are being conducted for exploring anti-*Blastocystis* alternatives. Proteases are attractive anti-protozoal drug targets, having documented roles in *Blastocystis*. Serine proteases are present in both hepatitis C virus and *Blastocystis*. Since drug repositioning is quite trendy, the *in vitro* efficacy of simeprevir (SMV), an anti-hepatitis serine protease inhibitor, against *Blastocystis* was investigated in the current study.

Methods: Stool samples were collected from patients, Alexandria, Egypt. Concentrated stools were screened using direct smears, trichrome, and modified Ziehl-Neelsen stains to exclude parasitic co-infections. Positive stool isolates were cultivated, molecularly subtyped for assessing the efficacy of three SMV doses (100,150, and 200 μg/ml) along 72 hours (h), on the most common subtype, through monitoring parasite growth, viability, re-culture, and also via ultrastructure verification. The most efficient dose and duration were later tested on other subtypes.

Results: Results revealed that *Blastocystis* was detected in 54.17% of examined samples. Molecularly, ST3 predominated (62%), followed by ST1 (8.6%) and ST2 (3.4%). Ascending concentrations of SMV progressively inhibited growth, viability, and re-culture of treated *Blastocystis*, with a non-statistically significant difference when compared to the therapeutic control metronidazole (MTZ). The most efficient dose and duration against ST3 was 150 μg/ml for 72 h. This dose inhibited the growth of ST3, ST1, and ST2 with percentages of 95.19%, 94.83%, and 94.74%, successively and viability with percentages of 98.30%, 98.09%, and 97.96%, successively. This dose abolished *Blastocystis* upon re-culturing. Ultra-structurally, SMV induced rupture of *Blastocystis* cell membrane leading to necrotic death, versus the reported apoptotic death caused by MTZ. In conclusion, 150 μg/ml SMV for 72 h proved its efficacy against ST1, ST2, and ST3 *Blastocystis*, thus sparing the need for pre-treatment molecular subtyping in developing countries.

1. Introduction

Blastocystis has a worldwide distribution, with a higher prevalence in developing countries reaching up to 60% compared to the developed countries (Khoshnood et al., 2015). It is more common among patients with irritable bowel syndrome (IBS) and immune-compromised patients (Mohamed et al., 2017). Genus *Blastocystis* is classified into more than 17 subtypes (STs) which may explain variations in symptoms and response to treatment (Coyle et al., 2012; Maloney et al., 2019; Stensvold and Clark, 2020). Subtypes related to infections in humans are one to nine, with subtype three being the most predominant human subtype in many countries (Abaza et al., 2014; Lepczynska et al., 2017).

Metronidazole (MTZ) is the drug of choice for *Blastocystis* treatment (Adao and Rivera, 2018). Most antimicrobial therapies

Abbreviations: IBS, irritable bowel syndrome; ST, subtypes; MTZ, Metronidazole; SMV, Simeprevir; PCR, Polymerase chain reaction; DMSO, Dimethyl Sulfoxide; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy; CV, central vacuole; MLO, Mitochondrion-like organelle.

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against *Blastocystis*, including MTZ, have documented side effects that are not tolerated by many patients. Moreover, drug resistance has been reported (Coyle et al., 2012; Sekar and Shanthi, 2013). Previous studies have reported subtype-dependent variations in drug susceptibilities; one of them reported the resistance achieved by ST3 (Rajamanikam et al., 2019). Hence, many studies were designed to explore *in vitro* and *in vivo* therapeutic alternatives for *Blastocystis* management (El Deeb et al., 2012; Al-Mohammed et al., 2013; Roberts et al., 2015).

The preliminary step of drug discovery is to select an enzyme that is essential in the biological pathways of an organism (Das et al., 2013). Proteases are attractive key enzymes, because of their role in the survival, metabolism, replication, and pathogenesis of parasites (Mckerrow et al., 2008). There are different types of proteases; of which, *Blastocystis*-derived serine proteases play a major role in the regulation of pro-inflammatory cytokine expression, protein kinase activation and also in the pathogenesis of IBS (Poirier et al., 2012; Lim et al., 2014). Since drug repositioning is quite trendy and serine proteases are present in both hepatitis C virus (HCV) and *Blastocystis* (Dunn et al., 2007; Alfonso and Monzote, 2011; Poirier et al., 2012; Izquierdo et al., 2014), the idea of trying serine protease inhibitor as a therapeutic alternative against *Blastocystis* has been encouraged.

Simeprevir (SMV) is a highly effective and safe anti-hepatitis C virus serine protease inhibitor, with tolerable adverse effects as it is less active against human proteases (Tanwar et al., 2012; Izquierdo et al., 2014). Hence, this study was designed to evaluate its *in vitro* efficacy against *Blastocystis*.

2. Material and methods

2.1. Stool samples collection, processing, and in vitro cultivation

The current study was conducted on 120 stool samples, which have been collected from patients with gastrointestinal symptoms from different departments and outpatient clinics of Alexandria University Hospitals and the Medical Research Institute, Alexandria, Egypt.

Each stool sample was screened for *Blastocystis* using saline and iodine smears, formol ether sedimentation concentration technique, and trichrome stain to exclude parasitic co-infections. Moreover, modified Ziehl-Neelsen acid-fast stain was used to exclude co-infections by intestinal coccidia (Garcia, 2007).

After excluding co-infection with other parasites, positive stool isolates were individually cultivated in a set of four culture tubes; each containing five ml of Jones' medium supplemented with 10% horse serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Culture tubes were incubated in an upright position at 37 °C and were daily monitored, for 72 hours (h) using iodine smear (El-Sayed et al., 2017).

By the end of the cultivation interval, culture sediment of each isolate was examined by iodine smear, then the four tubes were divided as follows; three tubes were further sub-cultured for conducting the drug susceptibility assay, while 0.5 ml of the fourth culture tube sediment was frozen at −20 °C for DNA extraction (Mokhtar et al., 2019).

2.2. DNA extraction and Blastocystis subtyping

Genomic DNA was extracted from thawed culture sediments with a DNA extraction kit, according to the manufacturer's directions (QIAamp; Qiagen Inc., Hilden, Germany) (Mohamed et al., 2017). Polymerase chain reaction (PCR) was carried out using three pairs of subtype-specific diagnostic primers as follows: STI (SB83) (F: GAAGGACTCTCTGACGATGA, R: GTCCAAATGAAAG GCAGC), ST2 (SB155) (F: ATCAGCCTACAATCTCCTC, R: ATCGCCACTTCTCCAAT) and ST3 (SB227) (F: TAGGATTTGGTGTTTGGAG A, R: TTAGAAGT-GAAGGAGATGGAAG) (Khademvatan et al., 2018).

Extracted DNA was used as a template for the amplification in a 50 μl reaction mixture containing 25 μl Dream Taq PCR Master Mix (2×), 50 pmol of each primer set pair (one ST at a time) and 19 μl nuclease-free water. The PCR reactions were performed in a thermal cycler (Eppendorf, Germany) which include denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extending for 1 min at 72 °C. Up to 35 cycles are required to amplify the DNA target, with an additional final extension cycle for 5 min at 72 °C (Abaza et al., 2014; Mohamed et al., 2017).

Amplification products were electrophoresed in 1.5% agarose gel (Primega, USA) and Tris-Borate-EDTA buffer, the gel was stained with ethidium bromide and was photographed by an ultraviolet gel documentation system. For each isolate, each primer pair of PCR amplification was done twice (Abaza et al., 2014).

2.3. In vitro antimicrobial susceptibility testing

2.3.1. Drugs

Metronidazole tablets (Flagyl®) (Sanofi Aventis Co.) were used as a therapeutic control. The drug was dissolved in phosphate-buffered saline (PBS). MTZ concentrations were adjusted to 10, 100, and 250 μg/ml (El Deeb et al., 2012; Roberts et al., 2015).

Simeprevir capsules (Olysio®) (Janssen) were tried against *Blastocystis* (Lin et al., 2009). The drug was dissolved in Dimethyl Sulfoxide (DMSO) solvent. Final concentrations of SMV were adjusted to a gradient concentration of 100, 150, and 200 μg/ml according to a preceding pilot study.

2.3.2. In vitro experimental design

Drug assay was primarily performed on the predominant molecularly verified subtype, then the most efficient dose and duration against this subtype were applied on the other tested subtypes. For each tested isolate, inocula of 1×10^4 *Blastocystis* /ml were further sub-cultured, at 37 °C for 72 h (Haresh et al., 1999). Subculture tubes were divided into four groups and were performed in triplicates. Group (I) was the non-treated infected control. Group (II) was the 1% DMSO solvent control. Group (III), the therapeutic control, was further subdivided into subgroups III (a, b & c); treated with MTZ at concentrations of 10, 100, and 250 μg/ml, successively. Group (IV), the experimental SMV-treated, was further subdivided into subgroups IV (a, b & c); treated with SMV at concentrations of 100, 150, and 200 μg/ml, successively.

2.3.3. Assessment of in vitro anti-Blastocystis activity of SMV

The activity of SMV has been evaluated after 24, 48, and 72 h, then was compared to the corresponding controls.

2.3.3.1. Parasite growth. Daily quantitation of *Blastocystis* was performed by a hemocytometer using iodine smears for three days (Yakoob et al., 2011).

2.3.3.2. Parasite viability. Viability of the retrieved *Blastocystis* was evaluated using eosin-brilliant cresyl blue dye. Where, viable *Blastocystis* completely or partially excluded the dye, while dead ones took it up (Haresh et al., 1999; El-Sayed et al., 2017).

Percentage inhibition of *Blastocystis* multiplication/viability in the experimental group (IV) and both the therapeutic control group (III) and solvent control group (II) in relation to the non-treated control group (I) was calculated according to the following formula:

$$\text{Multiplication/viability inhibition}(\%) = (a-b)/a \times 100$$

Where "a" is the mean number/mean viable intact *Blastocystis* in the non-treated control group and "b" is the mean number/mean viable intact *Blastocystis* in the tested group (experimental, therapeutic control, or solvent control) (El-Sayed et al., 2017).

Growth and viability profiles of each subgroup were recorded along the three studied intervals.

2.3.3.3. Parasite re-culturing. One hundred μl of culture sediments obtained from all groups was further re-cultured in fresh culture media for 72 h. The viability of re-cultured *Blastocystis* was evaluated using eosin-brilliant cresyl blue dye as mentioned before (Eida et al., 2008; Al-Mohammed et al., 2013).

2.3.3.4. Electron microscopic study (EM). The effect of SMV on *Blastocystis* ultrastructure was studied, using both; scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The study was performed on pooled subcultures treated with the most efficient dose and duration of SMV and was compared to the non-treated control group (I). Pooled subculture sediments were washed using phosphate-buffered saline pH 7.4 three times and centrifuged for 5 min at 2000xg. The resulting pellet was then fixed in buffered glutaraldehyde-phosphate 2.5% and processed for SEM and TEM studies (Zhang et al., 2012; Dhurga et al., 2016).

2.4. Statistical analysis

Data were analyzed using SPSS software package version 20.0 and described using mean ± standard deviation (SD). Comparison between different groups at different time intervals were done using One-way and Two-way ANOVA tests. Percentage inhibition by the used drugs at different durations were compared using the Chi-square test. Significance of the obtained results was judged at the 5% level (Kotz et al., 2006).

3. Results

3.1. Stool samples processing and in vitro cultivation

Screening of the 120 collected stool samples by light microscopic examination revealed that 65 samples were positive for *Blastocystis* (54.17%). Seven stool samples out of 65 positive samples (10.77%) showed concurrent parasitic infections being; *Giardia lamblia* in five samples (7.70%), *Cryptosporidium* oocysts in one sample (1.50%), and eggs of *Hymenolepis nana* in another sample (1.50%).

3.2. DNA extraction and Blastocystis subtyping

After excluding samples with mixed infection, all microscopically positive samples were culture positive. Out of the 58 positive stool cultures, 36 isolates (62.00%) were amplified with SB227 primer (526 bp), being identified as ST3 (Fig. 1a). Whereas five isolates (8.60%) were amplified with SB83 primer (351 bp), being identified as ST1 (Fig. 1b). Only two isolates (3.40%) were amplified with SB155 primer (650 bp), being identified as ST2 (Fig. 1c). Four isolates (6.80%) contained mixed infection with ST1 and ST3. Eleven isolates were not amplified with any of the used three primers (18.97%).

3.3. In vitro antimicrobial susceptibility testing

After excluding isolates containing mixed molecular subtypes and subtypes that were not identified with any of the used three primers, drug assay has been performed on ST3 as it was the most commonly detected subtype. Then, the most efficient dose and duration against ST3 was applied on ST1 and ST2. Results of this dose and duration were compared among the three tested subtypes.

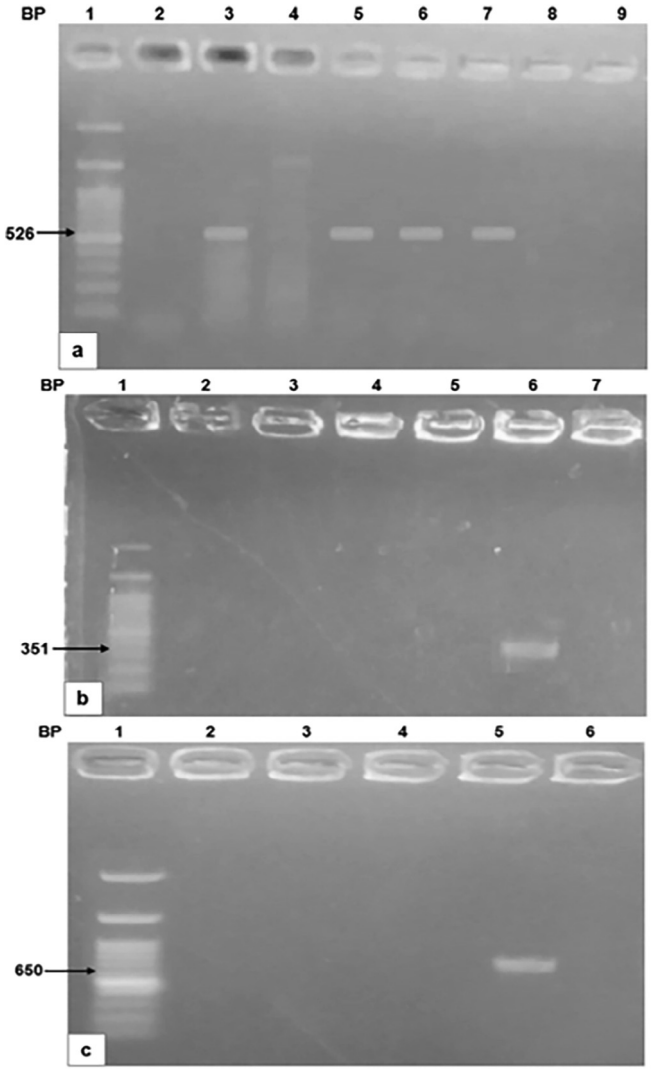

Fig. 1. Agarose gel analysis of PCR-amplified products of *Blastocystis*. **1a:** Amplification with SB227 primer (526 bp) (ST3), **1b:** Amplification with SB83 primer (351 bp) (ST1), **1c:** Amplification with SB155 primer (650 bp) (ST2).

3.3.1. Parasitological study (parasite growth, viability, and re-culture):
3.3.1.1. Assessment of ST3 Blastocystis' response to SMV. Vacuolar, granular and cystic forms were detected in groups (I), (II), and (III), whereas in the SMV-treated group (IV), granular form disappeared and amoeboid form was detected. Detected *Blastocystis* showed variations in their shape and size ranging from 1 to 3 μm (Fig. 2a, b, c& d). On using eosin-brilliant cresyl blue dye, viable *Blastocystis* completely or partially excluded the dye, appearing green, while dead ones absorbed it, appearing red in color (Fig. 2e, f, g & h).

Growth and viability profiles of the **non-treated control group (I)** showed a statistically significant increase in the mean *Blastocystis* count and viability at 48 h compared to 24 h, followed by a statistically significant reduction at 72 h as shown in Fig. 3.

In **group (II), DMSO** proved to have a non-statistically significant effect ($p > 0.05$) on *Blastocystis* growth and viability in comparison to the non-treated control group I, consequently, a

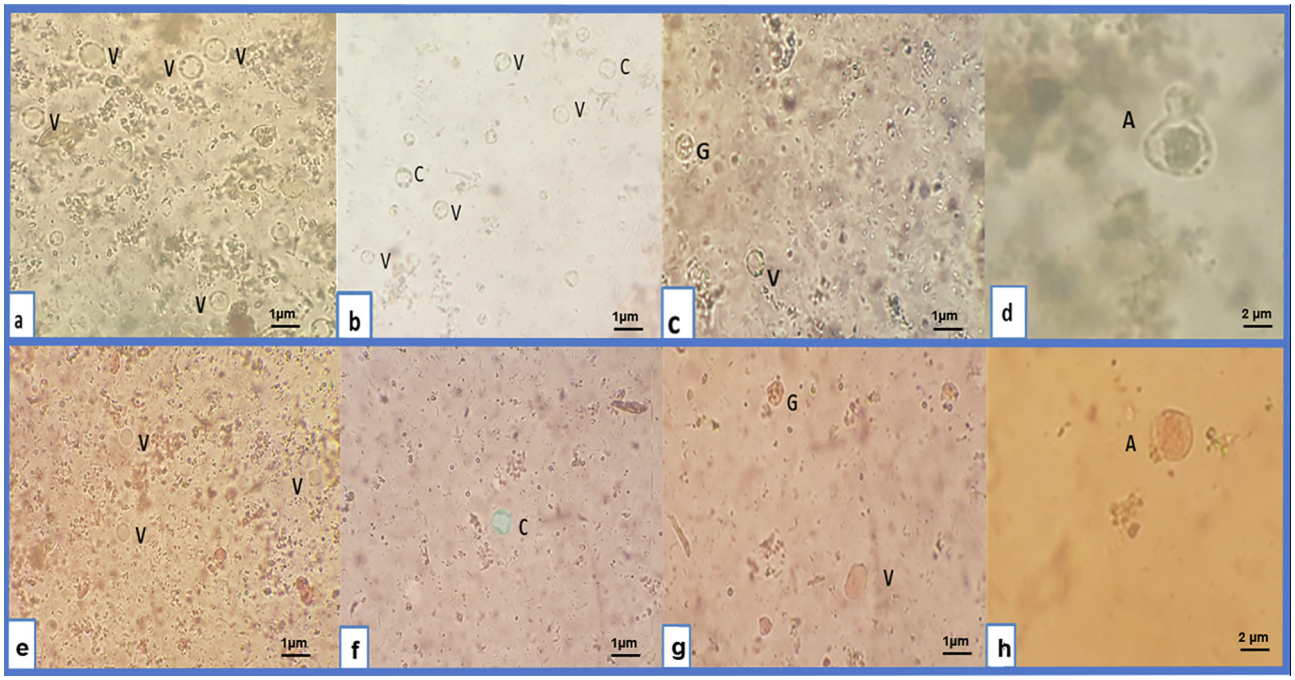

Fig. 2. Light microscopic findings of *Blastocystis* forms detected after drug testing by iodine smears and eosin-brilliant cresyl blue dye (×400). **2a:** Vacuolar (V) form by iodine smear, **2b:** Vacuolar (V) and cystic (C) forms by iodine smear, **2c:** Vacuolar (V) and granular (G) forms by iodine smear, **2d:** Amoeboid (A) form by iodine smear, **2e:** Viable vacuolar (V) form, **2f:** Viable cyst (C) form, **2g:** Non-viable vacuolar (V) and granular (G) forms, **2h:** Non-viable amoeboid (A) form.

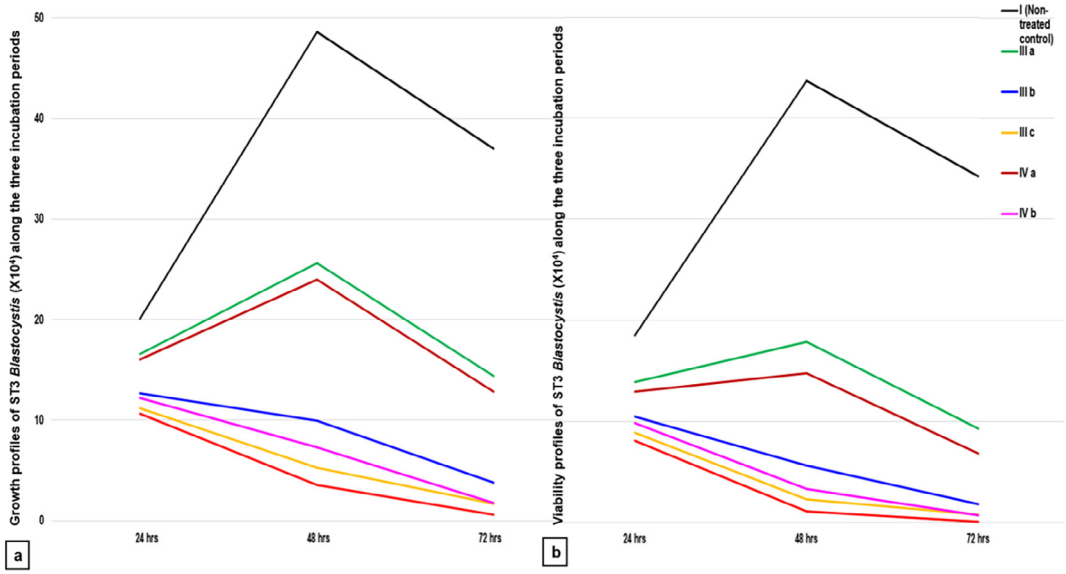

Fig. 3. Growth and viability profiles of ST3 *Blastocystis* along the three-times intervals for the SMV tested doses versus their respective non-treated and MTZ-treated controls. **a:** Growth profile **b:** Viability profile. I (non-treated control), III (MTZ-treated control): IIIa (10 µg/ ml), IIIb(100 µg/ml), IIIc(250 µg/ml), IV (SMV-treated): IVa(100 µg/ml), IVb (150 µg/ ml), IVc(200 µg/ml).

comparison of all groups was made only to the non-treated control group (I).

Regarding **group III and group IV** that were **treated with MTZ and SMV** respectively, there was a significant progressive decline in the mean count and viability of ST3 *Blastocystis* which was proportional to both; the increase in MTZ and SMV concentrations and the duration of exposure to the two drugs (Fig. 3). The highest inhibition of *Blastocystis* **multiplication** (98.35%) was recorded with 200 µg/ml SMV at 72 h. This inhibition was statistically significant when compared to the same dose at 48 h (92.64%). However, this inhibition was not statistically significant when compared to 150 µg/ml SMV at 72 h (95.19%) with (p = 0.256).

Blastocystis **viability** was affected in the same manner of growth (Fig. 3). 99.77% non-viable *Blastocystis* were recorded with 200 µg/ml SMV at 72 h. This dramatic inhibition of *Blastocystis* viability was statistically significant, when compared to the effect of the same dose at 48 h (97.57%). However, inhibition of *Blastocystis* viability was not statistically significant when compared to the dose of 150 µg/ml at 72 h (98.30%) with (p = 0.176) (Fig. 4), so the most efficient dose and duration was **150 µg/ml for 72 h**. This dose

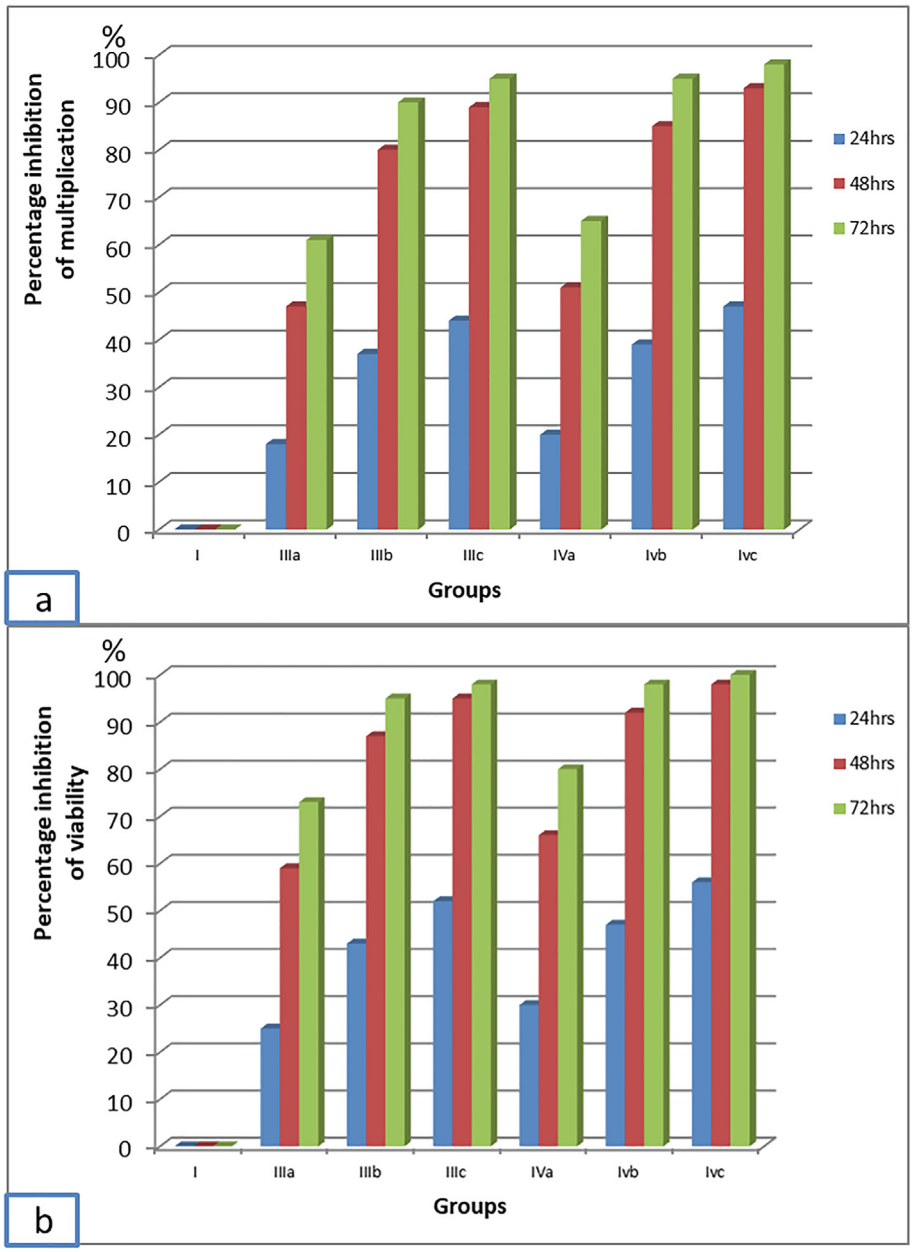

Fig. 4. Percentage inhibition of ST3 *Blastocystis* multiplication and viability by MTZ and SMV versus its respective non-treated control group**. 4a:** Percentage inhibition of ST3 *Blastocystis* multiplication, **4b:** Percentage inhibition of ST3 *Blastocystis* viability. I (non-treated control), II (Solvent control), III (MTZ-treated control): IIIa (10 μg/ ml), IIIb (100 μg/ml), IIIc(250 μg/ml), IV (SMV-treated): IVa(100 μg/ml), IVb(150 μg/ ml), IVc(200 μg/ml).

showed non-statistically significant viability inhibition in comparison to the intermediate dose of MTZ at 72 h (94.65%) with ($p = 0.242$).

No viable *Blastocystis* was detected after re-culturing of ST3 *Blastocystis* previously exposed to the intermediate and high doses of SMV at 48 h and 72 h (Fig. 5).

It was worth noting that two isolates molecularly belonging to ST3 didn't show a significant response to any dose or duration of MTZ, but responded to the most efficient dose and duration of SMV (data not shown) which requires further extensive verification studies.

3.3.1.2. Assessment of ST1 and ST2 Blastocystis response to SMV. Fig. 6 revealed that there was no significant difference between the achieved inhibition in *Blastocystis* growth, viability, and re-culture in both ST1 and ST2 *Blastocystis* when compared to ST3 on applying the most efficient dose and duration of SMV (150 μg/ml for 72 h) on them.

3.3.2. Electron microscopic study:

Using SEM, *Blastocystis* in the **non-treated control group (I)** was round or oval with a surface showing many indentations. Moreover, a fibrous surface coat attaching to cell surfaces of *Blastocystis* was shown in Fig. 7a and b. In **SMV-treated group (IV),** while the majority retained their spherical shape (Fig. 7c & d), some *Blastocystis* showed irregular amoeboid forms (Fig. 7f). Additionally, the surface of SMV-treated *Blastocystis* showed remarkable convolutions and folding (Fig. 7c), with occasional surface membrane pores in some of them (Fig. 7e).

Using TEM, *Blastocystis* in the **non-treated control group (I)** showed that the vacuolar form was round or oval in shape with a central vacuole and thin rim of cytoplasm with a nucleus of a

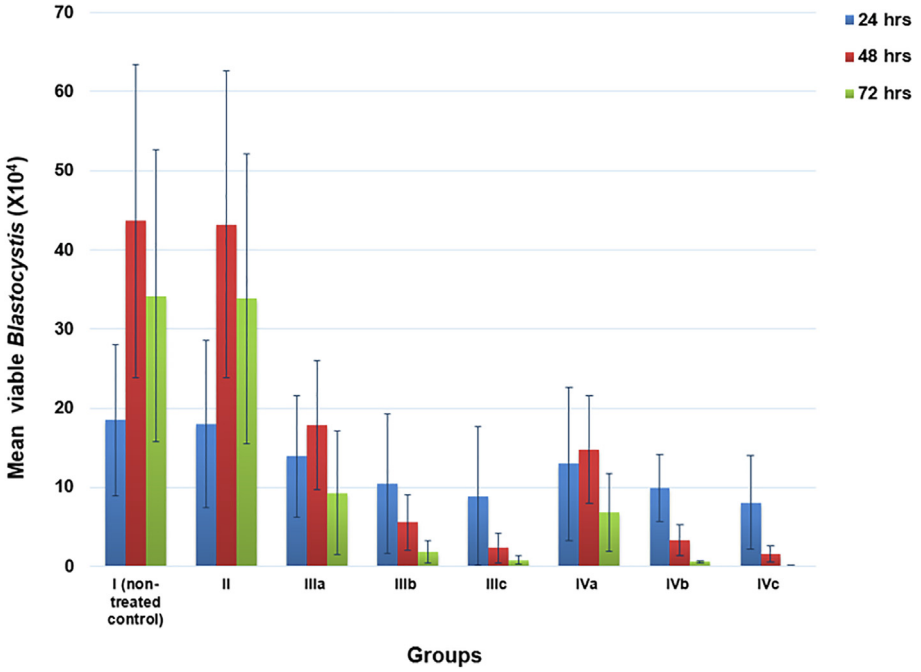

Fig. 5. Re-culturing of ST3 *Blastocystis* previously inoculated with SMV at different incubation periods versus its respective non-treated and treated controls. I (non-treated control), II (Solvent control), III (MTZ-treated control): IIIa(10 μg/ml), IIIb(100 μg/ml), IIIc (250 μg/ml), IV (SMV-treated): IVa(100 μg/ml), IVb(150 μg/ml), IVc(200 μg/ml).

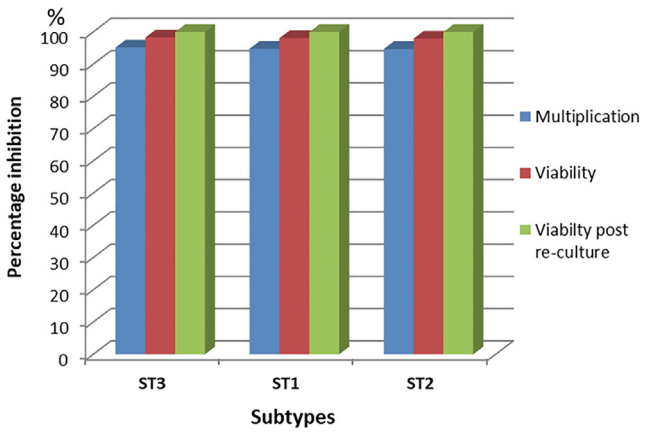

Fig. 6. Effect of 150 μg/ml SMV for 72 h on the percentage inhibition of multiplication, viability and viability post re-culture of ST1 and ST2 *Blastocystis* in comparison to ST3.

crescent band of electron opaque material at one pole (Fig. 8a). The granular form was round in shape and was similar in size to the vacuolar form. Yet, the central body was almost filled with granules of different electron densities and was surrounded by a peripheral rim of the cytoplasm (Fig. 8b). In the **SMV-treated group (IV)**, *Blastocystis* was electron-lucent and the central vacuole seemed devoid of any electron-dense particles (Fig. 8c). Some showed plasma membrane rupture with subsequent loss of intracellular contents (Fig. 8e). SMV-treated *Blastocystis* amoeboid form with central vacuole (CV) and mitochondrion-like organelle (MLO) is shown in Fig. 8d.

4. Discussion

Aiming at finding a promising anti-*Blastocystis* therapeutic alternative through drug repurposing, the current study explored the *in vitro* effect of SMV, an anti-HCV serine protease inhibitor, against *Blastocystis*. For fulfilling this aim, human stool samples were screened by light microscopy, positive samples were cultivated and molecularly subtyped for performing the drug assay on the identified subtypes.

Light microscopic screening revealed the **presence of *Blastocystis* in 54.17%** of the examined stool samples which have been collected from Alexandria, Egypt. Prevalence of 67.4% and 52% were previously reported in Alexandria in 2016 and 2019, respectively (Eassa et al., 2016; Elsayad et al., 2019), while in other Egyptian governorates prevalence ranged from 10% up to 53% (El-Shewy et al., 2002; El-Marhoumy et al., 2015; Farghaly et al., 2017). Epidemiological studies in several countries with different sanitation standards, revealed a wide range of *Blastocystis* prevalence ranging from 0.54% up to 63% (Beyhan et al., 2015; Duda et al., 2015; El Safadi et al., 2016; Osman et al., 2016; Ramírez et al.,2016; Seyer et al., 2017; Asfaram et al., 2019; Delshad et al., 2020; Zanetti et al., 2020)

Out of the 65 positive cases infected with *Blastocystis* in the current study, seven isolates were **co-infected with** *Giardia lamblia*, *Cryptosporidium* oocysts, and eggs of *Hymenolepis nana* (10.77%). *Giardia lamblia* was the most frequent concurrent parasite in the current work. This association was in accordance with that reported by Nascimento and Moitinho Mda (2005) and Elghareeb et al. (2015).

In the current study, molecular subtyping has been done using **ST1, ST2, and ST3** primers, since they are the most commonly detected STs in Egypt (Souppart et al., 2010; Abaza et al., 2014). **ST3 was the most predominant subtype** in this study (62%). Other authors agreed with this result, where the predominance of ST3 in Egypt was reported also in Cairo (61.9%) (Souppart et al., 2010), (44.54%) (Fouad et al., 2011) and Suez Canal (56.1%) (Abaza et al., 2014). Moreover, different epidemiological studies around the world reported that a majority of human *Blastocystis* infections was attributable to ST3 isolates in different countries with percentages ranging from 31.2% to 53% (Meloni et al., 2011; Forsell et al., 2012; Moosavi et al., 2012; Roberts et al., 2013; El Safadi et al., 2016; Ramírez et al.,2016; Seyer et al., 2017; Jiménez, et al., 2019). Nevertheless, fewer studies reported the predominance of ST1 and ST4 in other countries (Malheiros et al., 2011; Lee et al.,

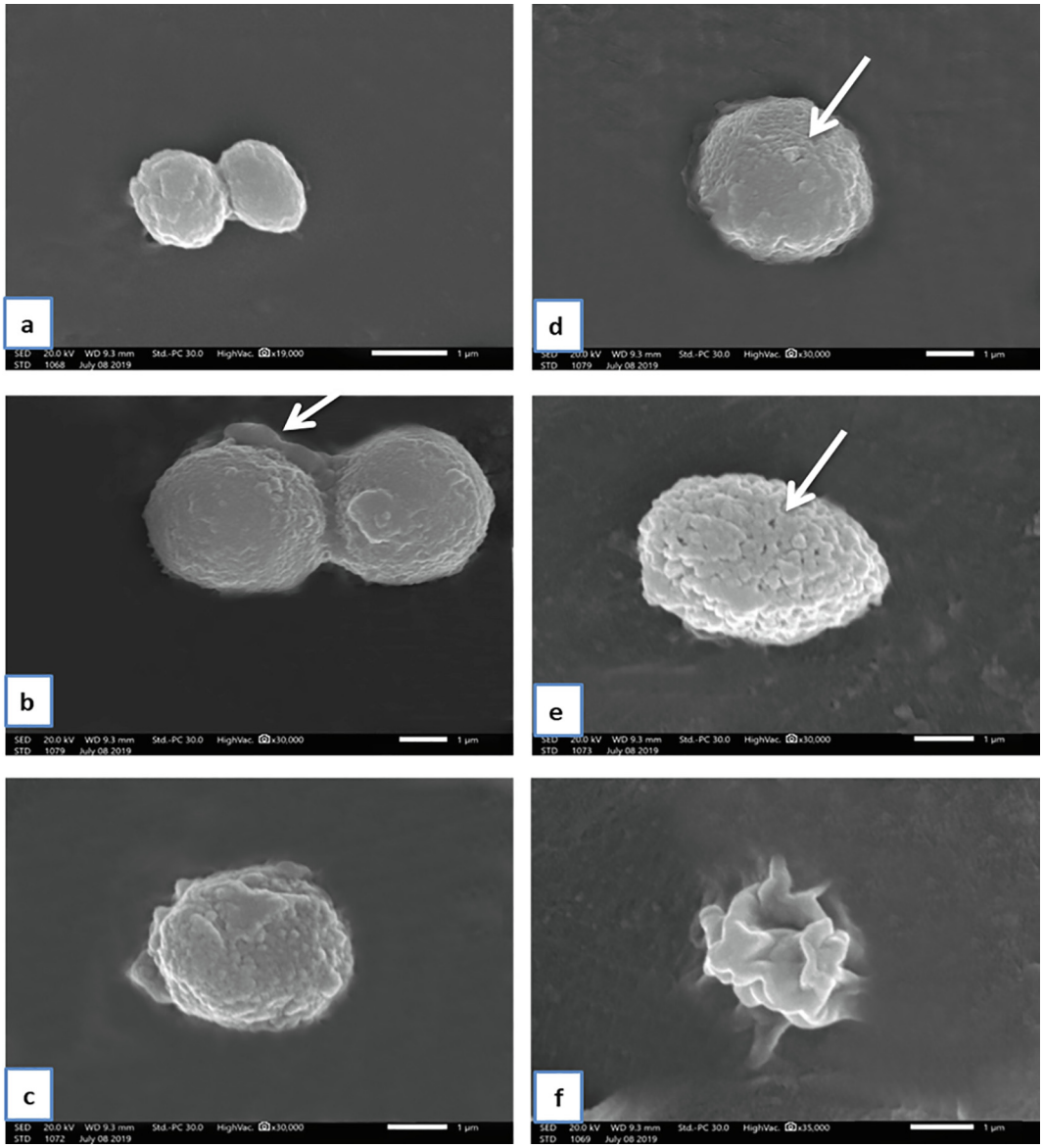

Fig. 7. (a-f): Scanning electron microscopy of the non-treated *Blastocystis* and SMV-treated groups. **a:** Non-treated *Blastocystis* showing its oval shape (X19000). **b:** Non-treated *Blastocystis* showing its round shape and fibrous surface coat attached to cell surfaces of *Blastocystis* (arrow) (×30000). **c:** SMV-treated *Blastocystis* showing remarkable convolution and folding (arrow) (×30000). **d & e:** SMV-treated *Blastocystis* showing remarkable convolution and folding and surface membrane pores (arrow) (×30000). **f:** SMV-treated *Blastocystis* amoeboid form (×35000).

2012; Thathaisong et al., 2013; Delshad et al., 2020; Zanetti et al., 2020). Prevalence variations of *Blastocystis* subtypes between different countries, and also within the same country, might be attributed to variable epidemiological conditions including; reservoirs and methods of transmission, prevailing local living conditions and customs (Li et al., 2007; Souppart et al., 2010).

In the current ***in vitro* drug assay, the non-treated control group (I)** showed that the mean *Blastocystis* count/ml was 20.11 × 10^4 ± 20.25 at 24 h. It significantly peaked to reach 48.61 × 10^4 ± 23.57 at 48 h, then the growth declined to 36.97 × 10^4 ± 20.36 at 72 h. In parallel, viability profile progressed in a similar manner, where viable *Blastocystis*/ml peaked to reach 43.69 × 0^4 ± 19.80 at 48 h and then declined to 34.19 × 10^4 ± 18.44 at 72 h. Many authors reported the same progress of growth and viability profiles of the untreated cultures (Yakoob et al., 2011; Al-Mohammed et al., 2013; Roberts et al., 2015).

Results of the **solvent control group (II)** revealed that, at all studied intervals, DMSO 1% did not induce any significant impact neither on *Blastocystis* growth nor on its viability profiles, as compared to the non-treated control group (I). Similarly, DMSO 1% used by Girish et al. (2015) showed no effect on *Blastocystis* growth. Likewise, other drug solvents such as 70% and 95% ethanol also showed no effect on parasite growth (Ramadan and Al Khadrawy, 2003; Vital and Rivera, 2009).

In the present study, **MTZ** was used as the standard **therapeutic control.** Results of **group (III)** demonstrated that ***Blastocystis* multiplication** was inhibited by 89.78% using the intermediate dose of MTZ at 72 h while using the highest dose (250 μg/ml) showed 89.14% and 95.43% percentages inhibition of multiplication at 48 h and 72 h, respectively. The **percentage inhibition of *Blastocystis* viability** was more than 90% with the intermediate dose at 72 h (94.65%) and with the highest dose at 48 and 72 h (94.71% and 97.72%, respectively). It has been reported that MTZ eliminated *Blastocystis* through inhibiting its nucleic acid synthesis (Nasirudeen et al., 2004; Raman et al., 2016). Different studies tested the sensitivity of *Blastocystis* to various doses of MTZ

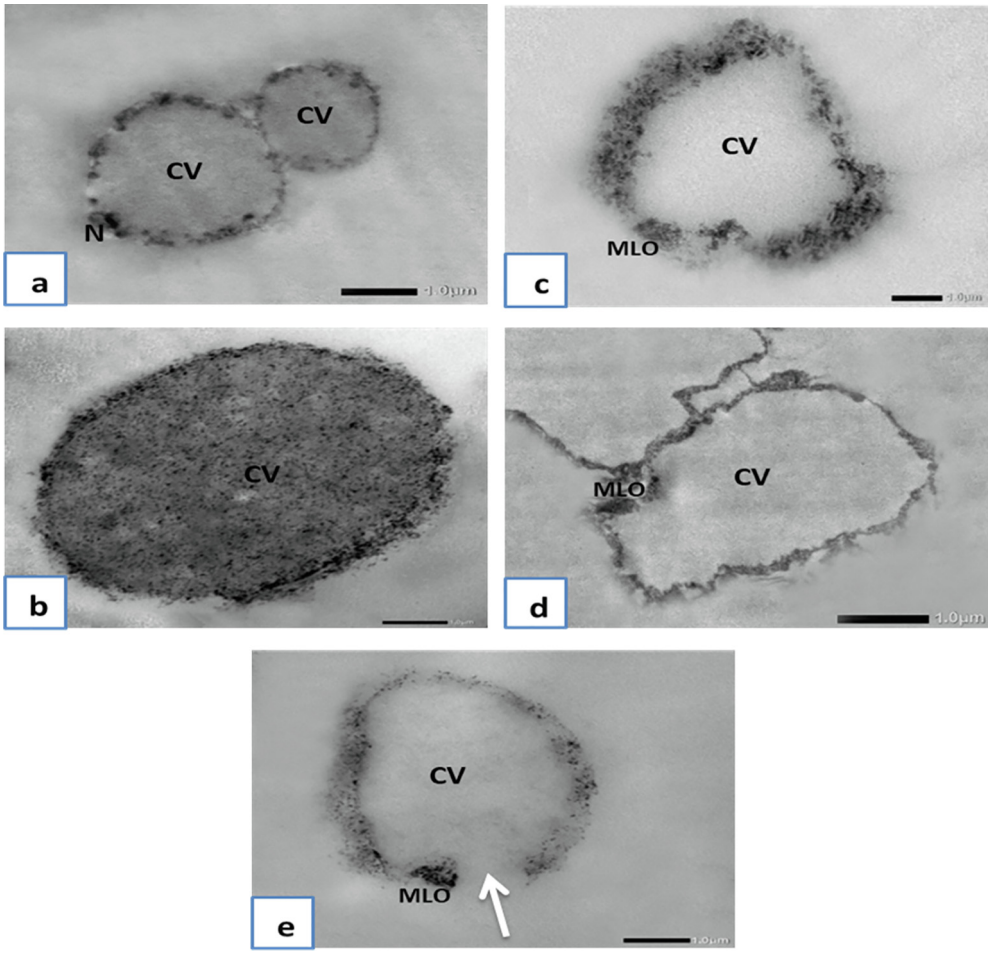

Fig. 8. (a-e): Transmission electron microscopy of non-treated *Blastocystis* and SMV-treated groups. **a:** Non-treated *Blastocystis* vacuolar form showing central vacuole (CV) and thin rim of cytoplasm with peripheral nuclei (N) (×6,000). **b:** Non-treated *Blastocystis* granular form showing central vacuole (CV) almost filled with granules of different electron densities and surrounded with peripheral rim of the cytoplasm (×8,000). **c:** SMV-treated *Blastocystis* vacuolar form showing central vacuole (CV) devoid of any electron-dense particles and showing mitochondrion-like organelle (MLO) (×6,000). **d:** SMV-treated *Blastocystis* amoeboid form showing central vacuole (CV) devoid of any electron-dense particles and showing mitochondrion-like organelle (MLO) (×6,000). **e:** SMV-treated *Blastocystis* showing central vacuole (CV) devoid of any electron-dense particles, and showing mitochondrion-like organelle (MLO) and plasma membrane rupture (arrow) (×6,000).

(Yakoob et al., 2011; El Deeb et al., 2012; Roberts et al., 2015; Raman et al., 2016; El-Sayed et al., 2017). Although total clearance of *Blastocystis* was not achieved by Roberts et al. (2015), even at 1000 μg/ml MTZ, other authors achieved it by lower concentrations (El Deeb et al., 2012; Mokhtar et al., 2016). Variability in response and susceptibility to the drug can be explained by different geographical locations or intra-subtype differences, which may be attributed to the presence of different alleles in each subtype (El-Sayed et al., 2017).

The *in vitro* effect of **SMV** on multiplication and viability of *Blastocystis* revealed that it induced progressive dose and duration dependent inhibitory effects. At 24 h, both growth and viability retardation have started. The highest **inhibition of *Blastocystis* multiplication** was achieved by the intermediate dose after 72 h **(**95.19%) and by the highest dose after 48 and 72 h (92.64% and 98.35%, respectively). In parallel, the highest **inhibition of *Blastocystis* viability** was achieved by the intermediate dose after 72 h (98.30%) and the highest dose after 48 and 72 h (97.57% and 99.77%, respectively). The impact of SMV on the inhibition of *Blastocystis* multiplication and viability was non-statistically significant when compared to MTZ at the respective concentrations and durations.

The current results could be presumably explained through the documented impact of protease inhibitors as antimicrobial agents. This is supported by Venturini et al. (2000), McKerrow et al. (2008) and Hussein et al. (2009). Several cysteine protease inhibitors proved to be influential against *in vitro Blastocystis*′ growth and viability. It was recorded that 99.74% inhibition of *Blastocystis* multiplication and 91% viability inhibition have been achieved by the highest dose of a cysteine protease inhibitor, at 72 h, and both parameters were abolished at 96 h (Eida et al., 2008). Similar results were reported by Al-Mohammed et al. (2013) who found that cysteine proteases inhibitors rendered *Blastocystis* non-viable. Protozoal growth retardation by serine protease inhibitors was successively documented (Dudley et al., 2008; Makioka et al., 2009). In *Blastocystis*, serine protease has been proven to play a critical effect on the pro-inflammatory cytokines expression and the protein kinase activation, as deduced from the *in vitro* incubation of *Blastocystis* with serine protease inhibitors (Lim et al., 2014).

For verifying the inhibitory extent of SMV on *Blastocystis* viability, **re-culturing** in fresh media revealed that the highest concentration of SMV (200 μg/ml), at the three studied intervals, and the intermediate concentration (150 μg/ml), at 48 and 72 h, were cytocidal to *Blastocystis*. On the other hand, the intermediate concentration (150 μg/ml), at 24 h, and the least concentration of SMV (100 μg/ml), at the three studied intervals, were only cytostatic, as *Blastocystis* resumed its growth after re-culturing. "Viable non-re-culturable cells" are these which have intact membranes,

that retain their metabolic activities, yet, lose their ability to produce progenies (Nyström, 2001; Allegra et al., 2008; Rousseau et al., 2018). In agreement with the current results, Eida et al. (2008) demonstrated that a high concentration of NaNO2, as an anti-protease, was cytotoxic to *Blastocystis* growth *in vitro*, while at low concentration, the incomplete inhibitory effect was found as *Blastocystis* resumed growth after NaNO2 had been ceased.

On correlating the SMV′s inhibitory effects on growth, viability and re-culture of *Blastocystis*, the most efficient dose and duration of SMV against ST3 *Blastocystis* turned out to be **150 μg/ml for 72 h**. On trying this dose and duration on **ST1 and ST2**, **growth** was inhibited by 94.83% and 94.74%, respectively, as compared to 95.19% in ST3. The **viability** of ST1 and ST2 was inhibited by 98.09% and 97.96%, respectively, as compared to 98.30% in ST3. No viable *Blastocystis* was detected on **re-culturing** of ST1, ST2, and ST3 previously exposed to this dose. These results showed that there was no significant difference between the inhibition of *Blastocystis* growth, viability and re-culture in ST1 and ST2 *Blastocystis* when compared to ST3. Similarly, Girish et al. (2015) reported uniform inhibition among different *Blastocystis* subtypes. On the contrary, other anti-*Blastocystis* protease inhibitors induced variable effects on different *Blastocystis* subtypes (Al-Mohammed et al., 2013; Mokhtar et al., 2019). Subtype dependent variation might explain the variable response of different *Blastocystis* subtypes to the same drug *in vitro* (Mokhtar et al., 2019).

A morphological study was done using both light microscopic examination and electron microscopic verification. Different *Blastocystis* forms were detected (Abou El Naga and Negm, 2001). Of them; vacuolar, granular and cystic forms were detected in the untreated cultures. The vacuolar form was the most commonly detectable form followed by the granular form. These results are in agreement with Souppart et al. (2009), Mehta et al. (2015) and Darabian et al. (2016). Granular form persisted after MTZ drug testing because, as reported, MTZ induces apoptosis, where granular formation is a self-regulatory mechanism of *Blastocystis* during apoptosis to produce high number of viable cells (Dhurga et al., 2016). This mechanism may underlie the resistance to MTZ as being stated by Haresh et al. (1999). However, after exposure to SMV, the granular form has disappeared and was replaced by the amoeboid form. The disappearance of the granular form may be attributed to the fact that serine protease inhibitors induce necrotic cell death (Al-Mohammed et al., 2013). The existence of amoeboid form has been formerly linked to drug-treated cultures (Kumar and Tan, 2013). It was described as a dying cell by Yason and Tan (2018) that could explain its presence after SMV drug trial.

In the present **SEM** study, SMV-treated *Blastocystis* verified the light microscopic findings that some of *Blastocystis* showed irregular amoebic forms, while others preserved their round or oval shape with remarkable convolutions and folding on their surfaces. This finding agreed with Raman et al. (2016) who treated *Blastocystis* with MTZ. Some treated *Blastocystis* showed membrane pores that could lead to cytoplasmic leakage, which was also previously detected by Yason et al. (2016) when *Blastocystis* was treated with LL-37 (a colonic antimicrobial peptide).

Transmission electron microscopy revealed that SMV, in the present study, induced *Blastocystis* necrosis and cell death. Necrotic cells were electron-lucent, the central vacuole seemed devoid of any electron-dense particles and some showed plasma membrane rupture. These abnormal findings were in agreement with the Nomenclature Committee on Cell Death that described the term "necrotic cell death" as being morphologically characterized by plasma membrane rupture and subsequent loss of intracellular contents (Nasirudeen et al., 2004; Kroemer et al., 2009; Al-Mohammed et al., 2013). The same necrotic findings were detected when *Blastocystis* was treated with proteases inhibitors by Al-Mohammed et al. (2013). On the other hand, authors reported the apoptotic effect of MTZ on *Blastocystis* (Nasirudeen et al., 2004; Al-Mohammed et al., 2013). Apoptotic cell death includes nuclear condensation, cell shrinkage, deposition of membrane-bound apoptotic bodies and heavy vacuolization appears (Nasirudeen et al., 2004; Al-Mohammed et al., 2013). Explanation of these discrepancies between the effects of MTZ and SMV by TEM may be due to different mechanisms of action of both drugs on *Blastocystis*. On entering the targeted cells, MTZ′s cytotoxic form disrupts the DNA of the parasite (Nasirudeen et al., 2004; Raman et al., 2016). On the other hand, SMV acts by inhibition of serine proteases, and the central vacuole of *Blastocystis* is a reservoir for proteases as was reported by Puthia et al. (2008).

5. Conclusion

Results of the present study proved the promising *in vitro* anti-*Blastocystis* effect of simeprevir. The most effective studied dose and duration of SMV (150 μg/ml dose for 72 h) proved its efficacy against ST1, ST2, and ST3, which are the most commonly reported subtypes in Egypt. This finding saves the need for molecular subtyping in developing countries, before starting *Blastocystis* treatment. Moreover, SMV-induced necrosis of the targeted organism is a promising advantage, versus the reported MTZ- induced apoptotic and granular formation, which underlays the risk of therapeutic resistance.

Further studies are recommended for subtype analysis of molecularly unidentified strains and for applying the effect of the most efficient dose and duration of SMV on them. Testing the precise effect of SMV on resistant isolates, their molecular verification, their biochemical analysis, and their electron microscopic study is recommended. Performing a biochemical study of protease level of ST1, ST2, and ST3 for further verification of the mode of action of SMV is mandatory.

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Ethical approval and Informed consent

Stool specimens were collected as part of routine clinical examination of patients, according to the national guidelines. Informed consents were sought from patients and approval from the institutional ethics committee was obtained.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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