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RESEARCH ARTICLE

ANTIPARASITIC ACTIVITY OF SPIRO-DERIVATIVE COMPOUND STABILIZED ON MULTI-WALLED CARBON NANOTUBES AGAINST *BLASTOCYSTIS HOMINIS* IN ALBINO MICE

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ABSTRACT

Herein, the medicinal action of a novel spiro-derivative compound ((2R,3R)-4-(4-bromophenyl)-2-(1,7,7-trimethylbicyclo[2,2,1] heptan-2-on-3-yl)-4-oxobutanoic acid, PSp-B) was tested against *Blastocystis hominis* (*B. hominis*) with enhancing its curativeness via being immobilized onto multi-walled carbon nanotube (MWCNTs). The infected mice were treated by 50 mg/kg/day drug dosage for five days. The parasitological, physiological and histopathological studies for *B. hominis* diseased albino mice were explored. Both PSp-B and PSp-B@MWCNTs showed considerable reduction in the number of *B. hominis* trophozoites in the intestinal content displaying 63.2 % and 76.2 %, respectively. Also, the number of cysts/gm stool in infected mice treated by PSp-B and PSp-B@MWCNTs were highly reduced by 84.7 % and 88.1 %, respectively. The serum Glutathione Reduced (GSH), Lipid Peroxide (MDA) and Nitric Oxide (NO) levels in PSp-B and PSp-B@MWCNTs treated group were ameliorated when compared to negative control group. The histopathological examination of the small intestinal sections in treated groups showed nearly similar character to that of positive control. It is plausible to mention that PSp-B@MWCNTs exhibited privilege Blastocystosis therapeutic performance rather than PSp-B. However, much more studies should be conducted to explore the mechanistic action of these drugs against such and alternative parasites.

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INTRODUCTION

Blastocystis hominis (*B. hominis*) is one of the most anaerobic protist dwell the human intestine that so far belongs to Stramenopiles group of organisms (Silberman *et al*., 1996). Such parasite attacks human being and preferentially colonize the gastrointestinal tract causing severe diarrhea, bloating and flatulence (Kaya and Cetin, 2007). Unfortunately, infection of human being by this parasite is facilitated by fecal-oral transmission pathway, whereas the contagion is solely caused by the cyst form of parasite (Yoshikawa *et al*., 2004). In accordance, developed and developing countries are seriously threatened by blastocystosis achieving infection rates of ~ 10 % and 100%, respectively (Tan *et al*., 2010). Interestingly, current information about this parasite is highly insufficient and several inquiries being stand as points of questionnaire such as pathogenesis of the disease (Carbajal *et al*., 1997) and exploration of efficient antiblastocystis agents (Nigro *et al*., 2003). Large scale of researcher's trials had been paid to emphasize the pathogenic role of *B. hominis* via studying the parasitic genotype and subtypes showing that the parasite could be present in immuno-depressed and immuno-suppressed humans with possible disappearance of symptoms (Coyle *et al*., 2012). The increasing demand for treatment of blastocystosis imposed several researchers to discover promising vaccines for such disease (Dinleyici *et al*., 2011). Diverse of anti-parasitic agents, e.g. metronidazole, nitazoxanide and paramomycin, were examined against *B. hominis* infection (Sekar and Shanthi, 2013). Metronidazole was the most commonly used antibiotic in treatment of blastocystosis either individually or conjugated with other drugs like paramomycin and cotrimoxazole (Tan *et al*., 2010). But, regrettably, the disease showed extensive revert after elapsing of the treatment period (Mirza *et al*., 2011). Nitazoxanide exhibited potent activity against blastocystosis displaying clinical and parasitological cure of 86 % (Rossignol *et al*., 2005). Paramomycin showed deprived impact on the parasite itself being engaged in destruction of gut bacterial flora, which sought to be essential in survival of *B. hominis*

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(Van Hellemond *et al*., 2013). Despite the efforts made to explore new anti-parasitic agents against *B. hominis*, still much work need to be performed in developing proficient drugs against blastocystis disease. Carbocyclic spiro organic compounds are bicyclic rings connected through just one atom. Such compounds have been exploited to provide tool compounds for biomedical study and to serve as scaffolds for the design of therapeutic agents (Smith, 2016). For example, spiranes emerged as promising candidate for treatment of mycobacterial and tuberculosis diseases attributable to their potential role in reduction of bacterial count in lung and spleen tissues (Kumar *et al*., 2008). They are also known as efficient anti-microbial and anti-inflammatory agents (Zhang *et al*., 2017), when such compounds tested in-vivo they have shown anti-inflammatory activity ranging from 20.4- 27.4% at the dose of 50 mg/kg body weight (Pareek *et al*., 2014).

Multi-walled carbon nanotube (MWCNTs) are allotropes of carbon that made of graphite constructed in cylindrical tubes with nanometer in diameter and several millimeters in length. Regarding to the pleasurable features of MWCNTs, particularly high surface area, advanced chemical stability, high biocompatibility, and progressive adsorbing behavior (Lopez *et al*., 2015), they could be positively used as deliverers to wide varieties of therapeutic molecules (drugs, proteins, antibodies, DNA, enzymes, etc.) (Hirlekar *et al*., 2009). They have been proven to be an excellent vehicle for drug delivery being capable to directly penetrate the cell membranes and keep the drug intact without conducting any metabolic processes unto achieve the targeted infected zones (Singh *et al*., 2012). The aim of the present study is to evaluate the medicinal action of novel spiro- derivative compound stabilized onto multi-wall carbon nanotube [4-phenyl-4-oxobutanoic acid] derivative, namely (2R, 3R)-4-(4-bromophenyl)-2-(1,7,7-trimethylbicyclo[2,2,1]heptan-2-on-3-yl)-4-oxobutanoic acid, (PSp-B@MWCNTs), against blastocystosis in infected albino mice. The therapeutic effect of such PSp-B@MWCNTs was examined via parasitological, physiological and histopathological analyses.

MATERIALS AND METHODS

Materials

(2R,3R)-4-(4-bromophenyl)-2-(1,7,7-trimethylbicyclo[2,2,1] heptan-2-on-3-yl)-4-oxobutanoic acid (PSp-B) were synthesized and purified according to EL-Hashash and Rizik, (2016). The chemical structure of PSp-B is shown in Fig. 1.

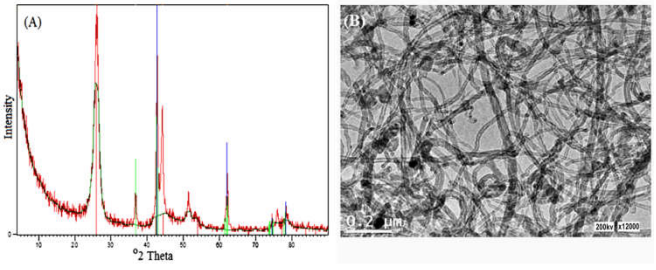

Fig. S1. (a) XRD spectra and (B) SEM image of MWCNTs.

Multi-walled carbon nanotube (MWCNTs) was synthesized by chemical vapor deposition method (Lopez *et al*., 2015) and supplied from the nanotechnology center of Egyptian Petroleum Research Institute (EPRI).

The produced MWCNTs is marked by 95 % purity and 4 % ash with diameters of 10 – 15 nm and lengths of 0.1 – 10 μm. Such results were evidenced by X-ray diffraction and scanning electron microscopy analyses, as being debated in Fig. S1, supplementary materials. Metronidazole was purchased from Rhone Poulenc Rorer/France company for pharmaceutical and chemicals. Other chemicals such as tetrahydrofuran were of analytical grade.

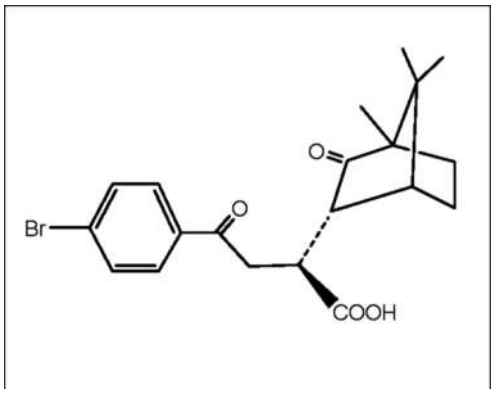

Fig. 1. Chemical structure of PSp-B.

Synthesis of spiro- modified carbon nanotubes

The MWCNTs (0.5027 g) was suspended in 80 ml tetrahydrofuran (THF) at 35^{o} C for 4 h. The spiro-derivative compound (PSp-B) was dissolved in minimum amount of THF, and then added to the MWCNTs suspension, whereas the weight ratio of PSp-B: MWCNTs: THF is 1: 3.3: 76. The mixture was vigorously stirred at 35^{o} C for 48 h to achieve complete adsorption of spiro-compound over MWCNTs. The black residue was filtered, washed several times by distilled water, ethanol and acetone, and dried at 80^{o} C for 4 h. The obtained powder was collected and saved under vacuum, and coadded by "PSp-B@ MWCNTs".

Animals

one hundred and sixty-two male albino mice (*Mus musculus*), 20 g were obtained from Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute (TBRI). The animals were maintained in an animal house at 25°C, with a relative humidity of 40–60%, and were fed a normal diet of commercial pellets and given filtered drinking water. All experiments were approved by an animal ethics committee and carried out at the SBSC/TBRI in accordance with international animal ethics guidelines.

Parasite

Isolation of *B. hominis* Stool samples were collected from patients attending the Parasitological Research and Diagnostic Laboratory Unit, Parasitology Department, Faculty of Medicine, Ain Shams University. The fresh stool samples were immediately examined for intestinal parasites by a wet smear with iodine stained and merthiolate iodine formaldehyde concentration technique (MIFC) (Melvin and Brooke, 1982). Samples were frequently screened and washed using normal saline.

Experimental infections and design

The acute toxic effect of PSp-B drug to albino mice was recorded 24 h post oral drug administration via intra-gastric

tube in an aqua-suspended form. Six drug dosages were used, 25 mg/kg and its folds up to 150 mg/kg. Three replicates, each of six mice, were utilized for every dose. Another three replicates were maintained without dosing as a control. The lethal dose (LD_{100}) was measured as described elsewhere (Litchfield and Willcoxon, 1949). Thirty albino mice were orally inoculated with 10,000 *B. hominis* cysts for each mouse according to previous works (Yoshikawa *et al*., 2004). Three weeks' post infection (pi), the mice were divided into five groups, i.e. each group contains six mice.

The understudied drugs were orally administrated for five successive days obeying the following protocol: (i) group was treated by metronidazole (MTZ), as a drug reference, in a dose of 200 mg/kg/day (Ismail *et al*., 2015), (ii) group was treated by suspension of MWCNTs (50 mg/kg/day), (iii) group was treated by proper un-toxic suspension of PSp-B drug (50 mg/kg/day) and (iv) group was vaccinated by PSp-B@MWCNTs drug (50 mg/kg/day). A negative control group (infected untreated) as well a positive control group (uninfected untreated) were maintained.

Parasitological studies

All mice were sacrificed 2 weeks post-treated. Quantitative estimation of the infection intensity in the stool samples of *B. hominis* infected mice was performed (Shlim *et al*., 1995). Different forms of *B. hominis* (fecal cyst and trophozoite) were counted in at least ten fields with estimation of the average number/high power field (HPF). The mean number of trophozoite/field in intestinal contents and the mean number of cyst/gm stool were determined in each experiment.

Physiological studies

The serum of sacrificed mice was obtained after two weeks of treatment for colorimetric measurement of Glutathione Reduced (GSH) applying the method of Beutler *et al*. (1963), Lipid Peroxide (MDA) utilizing the technique of Satoh (1978) and Nitric Oxide (NO) according to Montgomery and Dymock (1961) by using spectrophotometer. All reagent kits used in this study were purchased from Bio-diagnostic and Research Reagents /Egypt.

Histopathological studies

After scarification of mice, small intestine of all mice groups was removed and fixed in 10% neutral buffered formalin and processed to paraffin blocks. Sections with thickness 5µm were prepared and handled to stained with hematoxylin and eosin for histopathological examination (Moe *et al*., 1997).

Statistical analysis

The results were carried out using the mean ± standard deviation (SD). The percentage change between each two groups to be compared was assessed using the formula: (U -T) / U × 100 where, U and T are the mean values of negative control group and infected treated groups, respectively. Comparing the difference between the mean of any of the two groups were performed by unpaired 2 tailed students t-test (Sokal and Rohif, 1981). SPSS windows computer program (version 13.0) was used. The data was considered significant if P value was less than 0.05.

RESULTS

Parasitological studies of infected treated mice

The acute toxicity study is described in Fig. 2. The LD_{100} of PSp-B to albino mice 24 h post administration is 150 mg/kg. Thus, the picked dose of PSp-B for the treatment of *B. hominis* in albino mice is 50 mg/kg (one-third of the toxic dose), where the mice are of 100 % survival. Parasitological analysis of the treated infected mice by 50 mg/kg PSp-B and PSp-B@MWCNTs is represented in Fig. 3.

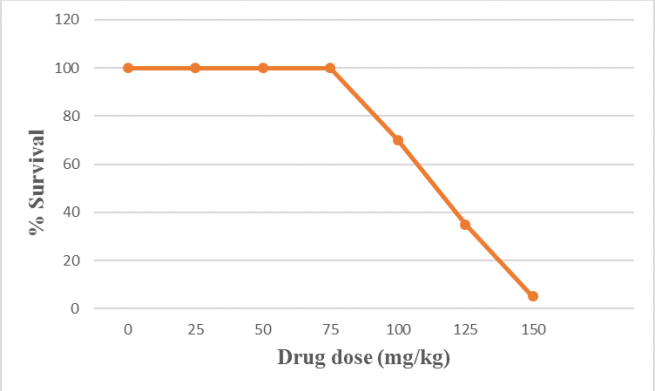

Fig. 2. Toxicity curve of PSp-B on adult albino mice after 24 hours of administration

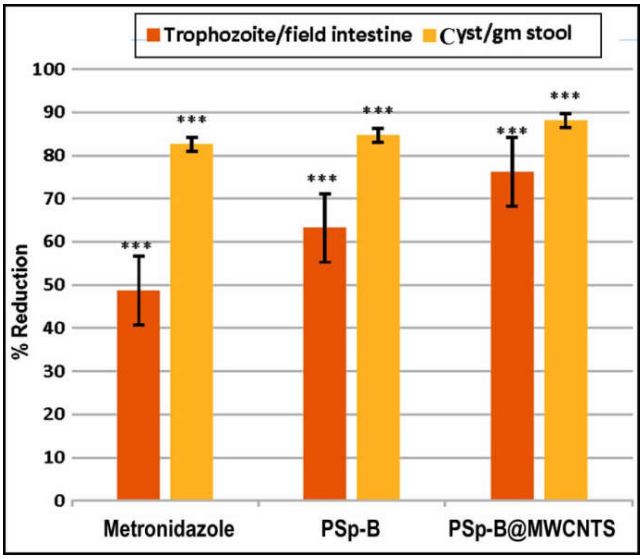

Fig. 3. Reduction rate (%) of B. *hominis trophozoites* and fecal cysts of infected mice treated with metronidazole, PSp- B and PSp-B@MWCNTs. Error bars represent the ± SD from three independent experiments. Highly significant differences relative to negative control is indicated by asterisk (***)

The results show highly significant reduction in the number of trophozoite and fecal cysts ($P<0.001$). The PSp-B treated group shows pronounced reduction in the number of trophozoite and fecal cysts, being 63.2 % and 84.7%, respectively. In case of PSp-B@MWCNTs treated group, the % reduction in the trophozoite and fecal cysts number become 76.2 % and 88.1%, respectively. The metronidazole treated group (Fig. 3) shows depression ($P<0.001$) in the number of trophozoite and fecal cysts by 48.7% and 82.6%, respectively. MWCNTs treated group shows similar result to that of negative control group.

Physiological studies of infected treated mice serum

Results of serum (GSH, MDA and NO) levels of the negative group and the various infected treated groups indicate considerable difference ($P < 0.001$) when compared to the positive control group, cf. Table 1.

Table 1. Physiological analysis of Blastocystis hominis infected mice serum 5 days' post treatment with 200 mg/kg of MTZ and 50 mg/kg of MWCNTs, PSp-B and PSp-B@MWCNTs after 3 weeks (p. i.)

Groups	GSH mg/dl	MDA nmol/ml	NO mol/L
Positive control	4.6 ± 0.3	12.1 ± 0.2	70.9 ± 1.5
Negative control	1.7 ± 0.1***	31.3 ± 1.2***	95.2 ± 4.3***
MTZ treated	2.3 ± 0.1***	15.8 ± 1.2***	85.2 ± 3.9***
MWCNTs treated	1.7 ± 0.2***	32.4 ± 1.5***	95.7 ± 3.2***
PSp-B treated	2.3 ± 0.1***	15.1 ± 1.8**	86.4 ± 2.5***
PSp-B@MWCNTs treated	3.3 ± 0.2***^^^	14.3 ± 0.8**^^	77.9 ± 2.9***^^

Data were expressed as mean ± SD; ***P < 0.001; **P < 0.01 for significant difference from positive control group. ^^^P <0.001; ^^P < 0.01 for significant difference from negative control group.

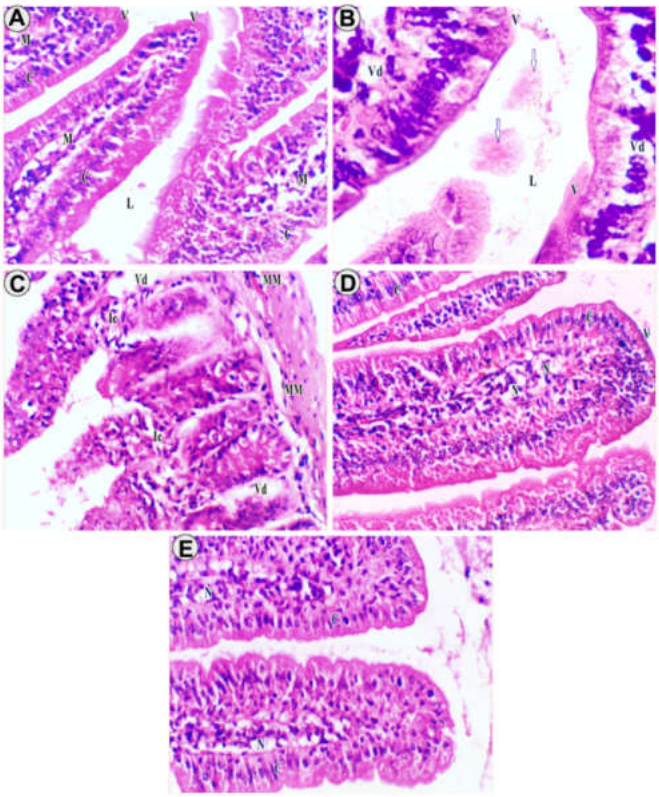

Fig. 4. Photomicrograph of small intestine sections (A) normal section of positive control group shows mucosa (M), columnar epithelia (C), lumen (L) and villi (V). H&E (× 400). (B) small intestine section of negative control group shows vacuolar degeneration (Vd), degenerated villi (V), lumen dilation (L) and *B. hominis* (white arrows). H & E (×1000). (C) small intestine section of MTZ treated group shows vacuolar degeneration (Vd), inflammatory cells (Ic) and degenerating in the muscle layer (MM). H & E (× 400). (D) small intestine section of PSp-B treated group shows semi- regular columnar epithelia (C), abundant villi (V) and necrotic foci (N) in mucosa. H & E (×400). (E) small intestine section of PSp-B@MWCNTs treated group shows healthy columnar epithelia (C) and a few necrotic foci (N). H & E (×400).

It is worth mentioning that no detectable difference is observed in the understudied serum levels in the infected MTZ, MWCNTs as well PSp-B treated groups when compared to the negative control one. Whilst, the infected PSp-B@MWCNTs treated group displays a highly significant increment in GSH level ($P < 0.001$) linked with noticeable depression in MDA and NO levels ($P < 0.01$), as compared to the negative control group.

Histopathological studies of infected treated mice

In the current study, histopathological examination of small intestinal sections from the various infected treated groups are represented in Fig. 4. Comparing to the negative control group, the intestinal tissues are clearly improved upon treatment with under investigated drugs. However, such improvement is still far from the normal tissue pattern of the positive control group. Positive control group (Fig. 4A) shows normal small intestinal structure with normal mucosa, columnar epithelia, lumen and villi. Negative control group as well MWCNTs treated group (Fig. 4B) exhibits cytoplasmic degeneration leading to cells fusion accompanied by deterioration in villi structure resulting in dilation of lumen with appearance of *B. hominis*. MTZ treated group (Fig. 4C) shows moderate healthy small intestine with vacuolar degeneration without fusion. Some inflammatory cells and degenerated muscular layers are appeared with remarkable loss of villi columnar epithelia. PSp-B treated group (Fig. 4D) possesses abundant ameliorated villi conjugate by semi-regular columnar epithelia with presence of undesirable necrotic foci in mucosa. PSp-B@MWCNTs treated group (Fig. 4E) develops advanced medicinal action onto the small intestinal tissues, where intact villi and healthy columnar epithelia appear linked with less fewer necrotic foci.

DISCUSSION

The present results of the therapeutic treatment by PSp-B and PSp-B@MWCNTs against blastocystosis show high significant difference compared to the negative control group. MTZ develops percentage reduction of 48.7% and 82.6% in both trophozoite and fecal cyst stages, respectively. For PSp-B drug, the % reduction in trophozoite and fecal cyst are relatively enhanced being 63.2%and 84.7%, respectively. For PSp-B@MWCNTs drug, the number of trophozoite and fecal cyst are highly reduced achieving 76.2% and 88.1%, respectively. This may be due to the active action of MTZ that may inhibit the growth of *B. hominis* (Zierdt, 1991). The chief role of PSp-B in retarding the number of different stages of *B. hominis* may be attributable to the inhibitory potential of spiro- as well keto acidic functional groups in restricting the growth of *B. hominis* (Zhang *et al*., 2017). Moreover, loading of such drug onto MWCNTs may encourage the targeted penetration of PSp-B through the host cell membrane toward the infected tissue and keep the drug almost intact with parasite. The medicinal effect of MTZ as well *Saccharomyces boulardii* against *B. hominis* infection were concurred to that obtained in the present study (Choi *et al*., 2011). Similar findings suggested that *Quillaja saponaria* (1000 µg/ml) could be useful in the treatment of Blastocytosis (EL-Gayar and Soliman, 2011). Ismail *et al*. (2015) studied the effect of MTZ alone and MTZ combined with either monolurine or lactobacillus against *B. hominis*. They found that poor % reduction of oocysts/gm stool was obtained using MTZ alone (30.7%), while superior % reduction was conducted by conjugating MTZ with either monolurine (96.6%) or lactobacillus (96.4%). Physiological examination of the infected groups reflects existence of highly significant difference in all the examined parameters compared to those derived from positive control group. Several previous studies

demonstrated that severe oxidative stress could be efficiently caused by *Blastocystis* infection (Chandramathi *et al*., 2010). Glutathione peroxidase (GSH), as a cytoplasm/mitochondrial enzyme needed for detoxification of H_2O_2 in most cells and protection of cell membrane from oxidative attack (Packer, 1994), suffer declining in its activity level upon infection of mice by *B. hominis*. Such decrement in GSH level is in confirmative with previous work, where the poor activity of GSH was remarkably caused by the marked reduction in the inhibitory action against oxygen toxicity (Sakaguchi *et al*., 1981). In an alternative study, examining the infection of *Plasmodium berghei* suggested that decreasing in GSH level was the key role in accelerating oxidative stress and damaging of host nDNA (Padín-Irizarry *et al*., 2016). Lipid peroxidation is a well-established mechanism of cellular injury in human, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of reactive carbonyl compounds such as malondialdehyde (MDA), which could be successfully used as an indicator for lipid peroxidation (Eser *et al*., 2003).

In this study, the infection of mice with *B. hominis* elevates MDA serum level, being incongruent with previous research (Eser *et al*., 2003). The elevation in MDA levels in *B. hominis* infection probably accelerate from free radical damage through deteriorating the protective defense system of cell (Eser *et al*., 2003). Nitric oxide (NO) plays multiple functions in the human body, such as maintenance of vascular tone (Gkaliagkousi and Ferro, 2011), modulation of epithelial barrier function (Kuebler *et al*., 2010), and neurotransmission (Sarti, 2004). Generation of nitrosative stress in response to microbes is also an important component of the human cellular defense arsenal (Lewis, 2010). The antimicrobial activity of NO has been reported for a wide range of prokaryotic and eukaryotic (Ramos, 2007) organisms. Infection of mice with *B. hominis* increases the NO level. This phenomenon possibly attributed to the innate immune response as well the sensated indiscriminative behavior of NO, which enthuse marked alteration in NO concentration within the host cells once a foreign organism was introduced in the gut flora (Mirza *et al*., 2011). In the current work, Treatment of mice by PSp-B and PSp-B@MWCNTs compounds adopt improvement in the GSH, MDA and NO levels comparing to those estimated in MTZ treated mice group. Such enhancement in the biochemical parameters are so far close to the normal levels detected from the positive control group. These findings may result from cellular damage, developed oxidative stress and deteriorations in cell metabolic processes that caused by Blastocystis infection (Chandramathi *et al*., 2010).

Histopathological examination of the small intestinal section of negative control mice group exhibits vacuolar degeneration without columnar epithelial lining of villi with presence of *B. hominis* forms at the intestinal surface, as being evidenced by previous work (Moe *et al*., 1997). Histopathological examination of the small intestinal section of MTZ treated mice group shows slight vacuolar degeneration, some inflammatory cells, degenerations in the muscular layers and remarkable loss of villi columnar epithelial cells linked most probable with absence of *B. hominis* in the intestine surface. Such results run in harmony with those found in literature (Ismail *et al*., 2015). Histological study of the small intestine of PSp-B treated mice group serves obvious amelioration in villi conjugate by semi-regular columnar epithelial cells with presence of undesirable necrotic foci in mucosa. While histological sections of the small intestine in PSp-B@MWCNTs treated mice group develops intact villi encompassing healthy columnar epithelial cells with presence of less fewer necrotic foci in mucosa. This may be attributed to the edema inhibition effect and the anti- inflammatory action of PSp-B drug (Lopez *et al*., 2015), which shown to be much more improved by loading PSp-B drug onto MWCNTs. Although MWCNTs possess poor therapeutic activity to blastocystosis, it remarkably enhances targeting of PSp-B drug toward damaged cells (Singh *et al*., 2012).

Conclusion

The concurrent study confirmed that PSp-B@MWCNTs possessed promising medicinal action against *B. hominis*, as being evidenced by the strong significance obtained from the parasitological, physiological as well histopathological studies. Thus, it is reasonably fair to accredit that MWCNTs laden with spiro- compounds are the futuristic candidate drugs for treatment of blastocystosis. However, further studies are still needed to understand the drug/parasite interaction and to well-demonstrate the medicinal action of spiro-derivative compounds.

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