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IN-VITRO STUDY ON THE EFFECT OF CHITOSAN, AND CHITOSAN NANOPARTICLES ON THE VIABILITY AND ULTRASTRUCTURE OF *BLASTOCYSTIS* SPECIES

By
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Abstract

Blastocystis is an anaerobic parasite that was identified as the most common eukaryotic organism reported in human fecal samples. Data on treatment options signifies that efficacious antimicrobial eradication of *Blastocystis spp*. is still far from straight forward**.** As well as Metronidazole, has shown some resistance. The study evaluated chitosan and chitosan Nano particles against *Blastocystis in vitro.* Blastocystis species in stool samples were cultured. Culture tubes were divided into three groups: Chitosan, Chitosan Nano particles and Metronidazole (reference drug), and positive control. Different criteria were used to evaluate the tested drugs on *Blastocystis* and assessing the viability before and after treatment by the use of Neutral red stain and recording structural changes. Also, TEMand the single cell gel electrophoresis (SCGE)/comet assay were used.

The results showed a direct relation between increase in the concentration of Chitosan and Chitosan nano-particles and exposure time and increase in the mortality rates. TEM showed morphological damage when exposed to tested drug, Comet assay showed direct relationship between the increase in the dose of Chitosan Nano particles NPs and the degree of DNA genotoxic destruction.

Key words: *Blastocystis species*, Chitosan, Chitosan nanoparticles, electron microscope

Introduction

Blastocystis spp. is an anaerobic parasite that inhabits the intestinal tract of humans and a wide range of animals. This emerging parasite with a worldwide distribution is often recognized as the most common eukaryotic organism reported in human fecal samples, and its prevalence has shown a dramatic increase in recent years (Shirvani *et al,* 2020). *Blastocystis* prevalence is higher in developing countries. In Egypt, prevalence rates, up to 67.4% were reported in humans (El-Badry *et al,* 2018). The non-specific symptoms of *Blastocystis* infection involve diarrhea, abdominal pain, fatigue, constipation, flatulence, and skin rash (Yakoob *et al,* 2004), but this parasite may also show an important role in numerous chronic gastrointestinal illnesses such as irritable bowel syndrome (Jones *et al,* 2009).

Despite such widespread of this intestinal protozoan, current knowledge about this parasite is incomplete and contradictory, including the use of available treatment options (Clark *et al,* 2013). *Blastocystis* is described as "an infection that is difficult to get rid of" although variable medications have been existing for several decades (Boorom *et al,* 2008*).* An accumulating data on treatment options denotes that efficacious antimicrobial eradication of *Blastocystis spp*. is still far from straight forward *(*Sekar *et al,* 2013), hence, the importance of research on another treatment option. As well as Metronidazole, the drug of choice has shown some resistance, lack of compliance from the patients and also taken for a long period of time. Also, pancreatitis, central nervous system toxicity at higher doses and alterable neutropenia were reported, which indicated alternative therapies of Blastocystosisis (El-Sayed *et al,* 2017).

Chitosan is a linear polysaccharide derived by the deacetylation of chitin and is made by treating the chitin shells of shrimp and other crustations with an alkaline substance such

as sodium hydroxide (Lim *et al,* 2021*).* Chitosan attracted great interest due to its wide range of antimicrobial and antifungal activity and reduced toxicity to mammalian cells (Said *et al,* 2012).

Nanoparticles are new promising drug carriers that proved to be effective in treating many parasitic diseases through overcoming the poor cellular permeability, nonspecific distribution, low bioavailability, and rapid elimination of anti-parasitic agents from the body (Sun *et al,* 2019; Younis *et al,* 2020).

This study aimed to evaluate the chitosan, and a combination of chitosan nanoparticle on the ultrastructure of *Blastocystis* spp. in vitro versus the drug of choice metronidazole using vital stain, transmission electron microscopy and comet assay.

Materials and Methods

This study was carried out in the Diagnostic and Research Unit of Parasitic diseases, Medical Parasitology Department (DRUP), the Biochemistry and Molecular Biology Unit, Faculty of Medicine, and department of Parasitology, Faculty of Veterinary Medicine, Cairo University.

Stool samples were examined for *Blastocystis* and excluding other parasites, Culture of stool samples to confirm the diagnosis, to study the different morphological forms of *Blastocystis* and to amplify *Blastocystis* organisms for subsequent use in the study and calculating the number of viable organisms in cultivated samples.

Subsequently *Blastocystis* in culture was exposed to different materials and the efficacy of these materials on *Blastocystis* was tested by using Transmission electron microscopic (TEM)**,** single cell gel electrophoresis (SCGE)/ comet assay.

Stool Samples were collected in dry clean wide mouth plastic containers with tight fitting lids and containing no preservatives. Specimens were kept free from contamination by urine or water. For safety, containers were put in plastic bags and were carefully transferred shortly after collection to the Parasitology department laboratory. Samples were examined within 1-2 hrs after being provided by the patient, if not, the specimen was excluded and not examined. All accepted stools samples were subjected to macroscopic examination and microscopic examination by using direct wet smear and Formol ether **c**oncentration technique. *Blastocystis* was identified depending on its taxonomic characters.

In the present study, measurement was done for 25-50 stages/sample and after cultivation. The whole size was allocated into the three size range**.** The cyst size was divided into three groups; small sized group ranging from 1-10µm, Medium sized group ranged from 11-20µm and large size was bigger than 21µm. Mean size of each group was calculated and percentage of each was examined culture and considered in the whole study.

In-vitro culture was done using Jone's medium for stool samples proved to be positive for *Blastocystis* only and other parasites-free. Positive samples were cultured in 5ml of modified Jones' media in the same day of sample collection. Culture tubes were incubated at 37°C with daily examined for 72 hours. Culture tubes were considered negative if no parasite was detected after 72hr of culture and incubation. Culture tubes were considered suitable for the drug assessment when parasites exceeded 1×103/ml vacuolar forms.

Quantitative assessment of *Blastocystis* cultures was done by using Neutral red (Vdovenko and Williams, 2000). Cells considered viable when they appeared with their morphological criteria with the thin rim of cytoplasm and peripheral nuclei and stained red. Number of viable *Blastocystis* per 25 organisms was counted. Five readings were taken and the average was obtained. The cultures of 70% vitality were used. Viable cultures were transferred to suitable tested tubes, the supernatants were removed by sedimentation and the chemicals were added to each tube (in triplicate). The tube was exposed to continuous agitation and under the

end of exposure time. Stocks of drugs with different concentrations were prepared and dissolved in distilled water (Zaki ***et al,*** 2022).

Culture tubes were divided into 4 groups each with 3 tubes. GA: Positive control (not treated). GB: Parasites and chitosan treated with concentrations: 10, 25, 50, & 100µg/ml. GC: Parasite treated with nano-chitosan at concentrations: 10, 25, 50, & 100µg/ml. GD: Parasite treated with Metronidazole as 150µg/ml. All tubes were incubated at 37°C and examined after 1hr, 3hrs, 6hrs, 12hrs & 24hrs. Treatment period of at least 1hr was used in previous standardized studies and had shown that this period is needed to induce the cytotoxic effect in *Blastocystis*.

At the end of each exposure time, the supernatant of the tubes containing the tested drug were removed out by centrifugation 1500 rpm for 3 min. The samples were washed 3 time using PBS & sedimented by centrifugation again, then Neutral red was added for the previously exposed sample, to test viability of *Blastocystis*.

Growth inhibition% was calculated: a-b/a x100. Where (a) was the mean number of parasites in control cultures and (b) was the mean number of parasites in treated cultures.

Calculations of LD_{50}, LD_{100}: Median lethal dose that killed 50% of the exposed parasite and median lethal dose that killed 100% were taken into consideration to determine each drug efficacy. Lethal concentration was determined by the mortality curve for each concentration (Adetunji *et al,* 2022). After recording the mortality rate in each exposure time, it was designed; LD_{50} & LD_{100} were obtained in graph.

To investigate the adverse effects of drugs on *Blastocystis;* good samples of cysts were exposed to drug different concentrations, and positive control samples were prepared for TEM inspection in lab FA-CURP, Faculty of Agriculture, Cairo university research park (Graham *et al,* 2007).

Comet assay was done to *Blastocystis* exposed to different concentrations of chitosan nano, metronidazole and positive control was done at the Animal Reproduction Research Institute (Giza, Egypt). DNA damage assessment was by using comet assay to investigate DNA damage in exposed *Blastocystis.* The damage was assessed in groups exposed to varying drug concentrations and in a control group that was not exposed. The DNA of *Blastocystis* was analyzed using Comet 5 image analysis software under a 40× fluorescent microscope. (Kinetic Imaging, Ltd., Liverpool, UK) .

Statistical analysis: Data were expressed as mean± standard deviation (mean ± SD). Graph drawings were done using Excel Software 2013, data were analyzed by using (ANOVA), the difference was considered significant when $p<0.05$ using SPSS 27(IBM.NY, USA)

Results

Control positive sizes were 4-10µm (6.67± 1.33), 11-20µm (15.66±2.32) & 22-31µm (26.3±2.4), small, medium & large. No cysts mortalities were recorded in exposed to concentrations lower than CNPS 10µg/ml.

Blastocystis exposed to CNPS at 10µg/ml caused mean mortality rate of 81.33% after 24 hrs, but at 100µg/ml of CNPS caused mortality rate of 99.33% after 24 hrs. Its exposed to CNPS 100µg/ml. caused increase in mortality rate of 74.33%, 77.33%, 85% & 92% after 1, 3, 6, 12 & 24hrs exposure respectively. CNPS 50µg/ml increased mortality rate from 61.66% to 91.33% after 1hr & 24hrs respectively. *Blastocystis* exposed to chitosan 25µg/ml showed mortality rate of 38%, 59%, 71.66%, 76.33% & 81.33% after 1, 3, 6, 12 & 24hrs ET, respectively. At 50-µg/ml increased mortality rate from 51.66% after 1hr ET to 85.66% after 24hrs ET. Exposed to 10µg/ml chitosan, mortality rate was 21% after 1hr ET, at 25µg/ml was 26.33%, at 50µg/ml rate was 34.33% and 45.33% at 100µg/ml.

Blastocystis exposed to 6hrs chitosan at 10 to100µg/ml, mortality rates increased from 50% to 85.33% and after 24hrs increased from 83.66% to 98.66% when exposed to 10

µg/ml & 100µg/ml respectively. The LD_{50} & LD_{100} sizes decreased versus susceptibility decreased, mainly in large cysts.

TEM: *Blastocystis* in positive control showed that vacuolar form was round or oval in shape with a central vacuole and thin rim of cytoplasm with a nucleus of a crescent band of electron opaque material at one pole. In treated cultures drug caused changes when exposed to 100µg/ml after 24hrs by CNPS led to cytoplasmic vacuoles with necrotic cells and disruption of normal morphology with outer surface alteration. Also, plasma membrane ruptured with loss of all contents led to electric density and central vacuole membrane destruction.

Single cell gel electrophoresis/comet assay: DNA destroyed *Blastocystis* different cysts size exposed to chitosan NPs 10µg/ml, chitosan NPs 100µg/ml for 24hr versus metronidazole 150µg/ml and positive control. DNA damaged *Blastocystis* exposed to chitosan NPs different concentrations after 24hr & positive control in blank RPMI media, showed a relationship between the CNPS increased dose and DNA degree of genotoxic destruction. The damage was presented by various DNA in tail segment at 20.76% with CNPS 100µg/ml. Tail length was 8.49µm in positive control, and 12,13µm when subjected to chitosan NPs 100µg/m. Tail moment varied from 0.796µm in positive control to 0.87µm with chitosan nano-particles 10µg/ml, &1.001µm with CNPS 100µg/ml & 1.18 µm with Metronidazole 150µg/ml. Olive tail moment was by migration of DNA fragments ranged from 0.946 with positive control to 1.7 with metronidazole 150µg/ml.

Details are shown in tables (1, 2, 3, 4, 5, 6, 7, 8 & 9) and figures (1, 2, 3, 4, 5, 6 & 7).

Table 1: Growth inhibition efficacy of CNPS on *Blastocystis* sizes (42% large, 31% medium, & 27% small).

Tested dose		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
10µg/ml	C1	35±0.4	40±2.9	48±1.8	70±4.2	82±3.2
	C2	33±1.6	39±3.1	45±2.3	70±3.7	79±1.7
	C3	33±2.8	38±0.8	46±1.5	72±1.9	79±0.5
Mean	33.66±1.6		39±2.67	46.33±1.86	70.66±3.26	80±1.8
25µg/ml	C1	45±1.2	70±1.8	79±3.9	87±1.2	94±3.2
	C2	47±3.5	69±2.6	76±2.4	89±2.8	93±2.5
	C3	44±2.5	70±3.2	81±4.2	90±0.8	91±1.3
Mean	45.33±2.4		69.66±2.53	78.66±3.5	88.66±1.6	92.66±2.33
50 µg/ml	C1	61±1.7	79± 3.9	86 ± 2.9	90 ± 1.8	97 ± 2.6
	C2	64±3.3	78±4.2	88±4.3	91±3.3	95±1.7
	C3	60±2.3	76±1.2	88±3.89	89±0.5	95±3.4
Mean	61.66±2.43		77.66±3.1	87.33±3.69	90±1.866	96.33±2.56
100 µg/ml	C1	74± 1.3	83 ± 3.6	88 ± 1.45	94 ± 2.3	98±1.9
	C2	76± 2.5	86±1.25	96±3.7	93±1.2	98±0.7
	C3	73± 3.6	85±2.56	92±2.9	95±0.9	100±2.46
Mean	74.33±2.46		74.66±2.47	92±2.68	94±1.466	98.33±1.68
Metronidazole 150µg/mL	18.92		62.65	70	100	100
Control group	2.5		4	6	6	15

Table 2: Growth inhibition efficacy of CNPS on *Blastocystis* sizes (17% large, 20% medium & 63% small)

Tested dose		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
10µg/ml	C1	32±1.4	35±1.6	41±1.9	50±0.9	62±1.5
	C2	29±2.6	36±0.6	39±4.5	49±1.7	64±3.3
	C3	28±0.8	33±2.5	37±3.2	54±2.25	65±5.7
Mean	33.66±1.6		34.66±1.56	39±3.2	51±1.61	63.66±3.5
25µg/ml	C1	40±0.2	59±4.6	65±0.9	79±0.46	85±5.5
	C2	42±2.5	58±0.46	64±5.5	77±4.5	86±4.1
	C3	39±1.5	59±1.2	65±2.7	78±1.9	87±3.5
Mean	45.33±1.4		58.66±2.08	64.66±3.03	78±2.28	86±4.36
50 µg/ml	C1	55±5.7	63± 0.6	72 ± 1.7	83 ± 0.9	92 ± 0.7
	C2	54±3.8	65±1.9	69±3.3	81±3.3	92±2.8
	C3	57±0.6	66±2.25	69±2.4	85±0.3	90±5.4
Mean	61.66±2.43		64.66±1.58	70±2.46	83±1.5	91.33±2.96
100 µg/ml	C1	70± 0.3	79 ± 1.3	85 ± 0.9	92 ± 2.8	95±1.4
	C2	71± 2.8	76±2.8	84±3.3	93±0.7	96±2.8
	C3	69±1.6	77±0.5	83±1.4	91±3.4	96±1.7
Mean	74.33±3.36		77.33±1.53	85±1.86	92±2.3	95.66±1.96
Metronidazole 150µg/ml	18.92		62.65	70	100	100
Control group	2		5	6	7	10

Table 3: Growth inhibition efficacy of CNPS on *Blastocystis* sizes (67% large, 20% medium, &13% small)

Tested dose		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
10µg/ml	C1	38±3.7	43±0.8	54±0.5	77±5.7	84±1.5
	C2	39±0.5	46±1.25	55±2.8	76±2.6	80±5.5
	C3	37±1.6	45±3.5	59±5.5	75±0.5	80±4.3
Mean	33.8±1.93		44.66±1.85	56±2.93	76±2.93	81.33±3.76
25µg/ml	C1	48±0.7	72±5.5	83±1.4	88±0.5	95±4.3
	C2	49±1.8	72±1.9	86±3.8	88±3.7	95±1.6
	C3	48±0.9	74±0.67	84±5.6	91±4.8	92±2.8
Mean	48.33±1.13		72.66±1.02	84.33±3.6	89±3	94±2.9
50 µg/ml	C1	67±0.8	84± 1.6	89 ± 1.5	92± 4.9	96± 1.6
	C2	68±2.5	83±2.4	90±3.2	93±2.2	98±2.8
	C3	68±3.5	84±5.4	90±1.45	92±1.9	97±5.9
Mean	67.66±2.26		83.66±3.13	89.66±2.05	92.33±3	97±3.43
100 µg/ml	C1	79± 2.6	85 ± 5.25	90 ± 0.5	96± 5.5	99±3.7
	C2	79± 1.7	88±0.5	96±2.8	96±4.3	100±4.8
	C3	77± 2.8	88±1.67	94±3.4	98±1.3	100±1.6
Mean	78.33±2.36		87±2.47	93.33±2.23	96.66±3.7	99.66±3.36
Metronidazole 150 µg/ml	18.92		62.65	70	100	100
Control group	2.5		4	6	6	15

Table 4: Growth Inhibition efficacy of chitosan on *Blastocystis* different sizes (30% large, 35% medium, & 35 % small)

Tested dose		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
10 µg/ml	C1	30±2.7	35±3.5	40±0.4	65±4.2	75±3.2
	C2	31±1.8	33±1.6	44±1.8	63±1.7	74±2.7
	C3	31±0.5	32±2.46	44± 2.9	62±2.9	73±1.5
Mean	30.66±1.66		33.33±2.52	42.66±1.7	63.33±2.93	74±2.47
25 µg/ml	C1	39±1.2	60±1.8	73±3.9	78±3.2	80±4.2
	C2	37±3.5	59±2.6	72±2.4	75±4.8	83±2.5
	C3	38±4.2	58±3.2	70±1.2	76±5.2	81±1.3
Mean	38±2.96		59±2.5	71.66±2.5	76.33±4.4	81.33±2.66
50 µg/ml	C1	51±1.7	69± 4.9	75 ± 0.9	80 ± 0.8	87 ± 4.6
	C2	54±3.3	68±1.2	76±4.3	81±2.3	85±1.7
	C3	50±5.3	66±2.5	72±1.89	79±4.5	85±5.4
Mean	51.66±3.43		67.66±2.86	74.33±2.36	80±2.53	85.66±3.9
100 µg/ml	C1	64±1.3	73 ± 2.6	78 ± 3.45	84 ± 4.3	94±1.9
	C2	66±2.5	72±4.25	77±1.7	85±1.2	96±1.7
	C3	63±5.6	70±1.56	75±4.9	86±0.9	97±3.46
Mean	64.33±3.13		71.66±2.8	76.66±3.35	85±2.13	95.66±2.35
Metronidazole150 µg/mL	18.92		62.65	70	100	100
Control group	2.5		4	6	6	15

Table 5: Growth inhibition efficacy of Chitosan on *Blastocystis* sizes (12% large, 22 % medium, &66% small).

Tested dose		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
10 µg/ml	C1	22±1.3	29±0.6	33±2.8	54±0.7	64±4.8
	C2	20±5.4	27±2.5	33±5.4	51±5.6	66±5.5
	C3	21±0.25	26±4.6	34± 3.7	52±3.5	63±2.8
Mean	21±2.31		27.33±2.56	33.33±3	52.33±3.26	64.33±4.36
25 µg/ml	C1	26±0.9	35±4.6	52±1.5	66±3.2	70±1.7
	C2	27±5.7	39±1.3	53±0.5	65±4.8	72±0.6
	C3	26±3.5	38±2.25	52±3.3	66±5.2	73±3.4
Mean	26.33±3.36		37.33±2.71	52.33±1.76	65.66±4.4	71.66±1.9
50 µg/ml	C1	31±5.0	46± 0.45	63±1.7	70±1.0	77±1.5
	C2	34±6.5	48±5.5	64±3.5	71±5.2	75±2.8
	C3	38±2.6	46±3.2	61±4.9	69±0.3	75±3.2
Mean	34.33±4.7		46.66±3.05	62.66±3.36	70±2.16	75.66±2.5
100 µg/ml	C1	44± 0.6	53 ± 1.4	68 ± 2.5	74±5.4	84±4.4
	C2	46± 1.4	52±5.5	66±3.3	72±0.8	86±5.0
	C3	46± 3.5	50±3.6	66±5.5	73±4.0	87±1.6
Mean	45.33±1.83		51.66±3.5	66.66±3.76	73±3.4	85.66±3.66
Metronidazole150 µg/ml	18.92		62.65	70	100	100
Control group	2.5		4	6	6	15

Table 6: Growth inhibition efficacy of chitosan on *Blastocystis* large sized cysts (70% large, 11% medium, &19 % small)

Tested dose		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
10 µg/ml	C1	38±0.9	45±0.8	48±5.3	75±1.5	84±5.6
	C2	39±5.6	43±2.25	50±4.4	76±0.9	84±3.5
	C3	36±3.25	44±5.6	52± 0.9	77±5.7	83±4.2
Mean	37.66±3.25		44±2.83	50±3.53	76±2.93	83.66±4.3
25 µg/ml	C1	44±2.6	66±3.3	77±1.4	84±2.0	90±0.5
	C2	47±4.2	66±1.4	78±3.2	82±1.5	88±1.3
	C3	48±1.5	65±5.3	79±4.9	84±5.7	89±4.6
Mean	46.33±2.76		65.66±3.3	78±3.16	83.33±4.4	89±3.33
50 µg/ml	C1	61±0.5	75± 2.3	79±0.5	85±0.4	95±0.7
	C2	58±1.4	75±4.5	79±3.5	86±2.4	95±2.9
	C3	59±4.7	74±5.25	82±2.6	87±5.3	96±4.7
Mean	59.33±2.2		74.66±4.01	80±2.2	86±2.7	95.33±2.76
100 µg/ml	C1	72± 2.4	77 ±1.8	84 ±1.5	89±1.25	98±0.8
	C2	75± 4.7	78±3.5	87±0.4	89±2.3	98±2.4
	C3	76± 3.3	80±2.6	85±3.45	90±5.6	98±5.6
Mean	74.33±3.46		78.33±2.63	85.33±1.78	89.33±3.05	98 ±2.93
Metronidazole150µg/ml	18.92		62.65	70	100	100
Control group	2.5		4	6	6	15

Table 7: Variation in LD_{50} & LD_{100} of CNPS to *Blastocystis* inoculum sizes.

Efficacy of CNPS	Conc. of tested materials	LD_{50}	LD_{100}
Blastocystis different sizes	10 µg/ml CNPS	7hrs	- - -
	25 µg/ml CNPS	1.5hrs	- - -
	Metronidazole 150µg/ml	2.5hrs	12hrs
Blastocystis mostly small in size.	10 µg/ml CNPS	12hrs	- - -
	25 µg/ml CNPS	2hrs	- - -
	Metronidazole 150µg/ml	2.5hrs	12hrs
Blastocystis mostly large in size.	10 µg/ml CNPS	5hrs	- - -
	25 µg/ml CNPS	1.15hrs	- - -
	Metronidazole 150µg/ml	2.5hrs	12hrs

Table 8: Variation in LD_{50} & LD_{100} of Chitosan to *Blastocystis* inoculum sizes.

Efficacy of chitosan	Conc. of tested materials	LD_{50}	LD_{100}
Blastocystis different sizes.	10 µg/ml chitosan	8hrs.	- - -
	25 µg/ml chitosan	2hrs.	- - -
	Metronidazole150µg/ml	2.5hrs.	12 hrs.
Blastocystis mostly small in size.	10 µg/ml chitosan	15hrs.	- - -
	25 µg/ml chitosan	6hrs.	- - -
	50 µg/ml chitosan	3.30hrs.	- - -
	100 µg/ml chitosan	2.15hrs.	- - -
	Metronidazole 50µg/ml	2.5hrs	12 hrs.
Blastocystis mostly large in size.	10 µg/ml chitosan	5.5hrs.	- - -
	25 µg/ml chitosan	hr.	- - -
	Metronidazole 50µg/ml	2.5hrs	12 hrs.

Table 9: Comet mean parameters and level of damage in DNA of *Blastocystis* exposed for 24hrs to different concentrations of CNPS compared control non-exposed *Blastocystis* and Metronidazole 150µg/ml.

Concentration	Mean mortality	DNA damage	Tail length (µg)	DNA in tail (µg)	Tail moment (µg)	Olive moment
Mean (1)	15%	16.76%	8.49	8.52	0.796	0.946
Mean (2)	80%	20.23%	8.83	10.6	0.87	0.98
Mean (3)	98.33%	20.76%	12.13	10.91	1.001	1.29
Mean (4)	100%	21.43%	15.625	11.48	1.18	1.7

Discussion

Blastocystis spp. is the commonest eukaryotic organism in human fecal samples (Salehi *et al,* 2017). In Egypt, the *Blastocystis* prevalence varied in different governorates. In Cairo, prevalence was 34.5% (El Deeb and Khodeer, 2013), in Dakahlia prevalence was 22.4% (El-Shazly *et al,* 2006), in Ismailia was 33.3-35.7% (Mokhtar and Youssef, 2018), in Gharbia was 53% (El-Marhoumy *et al,* 2015), in Alexandria rates varied between 67.4%, 52% &54.17% respectively (Eassa *et al,* 2016; Elsayad *et al,* 2019; Mossallam *et al,* 2021), in Sohag was 16.7% (El-Nazer *et al,* 2017), in Sharkia was 42.3% (Farghaly *et al,* 2017), in Beni-Suef was 53.6% (Hamdy *et al,* 2020) and in Kafr El-Sheikh was 39% (Abdo *et al,* 2021).

Metronidazole was considered the drug of choice alone or in combination with other drugs (Adao and River, 2018), it showed wide efficacy rates, and drug resistance as well as large doses caused many side effects (Rajamanikam *et al,* 2019). Besides, sometimes metronidazole in large doses didn't eradicate of the parasite (Stensvold *et al,* 2008).

Treatment of *Blastocystis* is a matter of debate due to its undefined pathogenicity, and nanoparticles are novel treatment as immune system stimulant drug with successful promising outcomes (Sarkar *et al,* 2022). Chitosan nanoparticle was used as one of the best nano-carriers in antibacterial, antifungal, and anti-parasitic drugs (Pan *et al,* 2019; Chabra *et al,* 2019). Chitosan had been widely studied as carrier to deliver biological curative nanoparticles to considerably increase their effects (Roshdy *et al,* 2019).

The current study showed a direct relation between the increase in the concentration of Chitosan, exposure time and increase in the rate of mortality. Exposing of *Blastocystis* to Chitosan 50µg/ml for 3hrs caused mortality rate of 67.66% that increased to 85.66% after 24hr. By increasing the concentration of Chitosan from 10µg/ml to 100µg/ml for 3hrs exposure, the mortality increased from 33.33% to 71.66%. This agreed with Rahimi-Esboei *et al*. (2013), who tested chitosan against Hydatid cysts *in vitro*. His results had signified that with the increase of chitosan concentration and exposure time, the viability of hydatic cysts decreased *in vitro*. The chitosan 50 µg/ml had a scolicidal effect of 89 % after 3 hours of exposure time and by increasing the concentration of chitosan to 100µg/ml it had 93% scolicidal effect after 3 hours of exposure time. Also, it agreed with Silva *et al*. (2021), who used chitosan oligosaccharides**,** while using the concentration of 100µg/ml of chitosan oligosaccharides exhibited inhibition by 19% against *Trypanosoma cruzi in vitro* after 72 hrs. By increasing the exposure time to 144 hours using the same 100µg/ml, mortality rate of 66.18%. Also, Teimouri ***et*** *al*. (2016) tested chitosan *in vivo* on survival time of mice infected with *Plasmodium berghei* using 10, 20, 40 & 80 mg/kg concentrations of chitosan which showed that as the concentration of chitosan increased the survival rate of mice was decreased.

In the present study, the maximum mean mortality rate with chitosan 100µg/mL was 95.66% after 24 hours exposure on an inoculum of variable sized *Blastocystis* cysts. The maximum mean morality rate was increased and reached 98% with large sized cysts using the same concentration and same exposure time.

The antiprotozoal activity of chitosan was also tested by other authors against *Leishmania infantum*. Chitosan showed mean mortality rate 100% growth of promastigotes at concentrations equal or superior to 1000µg/ml of chitosan (Salah *et al*, 2015). In contrast, C-6 oxidized chitosan derivative obtained from chemical chitosan oxidation that had been destroyed by enzymes systems such as endocellulase, hyaluronidase, hyaluronate lyase, chitinase, and other proteins, exhibited low antileishmanial against *L. infantum* **(**Salah *et al,* 2015). Yarahmadi *et al.* (2016) assessed the effect of chitosan on the viability of *Giardia lamblia* cysts that resulted in 100 % mortality rate after 180min of exposure at 400µg/ml of chitosan. Also, Tavassoli *et al*. (2012) found that 1250µg/ml of chitosan after 360 min exposure time completely inhibited the viability of *Trichomonas gallinae*.

Regarding Chitosan nanoparticles, the present study showed a direct proportional relation between the growth inhibition rates of *Blastocystis* when increasing the concentrations and exposure duration. Exposing *Blastocystis* to CNPS 10 µg/ml for 1 hour revealed mean mortality rate of 33.66 % which was increased to 80 % after 24 hours exposure time using the same concentration. The mean mortality rate increased with increasing the concentration from 10µg/ml to 100µg/ml of CNPS from 33.66% to 74.33%

respectively after 1hr exposure. This agreed with Zaki *et al*. (2022), who found that increased concentration and exposure time of CNPS 20µg/ml on *Blastocystis* gave a significant growth inhibition of 56.4% & 75.6% after 24hrs & 72hrs exposure times. CNPS 40µg/ml showed mortality rate of 73.1% & 93.8 % after 24 & 72hrs exposure time. Also, there was a significant decrease in parasites to 99.2% by 50µg/ml CNPS for 72hrs.

In the present study, the maximum mean mortality rate was 99.66 % when 100µg/ml CNPS was tested against an inoculum of mostly large sized cysts after 24hrs exposure time. This agreed with Taji *et al*. (2022), who studied the in vitro effect of CNPS with (75, 50, 25, & 12.5µg/ml), against *Blastocystis* viability after 24 & 48hrs, found that growth inhibition significantly increased to 85.33% with 12.5µg/ml to 100% with 75µg/ml. They added that lower CNPS concentration caused 100% growths inhibition due to long exposure time of 48 hours.

In the present study showed that CNPS had higher efficacy than chitosan. The LD_{50} of 10 µg/ml chitosan and 10µg/ml CNPS was 8hrs for chitosan and 7hrs for CNPS. The efficacy by comparing the mortality rate using 100µg/ml for both chitosan and CNPS after 24hrs caused increased from 95.66% to 98.33% respectively. This agreed with Elmi *et al.* (2021), who evaluated CNPS efficacy versus chitosan in a concentration dependent manner against *Plasmodium falciparum, Giardia lamblia and Trichomonas vaginalis,* found that the efficiency of the tested materials was dose dependent. The more the nano-compound concentration, the more the protozoa elimination rates as compared with chitosan with the same concentrations and exposure time. Afzal *et al.* (2019) used Mannosylated thiolated chitosan loaded poly nanoparticles for oral visceral leishmaniasis therapy on *Leishmania donovani* amastigotes, found that there was increased by 36-fold with the in vivo evaluation of antileishmanial activity reduction of parasite burden of infected BALB/c mice as compared to free PM. Also, Loiseau *et al.* (2020) used different chitosan nanoparticles formulations for visceral and cutaneous leishmaniasis *in vivo and in vitro* reported promising outcomes.

In the present study, the large sized *Blastocystis* cysts were more affected by the tested materials. Chitosan on different sized *Blastocystis* cysts, LD_{50} was reached after 8 & 2hrs at 10 µg/ml and 25µg/ml respectively. For small sized cysts, LD_{50} was reached after 15hrs, 6hrs, 3.30hrs & 2.15hrs with 10µg/ml, 25µg/ml, 50µg/ml & 100µg/ ml respectively. However, 1.5hrs ET for large sized cysts was reached with chitosan 10µg/ml ET &1hr ET for chitosan 25µg/ml. But, it failed to reach LD_{100} mortality against small sized cysts, but LD_{100} on different sized cysts reached 98% by100µg/ml after 24hrs ET. LD_{50} of 10µg/ml chitosan was reached after 15hrs against small sized cysts and was reached after 5.30 hrs against large sized cysts. However, LD_{50} of 10µg/ml CNPS was reached after 12hrs against small sized cysts and reached after 5hrs against large sized cysts. This agreed with Yaicharoen *et al*. (2006), who divided *Blastocystis* small sized cysts ranged from 1-10µm, medium sized cysts ranged from 11-20µm and large sized ones bigger than 21µm. Padukone *et al*. (2018) reported some evident variations in cysts size in cultures with a huge size of vacuoles ranged from 5 to 150µm.

In the present study, there was a high susceptibility of large cysts to the tested materials than small sized cysts which might be due to increase of the cysts surface area and increase in the number of negatively charged receptors on the cell wall. This agreed was Nagy *et al*. (2011), who declared that the permeability of the cell membrane of the cysts played an important role in increasing its the susceptibility to the drugs used.

In the present study, DNA damaged *Blastocystis* exposed to different Chitosan NPs concentrations after 24hrs in form of differences in DNA% in the tail segment that reached 20.76 % with CNPS 100µg/ml, tail length was fluctuating from 8.4µm with pos-

itive control to 8.8µm with CNPS 10 µg/ml, 12.13µm with CNPS 100µg/ml or 15.625µm damaged with Metronidazole 150µg/ml, tail moment varied from 0.796µm with positive control to 1.001 with CNPS 100µg/ml, and olive tail moment reported by migration of DNA fragments by agarose gel electrophoresis. Also, there was a significant difference as compared with data of positive control that showed variation degrees in different DNA parameters indicating the DNA damage increased with the increase of tested materials concentrations. This agreed with Kumar *et al.* (2015) and Attaullah *et al.* (2020), they assessed the DNA damage in dead *Trichinella spiralis* exposed to silver NPs at different concentrations for 12hrs versus positive control by using Comet assay. Ahmed *et al.,* (2023) used comet this technique as well in assessment of *Artemisia judaica* against of *Cryptosporidium parvum* oocysts showed DNA genotoxic damage.

In the present study, TEM of positive control showed that the vacuoles were round or oval in shape with a central vacuole and thin rim of cytoplasm with a dense opaque nucleus at one pole. This agreed with Zhang *et al*. (2012) and El Sayed *et al*. (2019), they reported that the vacuolar form as one of the common forms in fecal samples and culture, as oval in shape, with a central vacuole and scanty fine granules surrounded by the peripheral rim of cytoplasm and single nucleus.

In the present study, the CNPS 100µg/ml for 24hrs exposure was highly lethal against *Blastocystis* cysts that showed cytoplasmic vacuoles with necrotic cells with disruption of normal morphology and alteration of outer surface coat, plasma membrane rupture with loss of intracellular contents and destruction of central vacuole. This agreed with Mossallam *et al.* (2021), who found that the simeprevir affected *Blastocystis,* which central vacuole lacked any electro dense particles and some cysts showed ruptured plasma membrane with loss of all cellular contents.

Conclusion

The outcome data proved the potentially effective of chitosan and chitosan NPs on treating *Blastocystis hominis,* but chitosan NPs was the better one

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References

Abdo, SM, El-Adawy, H, Farag, HF, El-Taweel, HA, Elhadad, H, *et al,* 2021: Detection and molecular identification of *Blastocystis* isolates from humans and cattle in northern Egypt. J. Parasit. Dis. 45, 3:738-45.

Adao, DEV, Rivera, W. L, 2018: Recent advances in *Blastocystis* sp. research. Philipp Sci. Lett. 11:39-60.

Adetunji, CO, Inobeme, A, Olaniyan, OT, Anani, OA, Oloke, JK, *et al,* 2022: Application of biosurfactant for the management of *Plasmodium* parasites. In: Green Sustainable Process for Chemical and Environmental Engineering and Science. Academic Press.

Afzal, I, Sarwar, HS, Sohail, MF, Varikuti, S, Jahan, S, *et al,* 2019: Mannosylated thiolated paromomycin-loaded PLGA nanoparticles for the oral therapy of visceral leishmaniasis. Nanomed. 14, 4:387-406.

Ahmed, SA, Eltamany, EE, Nafie, MS, Elhady, SS, Karanis, P, *et al,* 2023: Anti-*Cryptosporidium parvum* activity of *Artemisia judaica* L. and its fractions: In vitro and in vivo assays. Front. Microbiol. 14:1193810. doi: 10.3389/fmicb.1193810.

Attaullah, MKZ, Zahoor, MA, Mubarik, MS, Rizvi, H, Majeed, HN, *et al,* 2020: Insecticidal, biological and biochemical response of *Musca domestica* (Diptera: Muscidae) to some indig enous weed plant extracts. Saudi J. Biol. Sci. 27: 106-16.

Boorom, KF, Smith, H, Nimri, L, Viscogliosi, E, Spanakos, G, *et al,* 2008: Oh my aching gut: irritable bowel syndrome, *Blastocystis,* and asymptomatic infection. Parasit. Vectors 1, 1:1-16.

Chabra, A, Rahimi-Esboei, B, Habibi, E, Monadi, T, Azadbakht, M, *et al,* 2019: Effects of some natural products from fungal and herbal sources on *Giardia lamblia* in vivo. Parasitology 146, 9:1188-98.

Clark, CG, van der Giezen, M, Alfellani, MA, Stensvold, CR, 2013: Recent developments in *Blastocystis* research. Adv. Parasitol. 82:1-32.
Eassa, SM, Ali, HS, El Masry, SA, Abd El-Fattah, AS, 2016: *Blastocystis hominis* among immunocompromised and immunocompetent children in Alexandria, Egypt. Ann. Clin. Lab. Res. 4, 2:92-8
El Deeb, HK, Khodeer, S, 2013: *Blastocystis* spp.: Frequency and subtype distribution in iron defiency anemic versus non-anemic subjects in Egypt. J. Parasitol. 99, 4:599-602.
El-Badry, AA, Abd El Wahab, WM, Hamdy, DA, Aboud, A, 2018: *Blastocystis* subtypes isolated from irritable bowel syndrome patients and co-infection with *Helicobacter pylori*. Parasitol. Res. 117:127-37.
El-Marhoumy, SM, Abd El-Nouby, K, Shoheib, ZS, Salama, AM, 2015: Prevalence and diagnostic approach for a neglected protozoon *Blastocystis hominis*. Asian Pacif. J. Trop. Dis. 5, 1:51-9.
Elmi, T, Rahimi Esboei, B, Sadeghi, F, Zamani, Z, Didehdar, M, *et al*, 2021: In vitro antiprotozoal effects of nano-chitosan on *Plasmodium falciparum, Giardia lamblia* and *Trichomonas vaginalis*. Acta Parasitol. 66, 39-52.
El-Nazer, MA, Shatat, MA, Omran, EK, Ali, NM, Ibrahim, MA, *et al*, 2017: *Blastocystis hominis* infection and its relation with other intestinal parasites among outpatients, Sohag, Egypt. Sohag Med. J. 21, 3:429-36.
Elsayad, MH, Tolba, MM, Argiah, HA, Gaballah, A, Osman, MM, *et al*, 2019: Electron microscopy of *Blastocystis hominis* and other diagnostic approaches. JESP 49, 2:373-80 .
El-Sayed, NM, Ramadan, ME, 2017: The impact of intestinal parasitic infections on the health status of children: an overview. J. Pediatr. Infect. Dis. 12, 04:209-13.
El-Shazly, AM, Awad, SE, Sultan, DM, Sadek, GS, Khalil, HH, *et al,* 2006: Intestinal parasites in Dakahlia Governorate, with different techniques in diagnosing protozoa. J. Egypt. Soc. Parasitol. 36, l3:1023-34.
arghaly, A, Hamza, RS, Abd El-Aal, NF, Metwally, S, Farag, SM, 2017: Prevalence, risk factors and comparative diagnostic study between immunofluorescence assay and ordinary staining techniques in detection of *Blastocystis hominis* in fecal samples. JESP 47, 3:701-8.
Graham, L, Orenstein, JM, 2007: Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat. Protocols 2, 10:2439-50.
Hamdy, DA, Abd El Wahab, WM, Senosy, S A, Mabrouk, AG, 2020: *Blastocystis* spp. and *Giardia intestinalis* co-infection profile in children suffering from acute diarrhoea. J. Parasit. Dis. 44:88-98.
Jones, MS, Whipps, CM, Ganac, RD, Hudson, NR, Boroom, K, 2009: Association of *Blastocystis* subtype 3 & 1 with patients from an Oregon community presenting with chronic gastrointestinal illness. Parasitol. Res. 104:341-5.
Kumar, A, Sharma, S, Verma, G, 2015: Insecticidal and genotoxic potential of *Acorus calamus* rhizome extract against *Drosophila melanogaster*. Asian J. Pharm. Clin. Res. 8:113-6.
Lim, C, Hwang, DS, Lee, DW, 2021: Intermolecular interactions of chitosan: Degree of acetylation and molecular weight. Carbohydr. Polym. 1, 259:1177-82.
Loiseau, PM, Pomel, S, Croft, SL, 2020: Chitosan contribution to therapeutic and vaccinal approaches for the control of leishmaniasis. Molecules 25, 18:4123-9.
Mokhtar, A, Youssef, A, 2018: Subtype analysis of *Blastocystis* spp. isolated from domestic mammals and poultry and its relation to transmission to their in-contact humans in Ismailia Governorate, Egypt. PUJ 11, 2:90-8.
Mossallam, SF, El-Mansoury, SA, Tolba, MM, Kohla, AA, Khedr, SI, 2021: In vitro susceptibility of human *Blastocystis* subtypes to simeprevir. Saudi J. Biol. Sci. 28, 4:2491-501.
Nagy, A, Harrison, A, Sabbani, S, Munson, Jr RS, Dutta, PK, *et al,* 2011: Silver nanoparticles embedded in zeolite membranes: Release of silver ions and mechanism of antibacterial action. Inter. J. Nanomed.18:33-52.
Padukone, S, Mandal, J, Rajkumari, N, Bhat, BV, Swaminathan, RP, *et al,* 2018: Detection of *Blastocystis* in clinical stool specimens using three different methods and morphological examination in Jones' medium. Trop. Parasitol. 8, 1: 33-40.
Pan, C, Qian, J, Fan, J, Guo, H, Gou, L, *et al,* 2019: Preparation nanoparticle by ionic crosslinked emulsified chitosan and its antibacterial activity. Colloids and surfaces A: Physicochem. Engineer. Aspects 568:362-70.
Rahimi-Esboei, B, Fakhar, M, Chabra, A, Hosseini, M, 2013: In vitro treatments of *Echinoc-*

occus granulosus with fungal chitosan, as a novel biomolecule. Asian Pacif. J. Trop. Biomed. 3, 10:811-5.

Rajamanikam, A, Hooi, HS, Kudva, M, Samudi, C, Kumar, S, 2019: Resistance to metronidazole in *Blastocystis* sp.: A pathogenic consequence. PloS One 14, 2:e0212542.

Roshdy, NN, Kataia, EM, Helmy, NA, 2019: Assessment of antibacterial activity of 2.5% NaOCl, chitosan nano-particles against *Enterococcus faecalis* contaminating root canals with and without diode laser irradiation: An in vitro study. Acta Odontol. Scand. 77, 1:39-43.

Said, DE, Elsamad, LM, Gohar, YM, 2012: Validity of silver, chitosan and curcumin nanoparticles as anti-*Giardia* agents. Parasitol. Res. 111, 2:545-54.

Salah-Tazdaït, R, Tazdaït, D, Harrat, Z, Eddaikra, N, Abdi, N, *et al,* 2015: Antiparasite activity of chitosan. In: Proceed. Inter. Conf. Chemical, Metallurgy and Environmental Engineering, Istanbul, Turkey.

Salehi, R, Haghighi, A, Stensvold, CR, Kheirandish, F, Azargashb, E, *et al,* 2017: Prevalence and subtype identification of *Blastocystis* isolated from humans in Ahvaz, South-western Iran. Gastroenterol. Hepatol. Bed. Bench 10, 3: 235-41 .

Sarkar, B, Mahanty, A, Gupta, SK, Choudhury, AR, Daware, A, *et al,* 2022: Nanotechnology: A next-generation tool for sustainable aquaculture. Aquaculture 546:737330.

Sekar, U, Shanthi, M, 2013: *Blastocystis* consensus of treatment and controversies. Trop. Parasitol. 3, 1:35-9.

Shirvani, G, Fasihi-Harandi, M, Raiesi, O, Bazargan, N, Zahedi, MJ, *et al,* 2020: Prevalence and molecular subtyping of *Blastocystis* from patients with irritable bowel syndrome, inflammatory bowel disease and chronic urticaria in Iran. Acta Parasitol. 65, 1:90-6.

Silva, NSD, Araújo, NK, Daniele-Silva, A, Oliveira, JWDF, Medeiros, JMD, *et al,* 2021: Antimicrobial activity of chitosan oligosaccharides with special attention to anti-parasitic potential. Marine Drug, 19, 2:110.

Stensvold, CR, Arendrup, MC, Nielsen, HV, Bada, A, Thorsen, S, 2008: Symptomatic infect ion with *Blastocystis* sp. subtype 8 successfully treated with the trimethoprim-sulfamethoxazole. Ann. Trop. Med. Parasitol. 102, 3:271-4.

Sun, Y, Chen, D, Pan, Y, Qu, W, Hao, H, *et al,* 2019: Nanoparticles for antiparasitic drug delivery. Drug Deliv. 26, 1:1206-21.

Taji, R, Sadraei, J, Pirestani, M, Bahadory, S, 2022: Investigating the effect of silver, chitosan, and curcumin nanoparticles on *Blastocystis* spp. and comparing it with metronidazole In Vitro. Inter. J. Enter. Pathog. 10, 4:125-8.

Tavassoli, M, Imani, A, Tajik, H, Moradi, M, Pourseyed, SH, 2012: Novel in vitro efficiency of chitosan biomolecule against *Trichomonas gallinae*. Iran, J. Parasitol. 7, 1:92.

Teimouri, A, Haghi, AM, Nateghpour, M, Farivar, L, Hanifian, H, *et al,* 2016: Antimalarial efficacy of low molecular weight chitosan against *Plasmodium berghei* infection in mice. J. Vector Borne Dis. 53, 4:312.

Vdovenko, AA, Williams, JE, 2000: *Blastocystis hominis*: Neutral red supravital staining and its application to in vitro drug sensitivity testing. Parasitol. Res. 86, 7: 573-81 .

Yaicharoen, R, Ngrenngarmlert, W, Wongjindanon, N, Sripochang, S, Kiatfuengfoo, R, 2006: Infection of *Blastocystis hominis* in primary schoolchildren from Nakhon Pathom Province, Thailand. Trop. Biomed. 23, 1:117-22.

Yakoob, J, Jafri, W, Jafri, N, Islam, M, Asim, BM, 2004: In vitro susceptibility of *Blastocystis hominis* isolated from patients with irritable bowel syndrome. Br. J. Biomed. Sci. 61, 2:75-7.

Yarahmadi, M, Fakhar, M, Ebrahimzadeh, M A, Chabra, A, Rahimi, B, 2016: The anti-giardial effectiveness of fungal and commercial chitosan against *Giardia intestinalis* cysts in vitro. J. Parasit. Dis. 40, 1:75-80 .

Younis, MS, Abououf, E, Ali, A, Abd Elhady, SM, Wassef, RM, 2020: In vitro Effect of Silver Nanoparticles on *Blastocystis hominis*. Inter. J. Nanomed. 15, 8167-73

Zaki, WM, Salem, HA, Madkhaly, AM, Oshiba, SF, 2022: The in vitro activity of chitosan nanoparticles versus metronidazole against *Blastocystis hominis*. JESP 52, 3:501-8.

Zhang, X, Zhang, S, Qiao, J, Wu, X, *et al,* 2012: Ultrastructural insights into morphology and reproductive mode of *Blastocystis hominis.* Parasitol. Res. 110:1165-72

Explanation of figures

Fig. 1: *Blastocystis hominis* with its signet ring appearance of nuclei after applying iodine stain (arrow).

Fig. 2: *Blastocystis* viable cyst on right side, vacuolar stained red (star) & dead one on left side, disintegrated, pale with irregular contour (arrow) (Neutral red X 400).

Fig. 3: *Blastocystis* left one living vacuolar stained red and sharply demarcated (star) & right dead *one* (arrows) faint in color with less demarcated contour (Neutral red X400).

Fig. 4: TEM cultured positive control, *Blastocystis* vacuolar with a central vacuole and electron dense organelle like structures surrounded by thin rim of cytoplasm with condensed glycogen, thick febrile surface coat.

Fig. 5: TEM of drug treated *Blastocystis* showed necrotic cells with cytoplasmic vacuolations with central vacuole membrane destruction.

Fig. 6: TEM of drug treated *Blastocystis* revealed damaged cell with loss of outer surface regularity.

Fig. 7: Comet assay, after 24hrs exposure showed damage in DNA: 1- Positive control showed sound not destroyed (DNA), 2- showed low level of damaged DNA in *Blastocystis* exposed to chitosan nano-particles (10µg/ml), 3- showed a more damaged level of DNA damage in *Blastocystis* exposed to CNPs 100µg/ml, & 4- Metronidazole 150µg/ml showed a high damaged level of DNA damage in *Blastocystis* .