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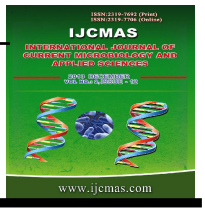

Original Research Article

Effect of Green Tea Extract and Cysteine Proteases Inhibitor (E-64) on Symptomatic Genotypes of *Blastocystis hominis in vitro* and in Infected Animal Model

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Keywords

Blastocystis hominis; E-64; green tea extract; viability; ultra-structures; *in vitro; in vivo.*

A B S T R A C T

Blastocystis hominis is an anaerobic enteric protozoon that inhabits the human intestinal tract. The effects of green tea extract (GTE) and E-64 on the growth and viability of three *B. hominis* symptomatic genotype subtypes (I, III and IV; four isolates per each genotype/subtype) were assessed *in vitro and in vivo*. All isolates showed susceptibility to both of GTE and E-64 *in vitro*. The cytocidal effects of GTE and E-64 were observed after 24 h at concentration ranged 20 - 35 and 25 – 35 mg/l while ranged 15-30 and 20 - 30 mg/l after 72 h, respectively. In addition, the ultra-structures of GTE and E-64 treated *B. hominis* by transmission electron microscopy demonstrated increase in size, cytoplasmic membrane injury and disposition of vacuolated nonhomogenous particles in the cytoplasm. Moreover, nucleus shifted periphery, expulsion of the cytoplasmic content then cell rupture were detected suggesting necrotic changes. The results of the *in vivo* experiments showed that, the cyst output numbers in GTE and E-64 treated infected rats were less than the number of cysts output in infected non treated rats groups and the duration of infection decreased in both of the majorities of treated rats at the first six days. The differences were statistically non significant between both groups. In conclusion, GTE and E-64 were useful in treatment of infected rats and increased the host resistance to *B. hominis*. These two drugs could be utilized as alternatives to metronidazole especially against resistant isolates

Introduction

Blastocystis hominis (*B. hominis*) is an anaerobic enteric protozoan that inhabits the human intestinal tract. Despite the fact that *B. hominis* was discovered almost 100 years ago, its clinical significant, pathogenicity and usefulness chemotherapeutic intervention remain unresolved (Stensvold *et al*., 2009).

Several studies have linked *B. hominis* with various gastrointestinal and extra-intestinal symptoms including diarrhea and abdominal pain in both immunocompetant and immune compromized hosts (Cirioni *et al*., 1999), irritable bowel syndrome (Hussein *et al*., 2008), chronic urticaria (Hameed *et al*., 2011) and palmoplantar pruritus (Kick *et al*., 2002). Moreover, the causative relation between *B. hominis* and cancer colon was proved *in vitro* cell lines (Chandramathi *et al*., 2010) and experimentally *in vivo* (Hussein *et al*., 2008). Previous genetic analysis of *B. hominis* showed that certain subtypes were predominant among symptomatic, others were among asymptomatic and some of them sharing between symptomatic and asymptomatic (Hussein *et al*., 2008; Stensvold *et al*., 2009).

The pathogenicity of *B. hominis* includes increasing epithelial permeability, inducing apoptosis of host intestinal epithelial cells and disruption of epithelial barrier function (Puthia *et al*., 2006; Hussein *et al*., 2008), modulation of immune response and cytokine release from colonic epithelial cells (Puthia *et al*., 2008). Recently, oxidative damage in rats inoculated with human *B. hominis* was reported (Chandramathi *et al*., 2009). Although metronidazole (MTZ) is the drug of choice to treat *B. hominis* infection, up to 40% resistance to such antimicrobial agent was recorded (Haresh *et al*., 1999; Yakoob *et al*., 2004). Moreover, MTZ was associated with cancer and undesirable secondary effects (Sangster *et al*., 2002). Recently extensive variations in drug sensitivities among two *B. hominis* subtypes 4 and 7 were recently published by Mirza *et al* (2011). Therefore, seeking out of novel effective agents is urgently required.

Cysteine proteases are essential for life cycle and pathogenicity of many parasites (Sajid and McKerrow 2002). E-64 (*N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine), a broad spectrum cysteine protease inhibitor, has been proposed as a possible therapeutic agent due its potent inhibitory activity, stability and permeability into cells and tissues (Powers *et al*., 2002). In addition, many studies showed that, E-64 inhibited the growth of different parasites in some biological experimental studies such as fascioliasis (Alacala-Canto *et al*., 2006), amoebiasis (Olivos-Garcia *et al*., 2004) and giardiasis (Hussein *et al*., 2009).

The use of medicinal plants by people in developing countries is popular because these products are safe, widely available at low cost and easy access as reported by Sawangjaroen and Sawangjaroen (2005) who tested five anti-diarrheic Thai medicinal plants against *B. hominis in vitro* and they found that the dichloromethane and methanol extracts of Acacia catechu resin and Quercus infectoria nut gall showed highest activity. The green tea polyphenols and its constitute epigallocatechingallate have a several effects such as inhibition of proteases and proteasome function, anti-inflammatory, cell cycle regulation, tumor invasion and to reducction oxygen-derived free radicals beside the anti-apoptotic effect on tumor cells (Tobi *et al*., 2002; Buttemeyer *et al*., 2003). Recently, anti adhesive effects of green tea extract (GTE) has been detected against many pathogens (Lee *et al*., 2009). Although many studies reported the inhibitory effects of GTE on bacteria and viruses, limited reports investigated its effect on parasites (Karori *et al*., 2008; Sharma *et al*., 2007; Hellmann *et al*., 2010).

The aim of the current study was to assess the effect of GTE and E-64 on twelve isolates of three *B. hominis* symptomatic genotype subtypes (I, III and IV) *in vitro* and in infected rats.

Materials and Methods

Sources of human clinical isolates

B. hominis were obtained from fecal samples of 12 gastrointestinal symptomatic patients and negative for others organisms and their genotype subtypes were previously identified in our laboratory (Hussein *et al*., 2008) to subtype I, III and IV. Metronidazole (MTZ) resistance isolates were considered when the dose 10 mg/l had no effect on *B. hominis* (Haresh *et al*., 1999). According to MTZ susceptibility/resistance, one isolate per each genotype/subtype showed resistance and the other nine isolates showed different degrees of susceptibilities with cytocidal effects ranged from 12.5 - 100 mg/l after 24 h and 6.25 - 50 mg/l after 72 h.

Green tea extraction

Aqueous Chinese green tea (Twinings, UK) extract was prepared according to Aboulmagd *et al*., 2011.

In vitro effect of GTE and E-64 on the growth and viability of *B. hominis* vacuolar form

Each stool sample containing *B. hominis* was cultivated in 3 ml of Jones' medium without rice starch and supplemented with 10% horse serum (Leelayoova *et al*., 2002). The culture was incubated at 37^{o}C and monitored daily for 96 hours by light microscopy after incubation of culture tubes at 37°C with 5% CO_2. Cultures were considered suitable for drug testing when *B. hominis* number/ml exceeded $1x10^3$ vacuolar forms. According to the method of Vdovenko and Williams (2000), GTE and E-64 were tested against twelve similar experiments' groups (one/isolate). Each experiment included different drug concentrations subgroups.

Each experiment subgroup was represented by 3 cultures tubes. Beside negative control group included *B. hominis* inoculated free Jones' medium. GTE was used at the following concentrations: 5, 10, 15, 20, 25, 30 and 35 mg/l while E-64 was used at 10, 15, 20, 25, 30 and 35 mg/l. The examinations were done after 24 and 72 h. The effect of different drugs on *B. hominis* was determined by detecting the cytostatic and cytocidal effect to all active form (vacuolar or central body) of the *B. hominis* by assessment of the viability of *B. hominis* which was evaluated using neutral red dye.

The cytostatic effect was considered when there was a reduction of *B. hominis* count in comparison to control while the cytocidal effect was defined as the lowest concentrations of GTE and E-64 lethal to all the vacuolar forms that were confirmed by no stained organism with neutral red and no growth 72 h later had been confirmed when 100 µl of the samples transplanted into fresh medium (Vdovenko and Williams, 2000). The inhibitory concentrations (IC_{50} and IC_{90}) for each drug were determined against each isolate genotype/subtype. They represent the concentrations of the drug that induced a 50% and 90% reduction in viable *B. hominis* in comparison to control. The number of *B. hominis* in each group at each time point was determined from the representative three culture tubes.

Transmission electron microscopy *in vitro* study

The effect of GTE and E-64 at IC_{90} was tested against one isolate of genotype/subtype I. One ml of cultured *B. hominis* vacuolar form was incubated in presence and absence of GTE or E-64 and harvested after 24 h according to Nasirudeen *et al* (2004). The cells were washed twice in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose and fixed in each of the following fixatives: 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h, followed by two washes in buffer; 2% osmium tetra oxide (OsO4) in 1% potassium ferrocyanide solution for 1 hr and two washes in 0.5 M sodium cacodylate buffer. The cells were processed (Moe *et al*., 1996) and examined by Joel 1210 TEM.

***In vivo* study**

Thirty six orally infected four-week-old male western rats inoculated with $4x10^7$ of the vacuolar form of *B. hominis* in 4-day-old axenic culture were used in each drug tested group according to Yoshikawa *et al* (2004). In each drug tested they were sub-divided into 12 subgroups according to isolate used (3 rats/each). In addition, one control group included 3 rats were inoculated by $4x10^7$ of the vacuolar form of *B. hominis* on culture medium. The treatment strategy of GTE was prepared according to Karori *et al* (2008). The tea infusion was prepared by adding 1 L of boiling water to weighted leaves of green tea and extracted for 10 min. After cooling, the aqueous extract was filtered and 10 g/L sucrose were added and given to the rats voluntary instead of water. The animals were investigated before the treatment giving and every three days to determine the effect on infection eradication. The dose that was given to the animal was 20 g green tea leaves/L of water.

Effect of GTE and E-64 *in vivo*

Quantitative estimation of the intensity of infection in stool samples of *B. hominis* infected rats was performed by examination of their stool samples every three days according to the methods of Shlim *et al* (1995). Different *B. hominis* forms were counted in at least 10 fields with estimation of the average/ high power field. *B. hominis* viability of morphological forms in stool samples of infected rats was evaluated using neutral red stain according to the method of Vdovenko and Williams (2000). Negative Blastocystis fecal samples of rats were cultured in the medium of Jones supplemented with 10% horse serum (Leelayoova *et al*., 2002). The culture was negative if the organism was not present until the 7^{th} day.

Statistical Analysis

IC_{50} and IC_{90} were calculated (concentration of the drug in X-axis and percentage of inhibition in Y-axis) using office XP (SDAS) software with linear regression. Chi square tests were used to test statistical significance for categorical data. P value ≤ 0.05 was defined as statistically significant.

Result and Discussion

The IC_{50} and IC_{90} of GTE and E-64 tested against 12 isolates of *B. hominis* genotype/symptomatic subtypes I, III and IV (four isolates/subtype) were determined *in vitro* and the results were depicted in Tables 1 and 2. No resistant isolates were

detected. The highest concentrations of GTE inducing the cytocidal effect and ICs were detected among isolates of genotype/subtypes I, three isolates (25%) were killed with 30 mg/l while the one isolate (8.3%) was killed with 35 mg/l (Table 1). Two and three isolates (16.7% and 25%) in genotype/subtype III and IV had cytocidal doses 25 and 20 mg/l at 24 and 72 h, respectively. The other two and one isolates (16.7% and 8.3%) of genotype/subtype III and IV had cytocidal doses 20 and 15 mg/l at 24 h and 72 h, respectively. On the other hand, the IC50 and IC 90 ranged 10 – 20 and 15 – 30 after 24 h contact while ranged 5 – 15 and 10 – 25 mg/l after 72 h, respectively.

As shown in Table 2, the highest concentration of E-64 inducing cytocidal effect (35 mg/l) was recorded against genotype/subtypes I after 24 h while the lowest concentration (20 mg/l) was demonstrated against genotype/subtype I and III by 72 h. On the other hand, the lowest IC_{50} (10 mg/l) was recorded after 72 h exposure against genotype/subtype I (one isolate), III (4 isolates) and IV (4 isolates). The IC90 ranged from 15 mg/l (against 5 isolates after 72 h) to 25 mg/l (against 7 isolates after 24 h) as mentioned in Table 2.

The ability of GTE and E-64 to eradicate *B. hominis* was evaluated in *vivo* and the results were summarized in Table 3. The majority of *B. hominis* infection (11 out of 12; 91.7%) was eradicated after 6 days while one isolate was eradicated after 9 days. Comparable results were recorded after administration of E-64. The differences were statistically non significant between both groups.

Transmission electron microscopy *in vitro* study of one *B. hominis* isolate (genotype/subtype I) cultured in normal growth medium showed classical *B. hominis* morphology (Figure 1). GTE induced necrotic cell death that is morphologically characterized by a gain in cell volume (oncosis), cytoplasmic membrane injury and disposition of vacuolated nonhomogenous particles in the cytoplasm, nucleus shifted periphery, expulsion of the cytoplasmic content then cell rupture were detected suggestive necrotic changes (Figure 2, A-H). Same pattern of necrosis was demonstrated in presence of E-64 (data not shown).

Currently most medications including antibiotics, anticancer drugs, proteases inhibitors and drugs against parasites are derivatives of natural compounds (Schafer and Wink 2009). Although several reports identified many plant extracts that are effective against the common intestinal protozoa such as *Giardia* and *Entamoeba* (Puthia *et al*., 2006), to the best knowledge of the authors, the effect of such plant extracts (especially green tea extract) on *B. hominis*, was not assessed before.

In the current study, all *B. hominis* isolates were susceptible to both GTE and E-64, although three of these isolates showed resistance to metronidazole. GTE showed cytocidal effects at low concentration especially after 72 h (15 mg/l). In addition, most isolates were completely eradicated after 6 days. These results are in accordance with the previously published studies which showed an inhibitory effect of GTE on haemoparasites *Plasmodium* and *Trypanosomes* (Sharma *et al*., 2007; Hellmann *et al*., 2010). In addition, several investigators identified the inhibitory effects of green tea alone in some bacteria (Lee *et al*., 2009) such as the inhibition of the membrane bound ATPase activity

Table.1 Effect of different doses of GTE (10, 15, 20, 25, 30 and 35 mg/l) on the tested isolates after 24 and 72 h exposure

Genotype/ subtypes	Isolates (n = 12) No. (%)	Cytocidal		IC_{50}		IC_{90}	
		24 h	72 h	24 h	72 h	24 h	72 h
I	1 (8.3)	35	30	20	15	30	25
I	3 (25)	30	25	15	10	25	20
III	2 (16.7)	25	20	15	10	20	15
IV	3 (25)						
III	2 (16.7)	20	15	10	5	15	10
IV	1 (8.3)						

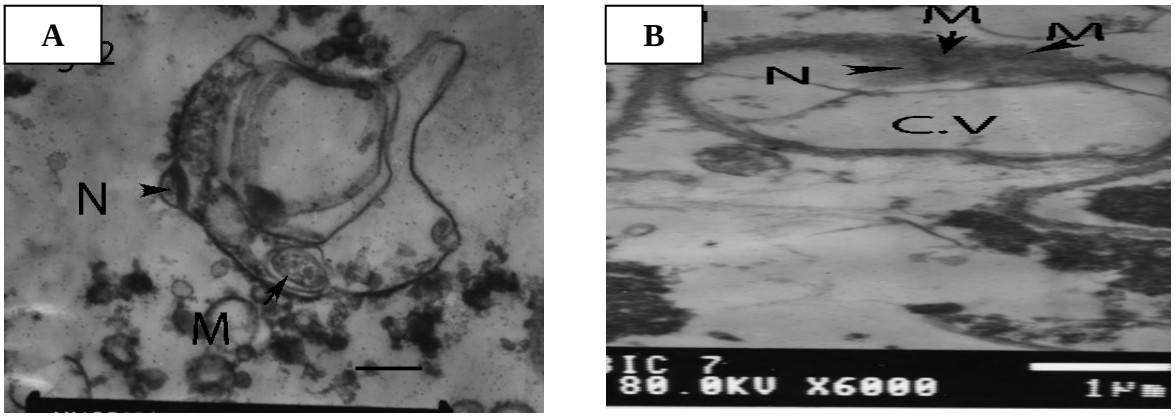
Table.2 Effect of different doses of E-64 (10, 15, 20, 25, 30 and 35 mg/l) on the tested isolates after 24 and 72 h exposure

Genotype/ subtypes	Isolates (n = 12) No. (%)	Cytocidal		IC_{50}		IC_{90}	
		24 h	72 h	24 h	72 h	24 h	72 h
I	2 (16.7)	35	30	20	15	25	20
I	2 (16.7)	30	25	15	10	25	20
IV	3 (25)						
III	4 (33.3)	25	20	15	10	20	15
IV	1 (8.3)						

Table.3 Comparisons between the days of infection eradication of *B. hominis* symptomatic genotypes subtypes among GTE and E-64 treated infected rats groups

Days of infection eradication	Genotype subtypes	GTE		E-64	
		No. (n = 12)	%	No. (n = 12)	%
Day 3	I	1	8.3	1	8.3
	III	2	16.7	3	25
	IV	3	25	3	25
	Total	6	50	7	58.3
Day 6	I	2	16.7	2	16.7
	III	2	16.7	1	8.3
	IV	1	8.3	1	8.3
	Total	5	41.7	4	33.4
Day 9	I	1	8.3	1	8.3
	III	0	0	0	0
	IV	0	0	0	0
	Total	1	8.3	1	8.3

Figure.1 Morphology of *B. hominis* grown in normal growth medium. A, Characteristic nuclear (N) morphology, a crescent band of electron-opaque material at one pole; B, Mitochondrion (M) organelles varied in number, containing low numbers of sacculations and central vacuole (CV) of *Blastocystis* vacuolar form, bar = 1µm.

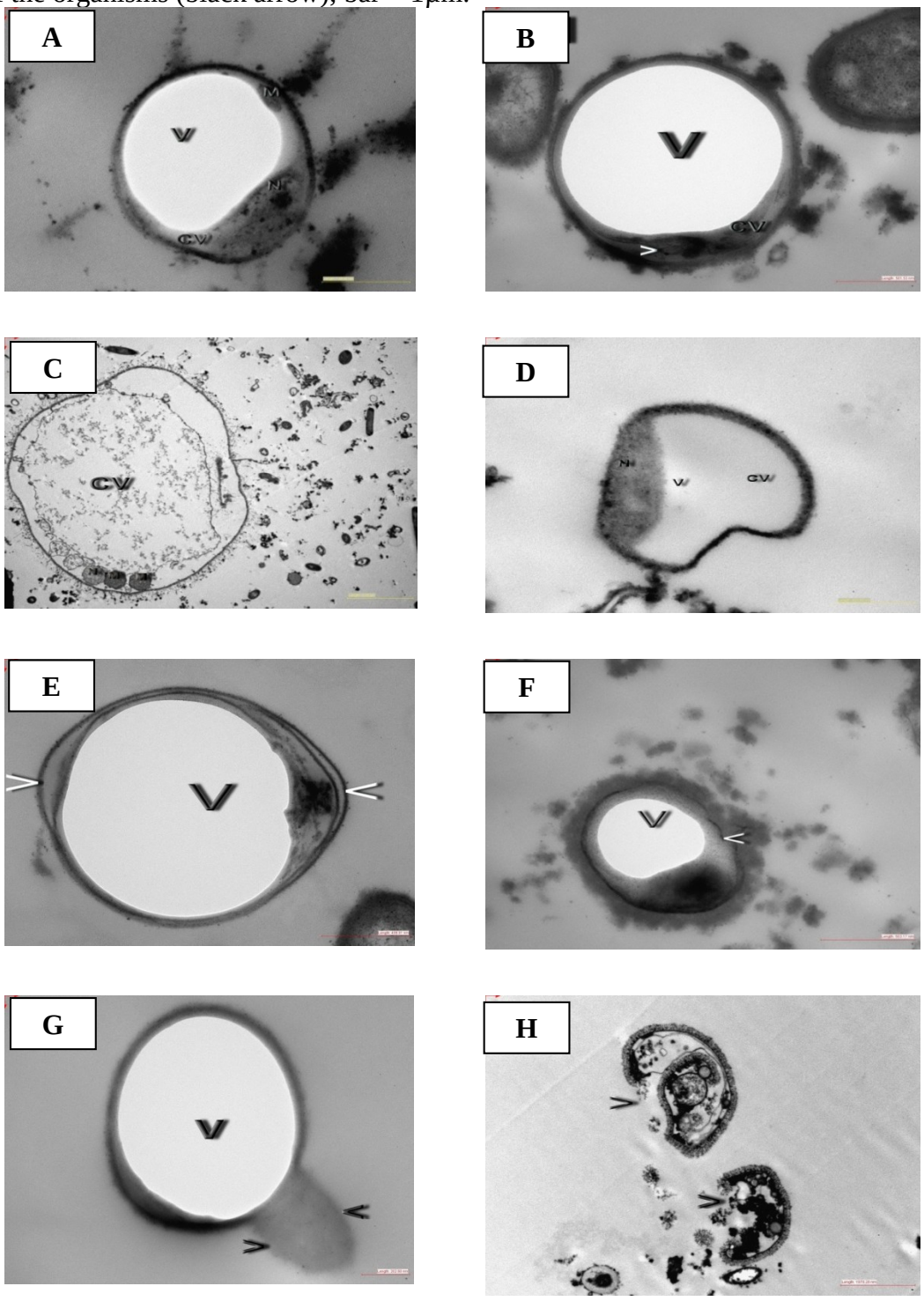


significantly at concentrations of more than 2 and 6 mg/ml in *Listeria monocytogenes* and *E. coli* O157:H7, respectively (Sivarooban *et al*., 2008) and streptococcal oral infection (Hassani *et al*., 2008). In addition, GTE in combination with others antibiotics had an synergistic inhibitory effects against *Staphylococcus aureus* (Peng *et al*., 2010, Aboulmagd *et al*., 2011). The explanation of these results may be due to the ability of GTE to penetrate the cell membrane of the organism inducing shape change, aggregation of vesicles and causing bursting through a large hole, separation of the plasma membrane and then killing of the organisms (Tamba and Yamazaki, 2005). This mechanism referred to tea catechins that bound to the lipid bi-layers causing aggregation of lipid vesicles and leaked contents from a suspension of vesicles (Tamba *et al*., 2007; Sun *et al*., 2009).

Very limited data are available regarding the effect of GTE in infected animal models. Karori *et al* (2008) confirmed the inhibitory effect of GTE on trypanosomes. In the current study, significant eradication of *B. hominis* was recorded after administration of GTE for treatment of infected rats. This effect may be due to green tea polyphenols and its constitute epigallocatechingallate that have several effects such as inhibition of proteases and proteasome function, anti-inflammatory, cell cycle regulation, tumor invasion and to reduce oxygen-derived free radicals beside the anti apoptotic effect on tumor cells (Tobi *et al*., 2002; Buttemeyer *et al*., 2003). In addition, green tea has anti adhesive effect against many pathogens (Lee *et al*., 2009). Moreover, Monbet *et al* (2010) proved that GTE increase the production of IgA and consequently, it can decrease the contact-independed apoptosis and disrupt barrier function produced by *Blastocystis* as shown by Puthia *et al* (2006).

The inhibitory effect of E-64 against tested isolates was clear and its activity either *in vitro* (cytocidal effect, IC50 and IC90) or *in vivo* (days required for infection

Figure.2 Effect of GTE on the morphology of *B. hominis*. A and B, normal nucleus (N) and mitochondria (M) and compressed of central vacuoles (CV), C, shape change and increase in size; D, aggregation of vesicles (V); E, separation of the plasma membrane (white arrows); F, injury of cell membrane (white arrow); G, bursting through a large hole (black arrows); F, killing of the organisms (black arrow); bar = 1µm.



eradication) was comparable to that of green tea extract. The inhibitory effect of E-64 is mediated by inhibition of cysteine protease secreted by *B. hominis* which breaks up IgA antibody allowing *B. hominis* survival and colonization in the human gut. To the best of the authors' knowledge, no reports are available regarding the *in vitro* or *in vivo* activity of E-64 against *B. hominis*. On the other hand, the effect of E-64 on some protozoa infection was emphasized by Zangger *et al* (2002) who proved that E-64 induces death of *Leishmania in vitro*. In addition, Hussein *et al* (2009) showed that, in *Giardia intestinalis* infected mice and E-64 treated, cysts output numbers were less than the number of cysts output in infected non treated mice. In addition, the histopathological changes, the number of infected mice and duration of infection decreased in E-64 treated mice than in non treated. Moreover, Olivos-Garcia *et al* (2004) reported that *Entamoeba histolytica* trophozoites injected into the portal vein of hamsters receiving a maintaining dose of E-64 failed to cause damage and rapidly eliminated.

In the present study, TEM *in vitro* study showed that, GTE and E-64 induced necrotic cell death. The TEM of the normal form of *B. hominis* (Fig. 1-2) showed a characteristic nuclear morphology which is a crescent band of electron-opaque material at one pole. Mitochondrion-like organelles were present containing low numbers of sacculate or tubular cristae. These abnormal findings were in agreement with the Nomenclature Committee on Cell Death (NCCD), that describe the term necrotic cell death morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents and plasma membrane permeabilization. On the contrary, apoptosis as a specific morphological aspect of cell death includes rounding up of the cell, reduction of the cellular volume (pyknosis), chromatin condensation nuclear fragmentation, plasma membrane blebbing and little or no ultrastructural modifications of cytoplasmic organelles (Kroemer *et al*., 2009). In agreement of these results TEM images showed that all tested isolates treated with 5% tea extract were injured or killed by the rupture of cell membranes and non-homogeneous disposition of cytoplasmic materials in a cell and cell killing was accompanied by depletion in the ATP pools of the cells due to cellular cytoplasmic leakage, indicating that the bactericidal activity of GTE results from damage of the cytoplasmic membrane (Sivarooban *et al*., 2008). In contrast, metronidazole induced apoptosis (programmed cell death, PCD) as shown by Nasirudeen *et al.,* (2004). The explanation of these controversies between the effects of MTZ and both of GTE and E-64 may be emphasized that the mechanisms of action of GTE and E-64 on living cells were similar. Recently Abdel-Hameed and Hassanin (2011) reported that proteases were recognized in symptomatic and asymptomatic patients infected with *B. hominis* genetic subtype 3. In addition, cysteine proteases play important functional roles in parasites ranging from cell cycle regulation (DNA replication and mitosis) to host-pathogen interactions (Concha *et al*., 2005) and the central vacuole of *Blastocystis* is a reservoir for cysteine proteases (Puthia *et al*., 2008). Both of the green tea components (polyphenols and its constitute epigallocatechingallate) and E-64 have inhibitory effect on proteases and proteasome function (Tobi *et al*., 2002;

Buttemeyer *et al*., 2003; Mirza and Tan 2009). These may be the main mechanisms of action of both GTE and E-64, in addition to the effect of GTE on the lipid bilayer which may explain the *B. hominis* abnormal ultrastructures identified (Tamba *et al*., 2007; Sun *et al*., 2009). Some studies emphasized that cysteine proteases has a positive role in inducing PCD (Jimenez-Ruiz *et al*., 2010). This may explain the presence of necrotic death instead of PCD in *B. hominis in vitro* treated with GTE and E-64 in the present study. Moreover, the intra-species and inter-species variability in cysteine protease among *B. hominis* isolates may explain these differences in ICs doses among different genotype/subtypes (Mirza and Tan, 2009) especially the highest ICs of the genotype/subtype I which is the most pathogenic in comparison with the subtypes III and IV (Hussein *et al*., 2008).

In conclusion, GTE and E-64 showed a good therapeutic effect either *in vitro* or *in vivo* against *B. hominis* human infection. These finding revealed that these two drugs are attractive therapeutic options for treatment of infections caused by MTZ resistant pathogens. Further studies are warranted to assess the clinical outcomes of such combinations.

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