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Hormetic Effect of Mixed *Subtypes 1&3* of *Blastocystis* sp. Antigen Exposure on N-Cadherin Expression in the HT-29 Colorectal Cancer Cell Line and Its Relationship with Cell Migration

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Abstract

Colorectal cancer is one of the most common malignancies and the second leading cause of cancer-related deaths worldwide. The disease is associated with intestinal protozoan infections, including *Blastocystis* sp. Mixed *subtypes 1&3* of *Blastocystis* sp. are frequently found in the fecal specimens of colorectal cancer patients in West Sumatera, Indonesia. The mechanism by which these protozoa influence the pathogenesis and course colorectal cancer is still unclear even it is debatable whether *Blastocystis* sp. are protecting or promoting cancer. This study aimed to determine the effects of mixed subtypes 1 and 3 of *Blastocystis* sp. antigen on N-cadherin expression and the migration of the HT-29 colorectal cancer cell line. It was *in vitro* experimental study and used a post-test only control group design. The experiment was conducted via a post-test only control group design. HT-29 cells were divided into 1 control group and 5 treatment groups and subjected to various concentrations of *Blastocystis* sp. antigen (0.005, 0.01, 0.05, 0.1 and 0.5 µg/mL). N-cadherin gene expression was measured via qRT-PCR, while cell migration was determined via a scratch assay and analyzed via Image J software. The effects of the antigens on N-cadherin expression and cell migration were analyzed via 1 Way Anova, followed by Tukey's test. The results revealed that the highest and lowest expression levels of N-cadherin were detected in the 0.5 and 0.01 µg/mL groups, respectively. The highest and lowest migration rates were found at 0.5 and 0.05 µg/mL, respectively. There was a significant difference in the expression of N-cadherin and the migration of HT-29 cells after the administration of mixed subtypes 1 and 3 *Blastocystis* sp. various concentrations. Furthermore, there was a correlation between N-cadherin and the migration of HT-29 cells. These results showed that *Blastocystis* sp. had promoted cancer at certain concentrations and indicated a hormetic effect after reaching optimal concentrations, influencing cell migration via N-cadherin.

Keywords: *Blastocystis* sp., Colorectal cancer, HT-29, N-cadherin, Cell migration, Sumatera, Indonesia

Introduction

Colorectal cancer is the third most common malignancy in the world after breast and lung cancer. According to the Global Cancer Observatory (GLOBOCAN), the number of new cases of colorectal cancer has reached 1.9 million. This disease constitutes 10 % of all malignancies and is the second leading cause of cancer-related deaths worldwide. In Indonesia, the prevalence of this disease in the last 5 years has continued to increase, reaching 104,235 or 37.3 % of all malignancies [1,2].

In general, colorectal cancer is often associated with *Blastocystis* sp. This protozoa lives in the intestines of humans and certain animals and is referred to as a potential zoonotic disease. There are several subtypes that infect humans, including 1,2,3,4,6 and 7 with mixed subtypes 1 and 3 being the most frequently found in

West Sumatera, Indonesia [3]. Eradication of these protozoa often fails because of drug resistance and the inability of the host's immune system to counter infection. Several epidemiological, *in vitro* and *in vivo* studies show that *Blastocystis* sp. plays a role in the oncogenesis process and influences the progression of colorectal cancer in a single subtype. Epidemiologic studies in Turkey, Saudi Arabia, Poland and Uzbekistan have reported high incidence of *Blastocystis* sp. infection in colorectal cancer patients. These studies concluded that *Blastocystis* sp. has an important role in the pathogenesis and progression of CRC. The dominant *Blastocystis* subtypes vary in each country. The dominant subtypes in Turkey, Poland and Saudi Arabia are subtype 4, subtype 1 and subtype 3 respectively. In the general population worldwide, it also shows that the dominance of *Blastocystis* sp. subtypes vary greatly and have different pathogenicity [4-6]. A study in a mouse model of colorectal cancer obtained by induction of azoxymethane (AOM) revealed that subtype 3 of *Blastocystis* sp. infection exacerbated carcinogenesis. Histopathological examination proved that *Blastocytis* sp. infection caused colonic aberrant crypt foci (ACF) and adenomas formation in infected rats and elevation of urinary advanced oxidative protein products (AOPP) and hydrogen peroxide [7]. *In vitro* studies on colorectal cancer cell lines HCT-116 revealed that separately of subtypes 1, 2, 3, 6 and 7 of *Blastocystis* sp. antigen highly increased transforming growth factor beta (TGF-β) and suppressed interferon γ and p53 gene expression in HCT116 cells, thus suggesting that it may have a supportive influence on the proliferation, invasiveness and metastatic properties of colorectal cancer cells [8]. *In vitro* studies with CCD-18Co and HCT116 showed that incubation of these tumor cells with *Blastocystis* sp. exposure could increase cell multiplication and reduce the effectiveness of 5-fluorouracil chemotherapy treatment [9]. Nowadays, the pathomechanism in cancer progression is still unclear. There is no research that investigates the effect of Mixed subtypes 1 and 3 of *Blastocystis* sp. on the Epithelial mesenchymal transition (EMT) process [3,6,10].

Epithelial mesenchymal transition (EMT) is a process characterized by epithelial cells losing their polarity and adhesion properties, allowing them to transform into mesenchymal cells that can easily migrate and invade. Generally, EMT occurs in several pathophysiological conditions in the human body, such as inflammation, wound healing, and cancer development. Under these conditions, E-cadherin expression is inhibited followed by an increase in N-cadherin, which causes epithelial cells to experience damage to intercellular adhesion. This process is known as cadherin switching. Cadherin is a transmembrane protein that acts as an adhesion molecule, and forms adherens junctions through a calcium-dependent mechanism [11,12].

N-cadherin is a key indicator to assess the ongoing EMT process. It is related to development, invasion and metastases cancer [13,14]. N-cadherin is widely used by clinicians as a biomarker to determine metastatic status and patient's prognosis [15]. At the cellular level, N-cadherin can increase the proliferation, viability and migration of cancer cells. High N-cadherin expression is associated with poor prognosis, increased invasion, and increased metastasis ability [12,14,16].

Materials and methods

This research was in vitro experimental study and used a posttest only control group design. It was approved by the Research Ethics Committee of Faculty of Medicine Universitas Andalas, Padang, West Sumatera, Indonesia (N0.513/UN16.2/KEP-FK/2023).

Isolation of *Blastocystis* sp.

Blastocystis sp. was isolated from stool samples of colorectal cancer patients at Dr. M. Djamil Hospital Padang, West Sumatra Province, Indonesia. The samples were screened via stool smears within a maximum of 6 h after collection to identify *Blastocystis* sp. without other gastrointestinal parasites. Approximately 50 mg of positive *Blastocystis* sp. stool samples were inoculated into 3 mL of Jones medium supplemented with 10 % horse serum. Parasite cultures were incubated at 37 °C in a CO_2 incubator and screened daily for 5 - 7 days. The samples were considered positive when vacuolar, granular, and ameboid forms of *Blastocystis* sp. were found and confirmed by PCR examination via universal primers and continued with subtype identification as shown in the table below.

Table 1 Primers for q-PCR.

Subtype	Primer	Product size (bp)	Sequence 5'- 3'	GenBank accession number	Reference
-	BL18SPPF1 F	342	AGTAGTCATACGCTCGTCTCAAA	MT645814.1	[17]
	BL18SR2PP R		TCTTCGTTACCCGTTACTGC		
1	SB83 F	351	GAAGGACTCTCTGACGATGA	AF166086	[18]
	SB83 R		GTCCAAATGAAAGGCAGC		
2	SB155 F	650	ATCAGCCTACAATCTCCTC	AF166087	[18]
	SB155 R		ATCGCCACTTCTCCAAT		
3	SB227 F	526	TAGGATTTGGTGTTTGGAGA	AF166088	[18]
	SB227 R		TTAGAAGTGAAGGAGATGGAAG		
4	SB332 F	338	GCATCCAGACTACTATCAACATT	AF166091	[3]
	SB332 R		CCATTTTCAGACAACCACTTA		
5	SB340 F	704	TGTTCTTGTGTCTTCTCAGCTC	AY048752	[3]
	SB340 R		TTCTTTCACACTCCCGTCAT		
6	SB336 F	317	GTGGGTAGAGGAAGGAAAACA	AY048751	[3]
	SB336 R		AGAACAAGTCGATGAAGTGAGAT		
7	SB337 F	487	GTCTTTCCCTGTCTATTCTGCA	AY048750	[3]
	SB337 R		AATTCGGTCTGCTTCTTCTG		

Blastocystis cultures are maintained *in vitro* and subcultured every 3 to 4 days. The basic aseptic methods were used during the maintenance of these parasite cultures. The medium solution for culture (Jones' medium) was put into an autoclave at 121 °C for 24 h. Before inoculating fecal samples, the culture medium was put back into the autoclave and incubated at 37 °C in the incubator. On the third and fourth days, culture samples were taken using sterile pipettes for microscopic examination.

Preparation of Jones' medium

The Jones' medium for culturing *Blastocystis* sp. consists of disodium phosphate (Na_2HPO_4), distilled water, monopotassium phosphate (KH_2PO_4), and yeast extract. The solution was sterilized via an autoclave at 121 °C - for 24 h. Horse serum was added under UVR-laminar flow until the total volume reached 100 mL. A total of 5 mL of medium was added to a 7 mL culture tube and stored at −20 °C until it was ready to be used. When inoculation is carried out, the medium is sterilized again [19].

Purification and isolation of antigen

Blastocystis sp. (1×10^5 parasites/mL) were purified from bacterially contaminated cultures via density gradient centrifugation. The cells were collected into 1 culture tube and washed twice with phosphate-buffered saline (PBS) for 5 min at 1,000 rpm. Phosphate-buffered saline commonly used in cell culture because of its stability and non-toxicity. A total of 5 mL of cell suspension was carefully layered onto 6 mL of Ficoll-Paque without shaking and rotated for 20 min at 1,800 rpm. The contaminated part was found immediately above a thick layer of yellowish-white lumps. These sections were gently isolated and washed with PBS. Antigens were extracted via the freeze-thaw method via liquid nitrogen and a 37 °C water bath. The lysate was left overnight at 4 °C, followed by filtration through a 0.22 µm pore size polyethersulfone (PES) filter to eliminate bacteria. The protein concentration was determined via NanoDrop system. Nanodrop was performed because the procedure is fast and simple, without additional reagents and it can be used for small sample volume. Measurement bias was resolved by

using buffers that do not affect the measurement. The lysate was stored at −20 °C until further use [18,19].

Confirmation antigen *Blastocystis* sp. via ELISA method

Confirmation of isolation of *Blastocystis* sp. antigen was performed via commercial Abnova Blastocystis sp. copro ELISA kit (Catalog No. KA0292). The procedures used 100 µL of negative control, 100 µL of positive control and 100 µL of specimen. The plate was covered and then incubated for 1 h at 37 °C and 100 % humidity. Washing was performed 5 times using 300 µL of wash buffer and then 100 µL of HRP horseradish peroxidase (HRP)-conjugate was added. The plate was covered and then incubated again for 1 h at 37 °C and 100 % humidity. Rewashing was performed 5 times using 300 µL of wash buffer and then 100 µL of tetramethylbenzidine (TMB)-substrate conjugate was added. The plate was covered and then incubated for 15 min at room temperature. A total of 100 µL of stop solution was added. Results were observed in absorbance at a wavelength of 450/620 nm.

Preparation of HT-29 cell

HT-29 (Catalog No. CL-0118 Elabscience) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). All media were prepared with 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 10 % heat-inactivated fetal bovine serum. The cell cultures were placed in an incubator at 37 °C in a humid environment (95 % air and 5 % CO_2) [22]. The cells were cultured in 30 wells (well plates) via exposure to different concentrations of *Blastocystis* sp. (0.005, 0.01, 0.05, 0.1 and 0.5 µg/mL), with 5 replications for each treatment concentration. *Blastocystis* sp. was exposed to colorectal cancer cells. The harvested colorectal cancer cell line (HT-29) (1×10^3 cells per well) in 100 µL of growth medium was placed into 96-well plates. After incubation, the samples were incubated 1 day and 1 night at 37 °C in an incubator containing 5 % CO_2. *Blastocystis* sp. at various concentrations (0.005, 0.01, 0.05, 0.1 and 0.5 µg/mL) were added to each well and incubated for 72 h. Each concentration was applied in 5 HT-29 cultures [20].

N-cadherin expression

There were 3 stages of examination, namely RNA isolation, cDNA synthesis, gradient PCR amplification, and quantitative real-time PCR (RT-PCR). Total RNA from the cultured cells was isolated via the use of 500 µL GENEzol Reagent (catalog #GZR200). The homogenizer used was 1 mL of GENEzol reagent per 50 - 100 mg of cells in the sample. Approximately 200 µL of chloroform was added, the tube was inverted, and the mixture was incubated for 5 min at room temperature. Subsequently, centrifugation was carried out at a speed of 12,000×g at a temperature of 4 °C for 15 min. The aqueous phase layer (top/clear) was transferred to a new sterile microtube, isopropanol (1:1) was added and the mixture was incubated again for 30 min at cold temperature. Centrifugation was performed at a speed of 12,000×g at 4 °C - for 10 min. The supernatant was discarded, and the pellet was washed with 350 µL of 70 % ethanol. The tube was turned upside down and vortexed slowly. Repeated centrifugation was carried out at a speed of 12,000×g at 4 °C - for 5 min, and the supernatant was discarded and vacuumed for 10 min. After vacuum, the pellet was resuspended in 25 - 40 µL RNAse-free water (depending on the number of pellets). The RNA concentration was measured via NanoDrop system.

cDNA synthesis was carried out via a synthesis kit (SensiFAST cDNA Synthesis Kit, Bioline BIO-65054). The sample was inserted into the PCR machine at 25 °C - for 10 min (primary annealing), 42 °C - for 15 min (reverse transcription), 85 °C - for 5 min (inactivation), and 4°C - for 5 min (cooling). cDNA can be used for real-time PCR or stored at −20 °C. cDNA synthesis used the SensiFAST cDNA Synthesis Kit from Bioline (BIO-65053). The primers used to detect N-cadherin gene expression (NCBI NM_001792.5) were 5'-ACAGTGGCCACCTACAAAGG-3' for forward and 5'-CCGAGATGGGGTTGATAATG-3' for reverse. GAPDH was used as a housekeeping gene with the primer sequence 5'-CATCATCCCTGCCTCTACTG-3' forward and 5'-CCAAATTGCTTGTCATACCAG-3' reverse. The primers were designed using Geneious 11.1 bioinformatics software. Gene expression examination used SensiFAST SYBR No-ROX reagent (BIO-98020) and BIO-RAD CFX96 real-time PCR machine.

RT-PCR

The material components in the PCR tube consisted of nuclease-free water, N-cadherin forward primer, N-cadherin reverse primer, SYBR GREEN, and cDNA. These components were inserted into the real-time PCR machine with an amplification range of 40 amplification cycles consisting of a 95 °C pre-denaturation step for 3 min. The core cycle consisted of 95 °C for 15 s, 53 °C for 30 s for E-cadherin, 55 °C for 15 s for N-cadherin, and 59.4 °C for 30 s for GAPDH. After completing PCR, the Ct or Cq value was obtained and processed via the Livak method.

Measurement of N-cadherin expression

Gene concentration measurements in this study were performed via the relative quantification method [23].

$$\Delta CT \text{ experiment} = CT \text{ experiment target} - CT \text{ experiment housekeeping}$$

$$\Delta CT \text{ control} = CT \text{ control target} - CT \text{ control housekeeping}$$

$$\Delta\Delta CT \text{ experiment} = \Delta CT \text{ experiment} - \Delta CT \text{ control}$$

$$\text{Comparison of gene expression levels} = 2^{\Delta\Delta CT}$$

HT-29 migration in scratch assay

Cell migration measurements were carried out via the scratch wound healing assay method. This method was carried out by making scratches manually on a cell monolayer via 100 µL sterile pipette tip perpendicularly via a plate lid that had been wiped with alcohol tissue on all plates growing on cells exposed to *Blastocystis* sp. supplemented with fetal bovine serum. The direction of the scratch and pressure applied must be the same for each well. The cells were washed with Phosphate Buffer Saline (PBS) until the scratched part had no attached cells and no floating cells. To minimize the limitations of manual scratching, a consistent technique in making wounds and 5 replications were applied. To minimize subjectivity, researchers used the same microscope (Zess Axiovert 40C), the same magnification, the same camera and the same settings for 5 fields of view.

Cell migration measurements were carried out by measuring the gap distance line (manual drawing) between opposite cells from an image of the object under a microscope within 72 h. The migration distance obtained was processed with the help of ImageJ software. Cell migration assessed by the average of 10 gap distance lines between opposite cells [24,25].

Statistical analysis

The SPSS version 29.0.2.0 application was used in data analysis. The normality of data distribution was tested via the Kolmogorov-Smirnov Test ($p = 0.750$ for N-cadherin and $p = 0.734$ for migration). Homogeneity was tested via the Levene's ($p = 0.266$ for N-cadherin and $p = 0.853$ for migration). The data were analyzed statistically via 1-way ANOVA to determine differences in N-cadherin expression and the migration of HT-29 cells after administration of several concentrations of antigen and Tukey's post hoc tests were performed. *p*-values of less than 0.05 ($p < 0.05$) were considered to indicate a significant difference. The correlation between N-cadherin and migration was subsequently analyzed by Pearson's bivariate correlation test.

Results and discussion

The results revealed that the highest mean N-cadherin expression was detected in the 0.5 µg/mL treatment group, and the lowest was detected in the 0.01 µg/mL group. This study indicates significant differences expressions of N-cadherin in various concentration of *Blastocystis* sp. in cell culture. These significant differences were detected in the 0.005 - 0.5, 0.01 - 0.5, 0.05 - 0.5 and 0.1 - 0.5 µg/mL (**Table 2**). The results showed that at certain concentrations, exposure to the *Blastocystis* sp. antigen inhibited N-cadherin expression. This inhibition continued until the optimum dose was 0.01 µg/mL. At the next dose, there was an increase in N-cadherin expression due to the hormetic effect, which was significantly influenced by the concentration of exposure. This phenomenon is characterized by a nonlinear exposure effect with increasing dose, namely, a high exposure dose results inversely proportional to a low dose. An increase was initially observed at a concentration of 0.05 µg/mL. Hormesis seen in "u-shaped" (**Figure 2(A)**) means low-dose caused inhibition of N-cadherin and high-dose cause stimulation. An "inverted u-shaped" of gap distance shown in **Figure 2(B)** was explained that small gap distance indicates high migration of cancer cells. It is clearly explained in **Figure 4** which is shown that N-cadherin expression are correlated with the HT-29 cell migration. It meant low N-cadherin expression inhibited

migration of HT-29 cells and implied that low antigen concentration could give beneficial effects to suppress cancer migration, invasion and metastases. This phenomenon occurs because of the response of HT-29 cells to stress. At low to moderate concentrations, an adaptive cell response occurs, which causes a temporary decrease in N-cadherin expression and induces a beneficial effect. At this stage, the cell is able to activate a protective signaling pathway that influences the expression of transcription factors or epigenetic regulators controlling N-cadherin expression [23-25]. Exposure to high concentrations of antigen can induce a stronger stress response, causing changes in the cell phenotype including high N-cadherin which can facilitate migration and invasion [29]. An increase in N-cadherin is an EMT marker that is associated with malignant transformation, clinical worsening, and prognosis, particularly for patients with colorectal cancer. A decrease in N-cadherin can cause cells to experience a reduction in malignant properties. A decrease in N-cadherin can cause cells to experience a reduction in malignant properties [14].

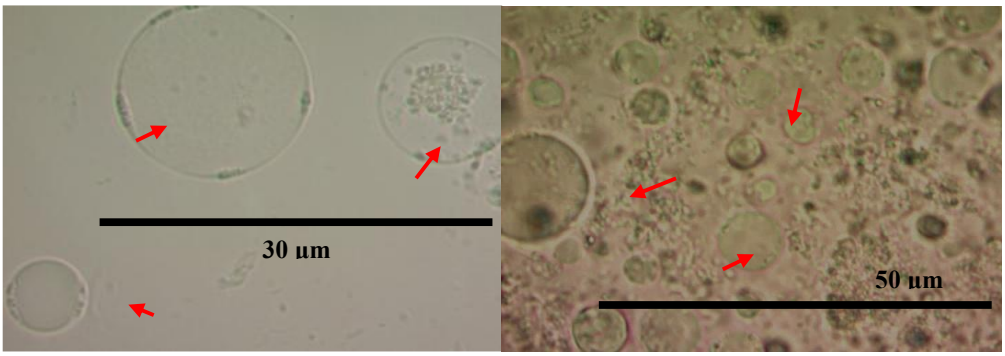
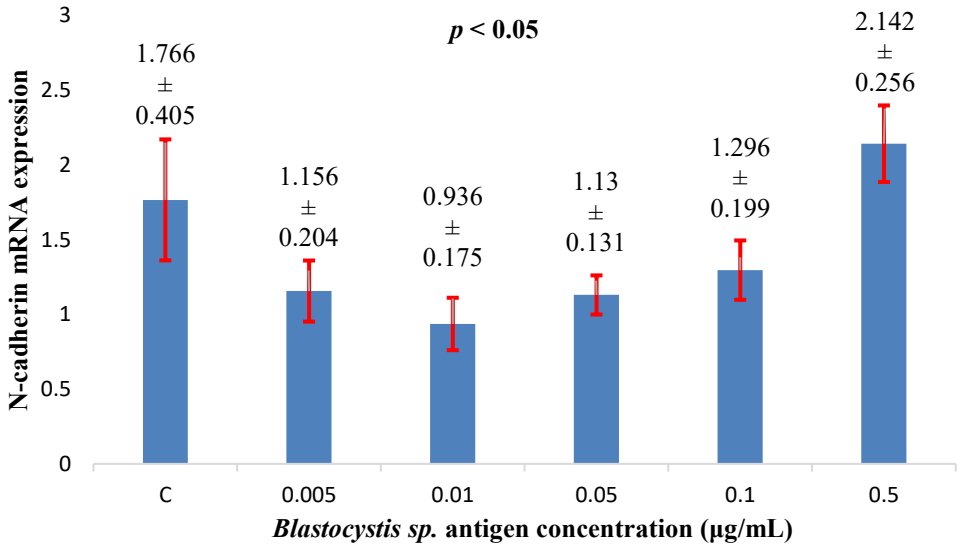
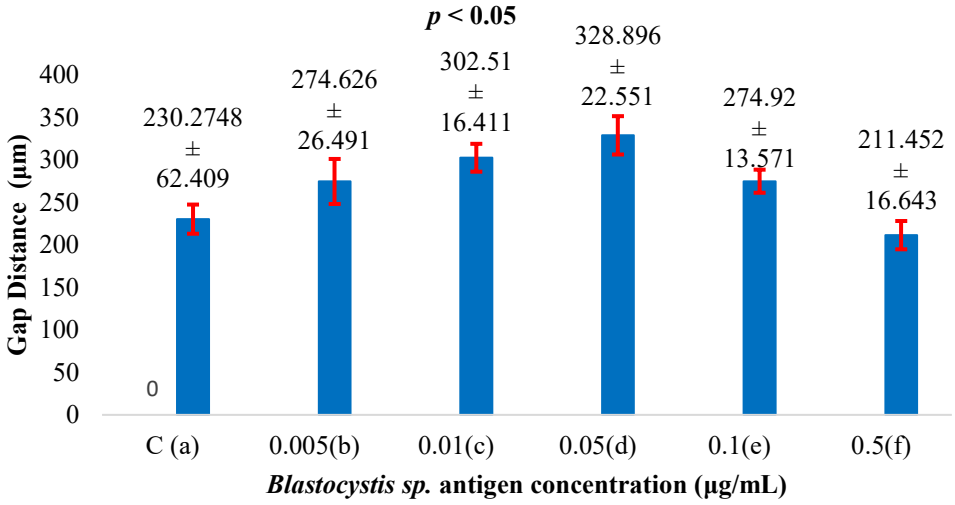


Figure 1 Morphology of *Blastocystis* sp. (vary in size 5 - 40 µm) in direct fecal smears and subcultured in Jones' medium. *Blastocystis* sp. were isolated from isolated from colorectal cancer fecal samples (a) and subcultured (b) under a microscope (1,000×).



(A)



(B)

Figure 2 Effects of mixed *subtypes 1&3* of *Blastocystis* sp. at different concentrations on N-cadherin mRNA expression (A) and the migration of HT-29 cells (B) after 72 h of treatment. The data shows the hormetic effects on N-cadherin mRNA expression and the migration of HT-29 cells in the control and treated groups. An inverse relationship between treatment concentration and N-cadherin mRNA expression was detected in the control group; (0.005 - 0.01 µg/mL), and a positive relationship was detected higher concentrations (0.05, 0.1 and 0.5 µg/mL). A positive relationship was detected between the treatment concentration and gap distance in the control group; (0.005 - 0.05 µg/mL) and an inverse relationship was detected at the next concentration (0.1 - 0.5 µg/mL). The data are expressed as the means ± standard deviations of 5 separate experiments. The data were analyzed via 1-way ANOVA. The results were considered statistically significant when $p < 0.05$ compared with the control.

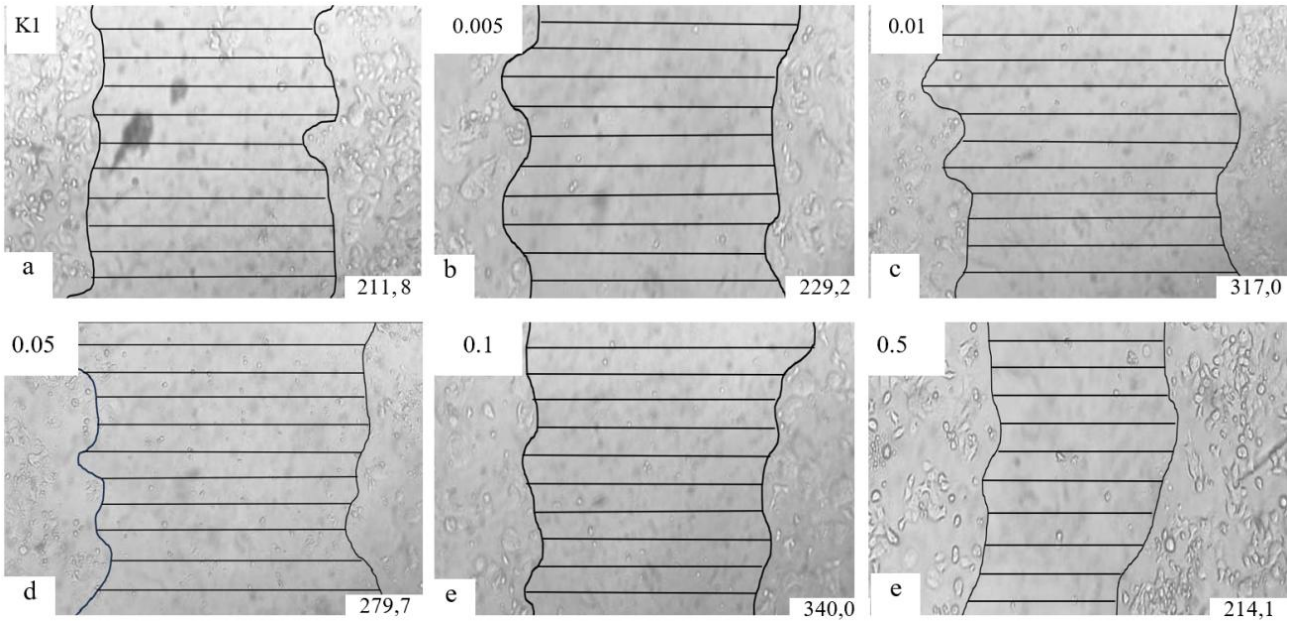


Figure 3 A qualitative scratch test was used to determine the effect of mixed subtypes of the 1&3 *Blastocystis* sp. antigens on the migration ability of HT-29 cells. Pictures were taken with ZeissAxiovert 40C microscope at 100× magnification and processed with imageJ software. Migration was determined by measuring the outer distance between tumor cells at 10 pair points and reported as the average value (µm). (a) is the control (untreated group) and (b-f) are the 0.005, 0.01, 0.05, 0.1 and 0.5 µg/mL treatment groups. Antigen exposure caused biphasic changes in tumor cell migration. The highest concentration of antigen had the greatest effect on the migration of tumor cells in the medium.

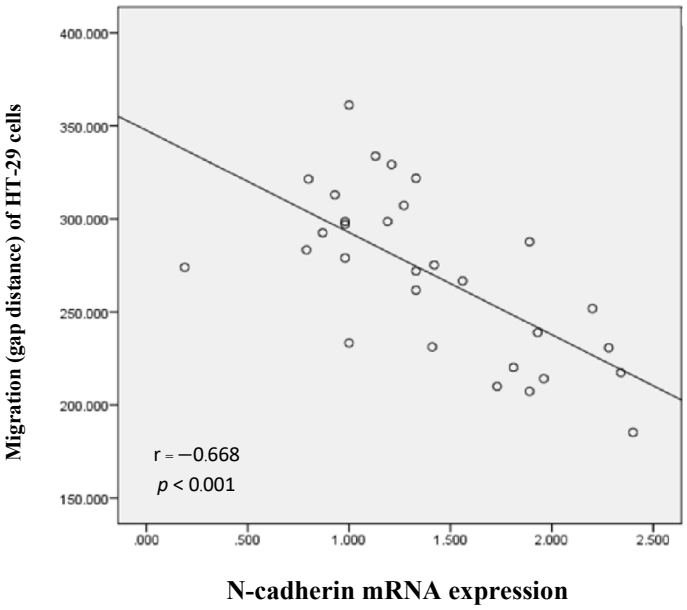


Figure 4 Correlation between N-cadherin expression mRNA and the migration of HT-29 cells. Pearson's correlation coefficient (r) and *p*-values are shown. The results reveal a negative correlation, where the increase in N-cadherin mRNA expression is followed by a decrease in gap distance (decreasing gap distance means increasing the migration of HT-29 cells).

Table 2 Tukey's test for N-cadherin expression.

Group	Control	0.005 µg/mL	0.01 µg/mL	0.05 µg/mL	0.1 µg/mL	0.5 µg/mL
Control	-	0.157	0.135	0.129	0.098	0.635
0.005 µg/mL	0.157	-	1.000	1.000	1.000	0.005*
0.01 µg/mL	0.135	1.000	-	1.000	1.000	0.004*
0.05 µg/mL	1.129	1.000	1.000	-	1.000	0.004*
0.1 µg/mL	0.098	1.000	1.000	1.000	-	0.003*
0.5 µg/mL	0.635	0.005*	0.004*	0.004*	0.003*	-

These results were similar with linalool exposure on B16F10 murine melanoma cell line. It revealed hormetic effects on epithelial-mesenchymal transition markers, migration, and angiogenesis. Stress response of B16F10 cells cause inhibition effect in low concentration (0.4 mM) and opposite response occurred in high concentration (0.8 mM) [30]. Another study also found that periostin has a hormetic effect on the development of pancreatic cancer cells. Low concentration of periostin reduced mesenchymal markers and cell migration and the opposite effect occurred in high concentration via AKT activation [31].

There are several studies related to hormetic response due to the stress response of cancer cells because of exposure to certain chemotherapeutic and radiotherapy agent. High dose irradiation of human breast cancer cell line MDA-MB-231 induced tumor proliferation and invasiveness via the transfer of exosomal miR-21 [32]. Low concentration of Withaferin A (WFA) could induce anti-migration and invasion abilities for oral cancer cells by triggering Reactive Oxygen Species (ROS) production and subsequently showed the opposite effect in high concentration [33]. Low-dose naltrexone inhibited cancer cell proliferation by blocking the opioid growth factor–receptor axis and enhancing the immune response against cancer cells and the other hand in high dose showed different effects [34].

Hormesis is a complex mechanism that is influenced by several interconnected biological

processes. Several studies have concluded that hormesis can occur as stress response through the activation of the NF-κB (nuclear factor kappa-B), MAPK (mitogen-activated protein kinase), AMPK (adenosine 5'-monophosphate (AMP)-activated protein kinase), mTOR (mammalian target of rapamycin), and PI3K/Akt (phosphatidylinositide 3-kinases/protein kinase B) signaling pathways and increased activity of cytoprotective proteins. All of these factors work together to overcome oxidative stress and inflammation. This process allows self-defense by cells or tissues and adapts by carrying out repairs and restoration [35].

The results of the present study revealed that the greatest mean migration of HT-29 cells was associated with the lowest gap distance in the treatment group at an exposure concentration of 0.5 µg/mL. The lowest degree of migration was found in the 0.05 µg/mL group. Statistical analysis revealed that exposure to *Blastocystis* sp. influenced HT-29 cell migration. These significant differences were detected in the control group −0.005, 0.01, 0.05, 0.1, 0.005 - 0.05, 0.005 - 0.5, 0.01 - 0.5, 0.05 - 0.1, 0.05 - 0.5 and 0.1 - 0.5 µg/mL (**Table 3**). This finding revealed that the administration of the antigen at concentration of 0.05 µg/mL caused a gradual decrease in cell migration. With increasing exposure concentration, the migration of HT-29 cells increased.

Table 3 Tukey's test for gap distance.

Group	Control	0.005 µg/mL	0.01 µg/mL	0.05 µg/mL	0.1 µg/mL	0.5 µg/mL
Control	-	0.015*	0.000*	0.000*	0.014*	0.642
0.005 µg/mL	0.015*	-	0.239	0.002*	1.000	0.000*
0.01 µg/mL	0.000*	0.239	-	0.290	0.249	0.000*
0.05 µg/mL	0.000*	0.002*	0.290	-	0.002*	0.000*
0.1 µg/mL	0.014*	1.000	0.249	0.002*	-	0.000*
0.5 µg/mL	0.642	0.000*	0.000*	0.000*	0.000*	-

The inhibitory effect on migration of *Blastocystis* sp. antigen at low concentrations is associated with low N-cadherin. This is supported by the gap distance curve having the same pattern as the N-cadherin expression curve and the results of the bivariate Pearson correlation test. N-cadherin is a cadherin that plays an important role in the migration process of cancer cells. N-cadherin activates Rho GTPase which is a protein that regulates the actin cytoskeleton in the process of cell division, migration, and extravasation of tumor cells to invade tissue. Rho GTPases also activate nucleation-promoting factors (NPFs), which trigger the Arp2/3 complex to regulate tumor cell migration and invasion [36].

Several studies revealed similar stimulation effects of antigen *Blastocystis* sp. in high dose on N-cadherin and migration of cancer cells *in vitro*. Exposure to the *Helicobacter pylori* (cagA+ vacA+) strain induces the EMT process in the RGM-1 epithelial cell line. This is characterized by increases in N-cadherin, vimentin, integrin-β1, and several other EMT markers. Increased expression of EMT markers facilitates the proliferation and migration of cancer cells [37]. Previous studies conducted on human papillary thyroid carcinoma IHH4 have shown that increasing N-cadherin expression leads to high migratory ability through the same pathway as elevated proliferative activity, namely, through the activation of the MAPK/Erk and PI3K/Akt pathways [38].

Migration is not only influenced by N-cadherin. For example, exposure of *Toxoplasma gondii* tachyzoites to various concentrations in the breast cancer cell lines MCF-7 and MDA-MB-231 showed that the inhibitory effect on proliferation and migration occurred through changes in BRCA1, MYC, and IL-6 gene expression [39]. Similar results were also reported in studies in which tumor cells were exposed to radiation. At low radiation concentration, there was a reduction in migration and invasion through the inhibition of JAK1/STAT3 signalling. Moreover, the opposite occurred under high-dose exposure [40].

This study only explained the effect of *Blastocystis* sp. antigen on N-cadherin and its

relationship with cancer cell migration. Further studies are needed to investigate other key EMT markers, such as E-cadherin, Vimentin, Fibronectin, ZO-1 and others to clarify their involvement in the EMT process. This study also cannot confirm the mechanism of the suppressive effect on N-cadherin and migration, so it is necessary to investigate proliferation, apoptosis, and senescence markers.

Conclusions

In conclusion, this study revealed that mixed subtypes 1 and 3 *Blastocystis* sp. could increase N-cadherin expression and the migration of HT-29 cells at high concentration. The results revealed a correlation between N-cadherin expression and cell migration. These findings indicate that *Blastocystis* sp. can promote cancer progression by increasing migration ability of colorectal cancer cells *in vitro*. Moreover, further studies were recommended to investigate this phenomenon in animal studies to observe interaction between tissue, organ, intestinal microbiota to *Blastocystis* sp. infection and explore activated pathways. It also needs specific analysis to strengthen the hormetic effect.

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