The Effect of Onion Peel Extract on Colorectal Cancer Cell Invasion Through L1CAM Expression

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Abstract

Colorectal cancer (CRC) is the major worldwide health problem from its high prevalence and mortality rates. Phytochemicals treatment or natural products have been used to prevent and treat various diseases including CRC. This study aimed to investigate the effects of onion peel crude extract on Caco-2 migration and invasion through the expression of L1CAM which cancer cells used for invasive migratory capability. The effects of the onion peel extract on Caco-2 colorectal cancer cells proliferation were investigated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell migration was performed by wound healing assay. Cell invasion was determined by transwell invasion assay and the expression of L1CAM was investigated by Immunofluorescence assay. The results shown 10 µg/ml of onion peel extract has no anti-proliferative effects. Treatment with various doses of onion peel extract for 48 h significantly ($P<0.01$, $P<0.001$) decreased cell migration rates in Caco-2 cells in dose-dependent manner and treatment with the onion peel extract significantly ($P<0.01$) decreased cell invasion rates for 48 h. Treatment with 5 and 10 µg/ml onion peel extract significantly ($P<0.001$) decreased L1CAM expression in Caco-2 cells. In conclusion, onion peel extract inhibited Caco-2 cells migration and invasion through reduced L1CAM expression. Further study is needed to clarify the molecular mechanisms of L1CAM and invasive capacity of colorectal cancer. The onion peel extract may have potential as a novel chemotherapy in anti-metastasis of colorectal cancer.

Keywords: Colorectal cancer, Migration, Invasion, Onion peel extract, L1CAM

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Introduction

Colorectal cancer (CRC) is the cancer that occurs in colon or rectum which is the major worldwide health problem from its high prevalence and mortality rates. In Thailand, it is ranked as the third most commonly diagnosed cancer in males and the second in females. Moreover, in the coming 15 years, a further 60% increase is expected with 13 million people estimated to die with cancer in 2030 (Ferlay et al., 2015; Kerr DJ, 2016; G. H. Lee et al., 2015); Petrelli F (2016)). In developed countries, the incidence rates of CRC are higher than developing ones due to the increase of aging population together with the poor dietary habits, smoking, low physical activity, obesity, heredity and familial factor (Samadder et al., 2014; Tuohy et al., 2014; Vasen, Tomlinson, & Castells, 2015). However, in the United States, incidence and mortality of CRC are slowly increased, whereas those in Thailand are continuously increased more than 10% every year and almost cases were found in severe stages (Thailand, 2016) which are associated with the invasion and metastasis (Institute, 2015; Society, 2017). The processes of migration and invasion are often categorized together in metastasis which is a hallmark of cancer and the leading cause of mortality among cancer patients from cancer progression (Douglas Hanahan and Robert A. Weinberg, 2011). The important process of tumor invasion is the epithelial-to-mesenchymal transition (EMT) associated with many signaling molecules, including L1 cell adhesion molecule (L1CAM) (Andrew GClark, 2015; Balkwill, 2001; Cano A, 2000; Glentis A, 2014; L1., 2014).

Phytochemicals treatment or natural products have been used to prevent and treat in various diseases including CRC (Gupta, Kim, Prasad, & Aggarwal, 2010). There are excellent sources of bioactive components exerting their health beneficial effects. Approximately, 50-60% of cancer patients in the United States use agents derived from different parts of plants or nutrients as either complementary or alternative medicine (Hu Wanga, 2012). Quercetin is a flavonoids present in many vegetables, fruits and beverages such as apples, berries, green tea and onion (Brito AF, 2015). Moreover, the outer dried protective layer or peel has significantly higher levels of flavonoids than inner flesh layers (J. Lee &Mitchell, 2011). It is presented as methylated, sulphated or glucuronidated metabolites in plasma followed by ingestion and metabolism after intake of onions at dietary or herbal extraction doses *in vivo* (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Quercetin, as a purified ingredient, plays important roles in inhibitory effects on cell growth and proliferation of various types of cancer including colon, breast, lung, liver, and oral cancer cells *in vitro* (Choi, Bae, & Ahn, 2008). It can suppress migration and invasion of colon cancer cells (Han, Song, & Zhang, 2016). Therefore, we will investigate the effects of the onion peel extract on colorectal cancer cell growth and invasion through L1CAM mediated cellular invasion.

Objectives

1. To investigate effects of the onion peel extract on colorectal cancer cell migration and invasion.

2. To study effects of the onion peel extract on L1CAM expression in colorectal cancer cells.

Research Methodology

1. Crude extract of onion peel

Crude extract of onion peel in this study was obtained from co-operation of Detox (Thailand) company under the project of "Quercetin from onion peel inhibit oxidative stress-induced colorectal cancer". Briefly, the onion peel was homogenized, extracted with 70% ethanol and lyophilized. The major compound of crude extract of onion peel is quercetin, which is confirmed by high performance liquid chromatography (HPLC).

2. Cell culture

Caco-2 human colorectal cancer cells were use in this study. Caco-2 cells were cultured in completed Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acid, 1% L-glutamine and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere that supplied with 5% CO_2 incubator.

3. Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used to evaluate the cell viability. $1.5x10^4$ cells of Caco-2 cells were seeded and maintained in 96-well plates for 24 h. Then the old media were replaced with 200 µl media containing onion peel extract at various concentrations for 24 h, followed by removing treated media and adding 200 µl of media containing 20 µl of 5 mg/ml MTT stock solution for 2 h prior to replacing MTT contained media with 100 µl of dimethyl sulfoxide (DMSO). The absorbance was detected at 570 nm with microplate reader (1420 Victor 2, Wallac, USA), the results were expressed as the percentage of viable cells (% cell viability) and the median inhibitory concentration (IC_{50}) of onion peel extract was analyzed. The non-toxic doses were used for further experiments (Teerapat RODBOON, 2015).

$$\% \text{ Cell viability} = (A_{sample}/A_{control}) \times 100$$

A_{sample} = Absorbance of experimental well

$A_{control}$ = Absorbance of negative control well

4. Wound-healing assay

Wound healing assay was used to study cell migration. $1x10^6$ cell/ml of Caco-2 cells were seeded in 6 well plates and incubated at 37°C and 5% CO_2 for 20 h. Then the cells were treated with 10 µg/mL mitomycin C (Naprod Life Sciences Pvt. Ltd.) for 2 hours and removed. Using scratcher (SPL life sciences) made a straight scratch and washed with PBS. The cells were treated with onion peel extract and incubated at 37°C and 5% CO_2. The data analysis was done by ImageJ from snapshot pictures 48 h as the percentage of area of cell migration (Honor L. Glenn, 2016).

$$\text{Area of cell migration (\%)} = (A_0\text{-}A_n) \times 100 / A_0$$

A_0 = Area of scratch line at initiation time

A_n = Area of scratch line at observed time

5. Transwell invasion assay

Cell invasion assay or Boyden chamber assay is used to study the ability of malignant cell to invade normal surrounding tissue. 24-well cell culture insert plate with 8.0 µm membrane pore site was used, 30 µl of extracellular matrix or ECM (Sigma-Aldrich) materials were added on top of the transwell membrane. $5x10^5$ Caco-2 cells were seeded on top of the extracellular matrix (ECM) with fetal bovine serum free media and added onion peel extract. Media with 20% fetal bovine serum was added in lower part. The seeded plate was incubated at 37°C and 5% CO_2 for 48 h. The invaded cells were stained with hematoxylin and eosin before obtained under light microscope (Justus, Leffler, Ruiz-Echevarria, & Yang, 2014).

6. Immunofluorescence assay

Indirect immunofluorescence was used for L1CAM expressions and its localization with a modified method. $1x10^5$ cells were seeded on cover slips and placed at the bottom of 6-well plates. Then seeded cells were incubated at 37°C and 5% CO_2 for 48 h. The culture media was removed and added fresh media with the onion peel extract. The cells were incubated for 24 h, fixed with cold absolute methanol, and washed with PBS. Fixed cells were permeabilized with 0.25% Triton X-100, and then primary antibody for L1CAM (Abcam) was added for 1.5 h and rinsed with PBS. The appropriate fluorescine isothiocyanate (FITC) conjugated secondary antibody was added for 0.5 h. Hoechst-33342 was used for counter staining. The cells were observed under fluorescence microscope (Olympus Model BX53, Japan), the results shown as an intensity of fluorescence were analyzed by Image J program of randomly 5 fields in triplicate (Groschwitz, Wu, Osterfeld, Ahrens, & Hogan, 2013).

7. Statistical analysis

Results were analyzed using SPSS software (version 18). Statistical comparisons was performed using one-way ANOVA as post hoc test the Tukey HSD formula. Data were presented as mean ± standard

error of the mean (SEM) of separate experiments (n=3). p-values less than 0.05 were considered to be significant.

Results

1. Effects of onion peel extract on cell proliferation

MTT assay was used to test the effects of various concentrations of onion peel extract (0.1, 1, 10, 100, 1000 and 10000 µg/ml) in Caco-2 cells for 24 h. Caco-2 cell viability did not change in cells that treated with 0.1, 1 or 10 µg/ml onion peel extract, indicating that onion peel extract at 0.1, 1 and 10 µg/ml has no anti-proliferative effects as shown in Figure 1A. Treatment with 0.1, 1, 10, 100, 1000 and 10000 µg/ml onion peel extract for 24 h decreased Caco-2 cell survival rates to levels that were 101.62±4.85%, 101.19±1.58%, 101.02±3.70%, 63.06%±1.51% ($p<0.001$), 53.33±2.53% ($p<0.001$), and 40.1±2.3% ($p<0.001$) of those in cells untreated, respectively. We next assessed the effects of onion peel extract at non-antiproliferative effects (2.5, 5, 7.5 and 10 µg/ml). Treatment with 2.5, 5, 7.5 and 10 µg/ml onion peel extract for 24 h decreased Caco-2 cell survival rates to levels that were 101.14±2.6%, 101.13±3.18%, 101.06±3.02% and 101.01±5.12% of those in cells untreated, respectively as shown in Figure 1B. We next assessed the effects of onion peel extract at 5 and 10 µg/ml (maximum non-antiproliferative dose) on cell migration, cell invasion and L1CAM expression in Caco-2 cells.

Figure 1A.

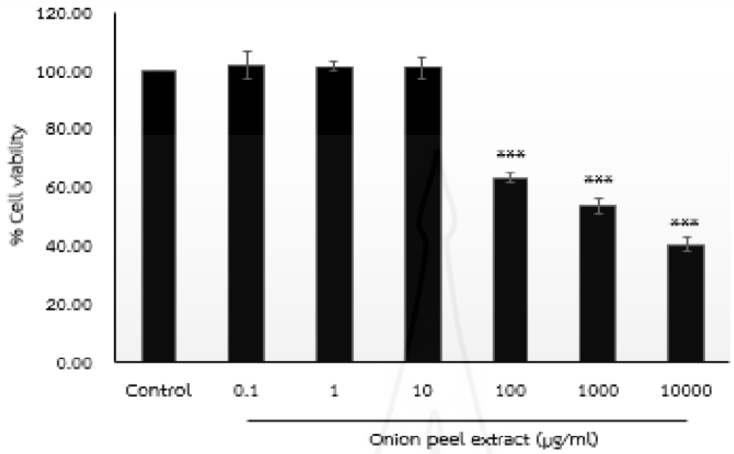


Figure 1B.

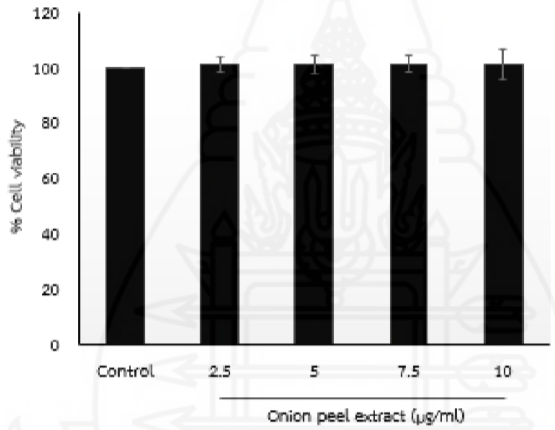


Figure 1. Effects of onion peel extract on Caco-2 cell proliferation. **A.** Caco-2 cells were seeded in a 96-well plate. After incubating for 24 h, the cells were treated with onion peel extract at different concentrations (0.1, 1, 10, 100, 1000 and 10000 µg/ml). **B.** Caco-2 cells were seeded in a 96-well plate. After incubating for 24 h, the cells were treated with onion peel extract at different concentrations (2.5, 5, 7.5 and 10 µg/ml) and then incubated for 24 h. Cell viability was detected by MTT assay. The line graph represents means ± standard error deviation (SE) of the three experiments, ***p<0.001.

2. Onion peel extract inhibited Caco-2 cell migration

To evaluate the effects of onion peel extract on colorectal cancer cell migration, we performed wound healing assay. For this experiment, Caco-2 cells were treated with no anti-proliferative effects doses (5 and 10 µg/ml) of onion peel extract for time intervals (0 and 48 h). The treatment with various doses of onion peel extract for 48 h significantly decreased cell migration rates in Caco-2 cells in dose-dependent manner as shown in Figures 2A and B. The treatment with 5 and 10 µg/ml onion peel extract for 48 h

decreased Caco-2 cell migration rates to levels 93.80±1.10% and 68.19%±4.55% ($p<0.001$) for 48 h of those in cells untreated, respectively.

Figure 2A.

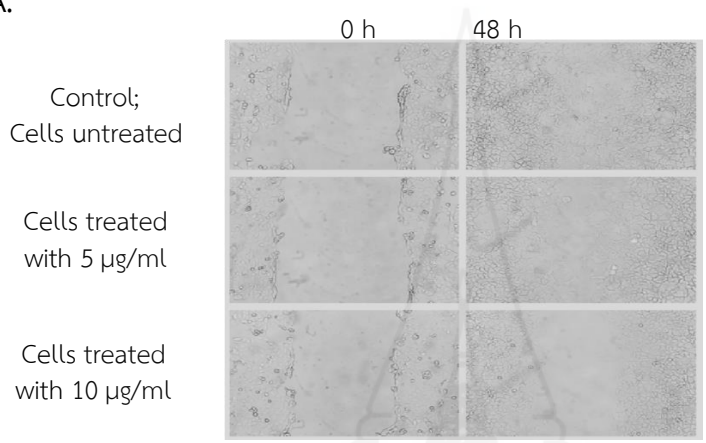


Figure 2B.

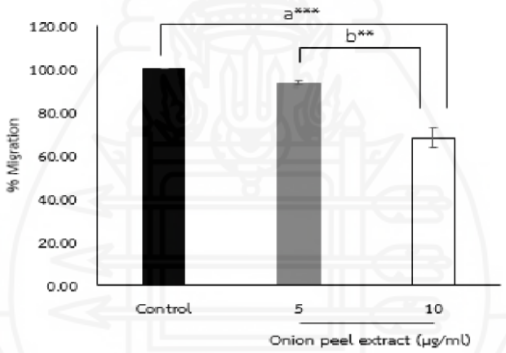
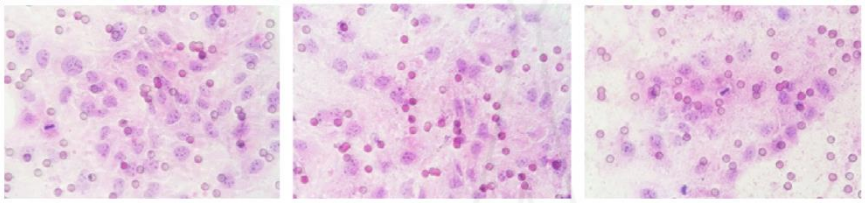


Figure 2. Onion peel extract inhibits Caco-2 cell migration. **A.** Representative images captured under an inverted microscope with 10x magnification after 0 and 48 h treatment with onion peel extract at 5 and 10 µg/ml. **B.** Caco-2 cells were seeded in a 6-well plate and then treated with onion peel extract for 48 h. Cell migration was detected by wound healing assay in cells treated with no anti-proliferative effects doses of onion peel extract (5 and 10 µg/ml) for 48 h. The bar graph represents means ± standard deviation (SD) of the three experiments, **$p<0.01$ and ***$p<0.001$, a = compared with control and b = compared between doses.

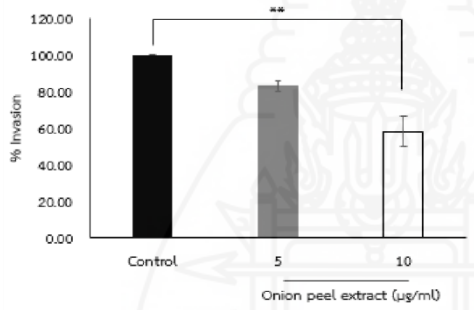
3. Onion peel extract inhibited Caco-2 cell invasion

We also examined cell invasion capacity with a three-dimensional Matrigel-coated filter after the Caco-2 cells were treated with onion peel extract. The cells treatment with onion peel extract significantly

decreased the cell invasion rates, when compared with the control cells, suggesting that onion peel extract inhibits Caco-2 cell invasion in colorectal cancer *in vitro* as shown in Figures 3A and B. The treatment with 5 and 10 µg/ml onion peel extract for 48 h decreased Caco-2 cell invasion rates to levels 83.10±3.10% and



Control; Cells untreated | Cells treated with 5 µg/ml | Cells treated with 10 µg/ml



58.30%±8.14% ($p<0.01$) of those in cells untreated, respectively.

Figure 3A.

Figure 3. Onion peel extract inhibits Caco-2 cell invasion. **A.** Representative images captured under a light microscope with 40x magnification after 48 h of treatment with onion peel extract at 5 and 10 µg/ml. **B.** For cell invasion assay, Caco-2 cells were seeded in the upper chamber of a transwell apparatus in serum-free media for 48 h. Cell invasion was detected by transwell assay. Invading cells were stained with Hematoxylin and Eosin. The bar graph represents means ± standard deviation (SD) of three experiments, **$p<0.01$.

Figure 3B.

4. Onion peel extract downregulated L1CAM expression in Caco-2 cells

To determine the expression of L1CAM, we assessed the effects of onion peel extract on L1CAM expression by immunofluorescence. We treated Caco-2 cells with 5 and 10 µg/ml for 48 h. The results demonstrated that the treatment with 5 and 10 µg/ml onion peel extract significantly decreased the L1CAM in Caco-2 cells, when compared with the control as shown in Figures 4A and B. The treatment with 5 and

10 µg/ml onion peel extract for 48 h decreased the L1CAM expression to levels 41.46%±2.64 ($p<0.001$) and 35.34%±1.53% ($p<0.001$) of those in cells untreated, respectively.

Figure 4A.

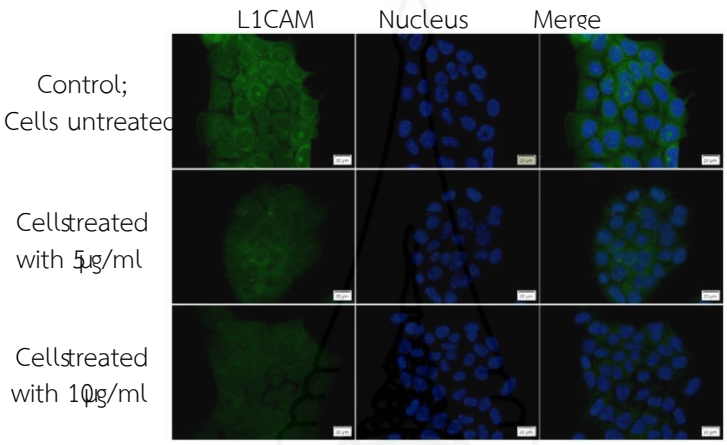


Figure 4B.

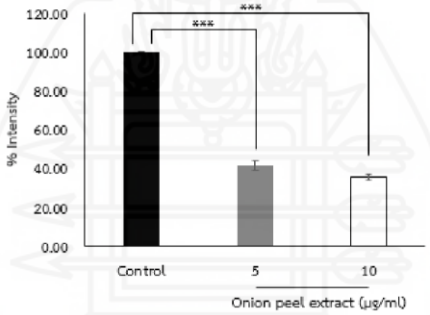


Figure 4. Onion peel extract downregulates L1CAM expression in Caco-2 cells. **A.** Representative images of L1CAM immunofluorescence staining after treatment with onion peel extract at 5 and 10 µg/ml for 48 h. L1CAM (green) and Hoechst-33342 (blue). Scale bar, 20 µm. **B.** Caco-2 cells were treated with onion peel extract at 10 µg/ml and then incubated for 24 h. Changes in L1CAM expression were detected by Immunofluorescence. The bar graph represents means ± standard deviation (SD) of the three experiments, ***$p<0.001$.

Discussion

Quercetin is a natural flavonoid compound that has attracted increased interest and attention due to its anticancer activity including induced apoptosis and anti-metastasis activity (Khan et al., 2016). *In vitro* and *in vivo* studies have verified that quercetin effectively inhibits various cancers via different mechanisms including colorectal cancer (Hashemzaei et al., 2017). The important process of tumor invasion is the epithelial-to-mesenchymal transition (EMT) initiated from tumor cells which lose their epithelial features and acquire a mesenchymal phenotype leading to invasive and migratory behavior of cancer cells. Previous studies showed that L1CAM is involved in EMT via integrin binding and activating NF-kB in pancreatic ductal adenocarcinoma (Tischler et al., 2011). In human tumor, L1CAM expression related to cellular properties such as migration, invasion, growth, metastasis and chemo-resistance that associated with poor prognosis (Fogel et al., 2003; Kiefel, Pfeifer, Bondong, Hazin, & Altevogt, 2011).

This study aims to investigate the effects of onion peel crude extract on Caco-2 migration and invasion through the expression of L1CAM, in which cancer cells used for invasive migratory capability. Our finding shows that the major component of onion peel extract was quercetin confirmed using high performance liquid chromatography (data not shown). Moreover, the effects of onion peel crude extract inhibited cellular migration and invasion. These results suggested that quercetin could inhibit Caco-2 migration and invasion. Also shown in previous studies, quercetin inhibited cell migration and invasion in various cancers including human osteosarcoma cells, prostate cancer cell line, oral cancer cells (Lai et al., 2013; Lan et al., 2017; Senthilkumar et al., 2011).

Although L1CAM has been shown to be a prognostic marker in various cancers and has been suggested to play a role in epithelial mesenchymal transition (Schrevel et al., 2017), L1CAM-dependent signalling capabilities are still incompletely understood. Herein we first demonstrate the localization of L1CAM expression in Caco-2 cells by immunofluorescence assay. The expression of L1CAM mainly expresses around nucleus with corresponding to their function (Kiefel et al., 2012). Our finding shows the effects of onion peel crude extract inhibited L1CAM expression. This result suggested that onion peel crude extract inhibited Caco-2 cells invasion through reduced L1CAM expression. Previous studies have shown that L1CAM expression enhances cell motility, increases cell invasion, augments tumor growth and metastases formation (Gavert, Ben-Shmuel, Lemmon, Brabletz, & Ben-Ze'ev, 2010; Gavert et al., 2005; Tischler et al., 2011). L1CAM promotes EMT with increased characteristics of cancer initiating cell and paclitaxel resistance in human endometrial cancer cells (Chen, Gao, & Liu, 2018).

In conclusion, onion peel extract inhibited Caco-2 cells migration and invasion through reduced L1CAM expression. Further study is needed to clarify the molecular mechanisms of L1CAM and invasive

capacity of colorectal cancer. Onion peel extract may have potential as a novel chemotherapy in anti-metastasis of colorectal cancer.

Suggestion

Phytochemicals treatment or natural products have been used to prevent and treat various diseases including colorectal cancer. There are excellent sources of bioactive components exerting their health beneficial effects, and very often, these sources are materials for gourmet food consumptions. Quercetin is flavonoids present in many vegetables, fruits and beverages such as onion, which polyphenolic compounds that may become potential therapeutic agents. These results suggested that onion peel extract with quercetin rich component could inhibit Caco-2 migration and invasion. Onion peel extract may have potential as a novel chemotherapy in anti-metastasis of colorectal cancer.

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