Solvent fractionation of *Clinacanthus nutans* (Burm.f.) Lindau and characterization of its fractions for anti-apoptosis activity in bovine endothelial cells

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Abstract

Clinacanthus nutans (Burm.f.) Lindau belongs to the Thai national list of essential medicine (NLEM). This plant has been used in the treatment and prevention of a variety of symptoms such as anti-viral, anti-bacterial, and anti-inflammatory activity. In this study, we investigated the effects of different extract fractions of *C. nutans* on anti-cell death. Firstly, the *C. nutans* fractionation was performed by sequential extraction using different organic solvents including hexane, dichloromethane, ethyl acetate, and water, respectively. We further investigated its anti-apoptosis activity in bovine endothelial cells (CPAE) after the treatment of *Escherichia coli* lipopolysaccharide (LPS) by using PrestoBlue™ cell-viability assay. The result showed that LPS strongly induced cell deaths with the CC50 values of LPS of 57.62 ng/mL. Interestingly, all fractions of *C. nutans* extract significantly lower the LPS-induced cell death in a dose-dependent manner. The ethyl acetate fraction was the most effective to protect cell death. At 125 µg/mL, it could protect more than 80% of the cells. Furthermore, the bioactive compound glyceryl 1,3 distearate ($C_{39}H_{76}O_5$) was measured in *C. nutans* extract by using TLC analysis which could be the major compound that contributed to the anti-apoptosis activity. Our finding could lead to the development of *C. nutans* extract to treat inflammation during bovine mastitis.

Keywords**:** *Clinacanthus nutans (Burm.f.) Lindau, Bovine mastitis, Cell death, glyceryl 1,3 distearate, TLC analysis, Sequential extraction*

1. Introduction

Clinacanthus nutans (Burm.f.) Lindau (Phaya Yo or Saled Pangpon Tua Mea in Thailand). belonging to the *Acanthaceae* family has been used in traditional herbal medicine in Thailand and Southeast Asia. *C. nutans* has been widely studied for its pharmacological properties and strong anti-viral, anti-inflammatory, anti-bacterial, anti-apoptotic, anti-tumorigenic, and stimulating immunity activities (Pongmuangmul et al., 2016; Mai et al., 2016; Zulkipli et al., 2017; Panya et al., 2020). Every part of this plant has been used for treatment, but the most popular was the leaf that has been reported for treatments of herpes simplex virus types 1 and 2 (Pongmuangmul et al., 2016), skin rashes, burns, fever, snakebite, and some types of cancers (Aslam et al., 2015).

Bovine mastitis is an inflammatory response of the mammary gland caused by the infection of bacteria that invaded the teat canal of the mammary gland. Mostly, bovine mastitis was caused by various bacteria species i.e., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis* (gram-positive) *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (gram-negative) (Erskine, 2020; Strandberg et al., 2005). The lipopolysaccharide (LPS), an endotoxin component of gram-negative bacterial cell walls such as *E. coli* has been reported to promote the immune response of bovine mammary epithelial cells and its effect was stronger than lipoteichoic acid (LTA) from *Staphylococcus aureus* (Zbinden et al., 2014). LPS is a highly inflammatory molecule that activates a wide range of endothelial responses, including the upregulation of cytokines, and chemokine resulting in endothelial cell death through the apoptosis pathway (Bannerman & Goldblum, 2003; Panya et al., 2020). When the bacteria enter

through the teat canal and multiply inside the gland, it causes cell death and inflammation of the mammary gland which can alter either quality or quantity of milk production and highly impacts the financial loss. Currently, the treatment of bovine mastitis is generally used antibiotics such as streptomycin, ampicillin, cloxacillin, penicillin, and tetracycline (Bhosale et al., 2014). However, people were concerned the rapid increase in bacteria resistance and drug residues in the milk would be transferred to humans in addition to the cost of the treatment. Those facts lead the huge attempts to find an alternative treatment to replace antibiotics. Previously, we discovered that *C. nutans* crude extract exerted the most effective anti-bacterial and anti-cell death activity among 14 herbal extracts in LPS treated bovine endothelial cells (CPAE) (Panya et al., 2020). In this present study, we further characterized the anti-cell death effect of different extract fractions of *C. nutans* including hexane fractions, dichloromethane fractions, ethyl acetate fractions, and water fractions in LPS induced cell death by using a cell viability assay. The presence of bioactive compound glyceryl 1,3 distearate ($C_{39}H_{76}O_5$) was confirmed in these fractions by using TLC. Our finding the potent extracts fractions represent the frontier of new therapies for bovine mastitis treatment.

2. Objectives

1. To investigate the anti-cell death effect of *C. nutans* fractions in LPS stimulated-bovine endothelial cells

2. To investigate the effect of glyceryl 1,3 distearate ($C_{39}H_{76}O_5$), a bioactive compound, in *C. nutans* extracts on anti-cell death activity

3. Materials and Methods

3.1 Herbal leave extracts

C. nutans was purchased from the organic market, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand, 2019. Dr.Narin Printarakul, taxonomist, Department of Biology, Faculty of Science, Chiang Mai University identified and prepared voucher specimen No.CN001-003 at the Department of Biology. The mature leaves were separated, cleaned, and dried in a hot air oven at 50 °C for 2 days. Using the grinder, the plants were ground into powder. 70 % ethanol (a mixed herb with 70 % ethanol 1:20 ratio) was used to extract the herb powder, which was shaken at 160 rpm/min for 12 hours at room temperature. The extracts were filtered with Whatman No. 1 filter paper before being evaporated with a rotary evaporator and were thoroughly dried in a water bath at 95°C to eliminate the solvent extracts. The extracts were stored at 4°C until use, in which the extracts were dissolved using DMSO for treated cells.

3.2 Fractionation of *C. nutans* crude Extracts

The 70% ethanolic *C. nutans* crude extract was dissolved in water with 5% Methanol (MeOH) in the total volume. Sequential extraction was used to produce 4 extract fractions of the fractionated crude extract: hexane fraction (C_6H_{14}), dichloromethane fraction (CH_2CI_2), ethyl acetate fraction ($C_4H_8O_2$), and water fraction (H_2O) respectively. Using a separatory flask, the MeOH portion was partitioned with hexane at a ratio of 1:1 for 3 times. The hexane was separated first, then followed by dichloromethane, ethyl acetate, and water in a ratio of 1:1. After that, a water bath at 95°C was used to eliminate the solvent extracts. All parts of the extracts were evaporated and stored at 4 °C until use and the extracts were dissolved by DMSO for treated cells.

3.3 Cell Lines and Reagents

The bovine endothelial cell lines (CPAE; CCL209TM), were cultured in minimal essential medium (MEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% L-glutamine, 1%antibiotics, and 20% (v/v) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 ºC under 5% CO_2 humidified atmosphere. In the experiment, the cells were plated a day before the experiment.

3.4 Cell viability

The CPAE cells were plated in 96-well plates at a density of approximately 7,000 cells/well a day before the experiment and were treated cells with LPS concentrations at 6.25-100 ng/mL (2-fold dilution) (LPS derived from *E. coli* 0111:B4, Sigma-Aldrich, St. Louis, MO, USA), *C. nutans* crude extract and *C. nutans* fraction extract concentration at 15.63-1,000 µg/mL (2-fold dilution) for 24 hours at 37 ºC under 5% CO_2 humidified atmosphere and was determined % cell viability by PrestoBLUE™ cell viability reagent (Thermo Fisher Scientific, MA, USA) at 570 nm and 595 nm by a microplate reader (EZ Read 2000, Biochrom, Cambridge, UK). The % cell viability was calculated compared to that of normal cells and the values of CC50 (half-maximal cytotoxicity concentration) by regression analysis. After that, LPS was treated (at final concentrations of 50 ng/mL in each sample) in combination with all extract fractions concentration at 3.90-125 µg/mL (2-fold dilution) in CPAE cells. The % cell viability was calculated using the formula below.

% Cell viability = [(OD570-OD595) treated cells/(OD570-OD595) non-treated cells] X 100

3.5 Thin-layer chromatography (TLC) analysis

The extracts of *C. nutans* and its bioactive compounds were investigated on the TLC plate and were developed in the mobile phase; formic acid: water: methanol: ethyl acetate: chloroform: hexane in a 0.1:0.1:2:2:3:3 ratio. After that, spray the TLC plate with 10% H_2SO_4 dissolved in methanol and heat the TLC plate at 120 ºC on a hot plate for 5 minutes. The retention factor (Rf) was measured using the CAMAG Linomat 5 (Camag Chemie-Erzeugnisseund Adsorptionstechnik AG, Muttenz, Switzerland) under UV light at 366 nm.

4. Results and Discussion

4.1 The effect of LPS on inducing cell death in CPAE cell lines

In this experiment, we investigated the cytotoxic effect of LPS of *E. coli* in the CPAE cell line. Firstly, the cells were treated with LPS at concentrations of 6.25-100 ng/mL (2-fold dilution) for 24 hours. The result showed that LPS could lower the cell viability significantly in a dose-dependent manner by lowering the percentage of cell viability to 75%, 47.33%, and 37.66% after treatment of LPS at concentrations of 25, 50, and 100 ng/mL, respectively. The half-maximal cytotoxic concentration (CC50) was analyzed using the non-linear regression which showed the LPS caused the cell death with the CC50 of 57.62 ng/mL in CPAE cells (Figure 1). In accompanied with the previous reports, LPS could dramatically cause endothelial cell death. The pathogenesis of LPS on endothelial cells (ECs) after binding and activating TLR4 of ECs resulted in increased releasing of various inflammatory cytokines (*IL6* and *TNF-α*) and lead to mortality of cells (Yi et al., 2019). Interestingly, LPS mediated cytotoxic effect was dependent on the species of endothelial cells. Based on our study, bovine endothelial cells, CPAE, were very sensitive to LPS compared with other species such as endothelial isolated from human umbilical vein, goat aortic, and canine vena cava endothelial cells that have been reported to resist the LPS at the concentration up to 100 µg/ml (Harlan et al., 1983).

4.2 The anti-cell death effect of *C. nutans* crude extract and its fractions in LPS-treated CPAE cells.

We performed the sequential extraction of the *C. nutans* extract following the polarity of solvent from low polar to high polar including hexane fractions, dichloromethane fractions, ethyl acetate fractions, and water fractions, respectively. We investigated the cytotoxic of extracts fractions in CPAE cells. The result showed that at the concentration of 15.63-125 µg/mL, all fractions except dichloromethane yielded cell viability of more than 80% (Figure 2).

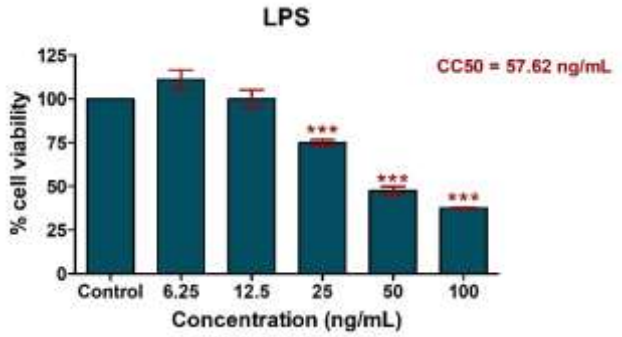

Figure 1 The effect of LPS on CPEA cells death when LPS-treated cells for 24 hours

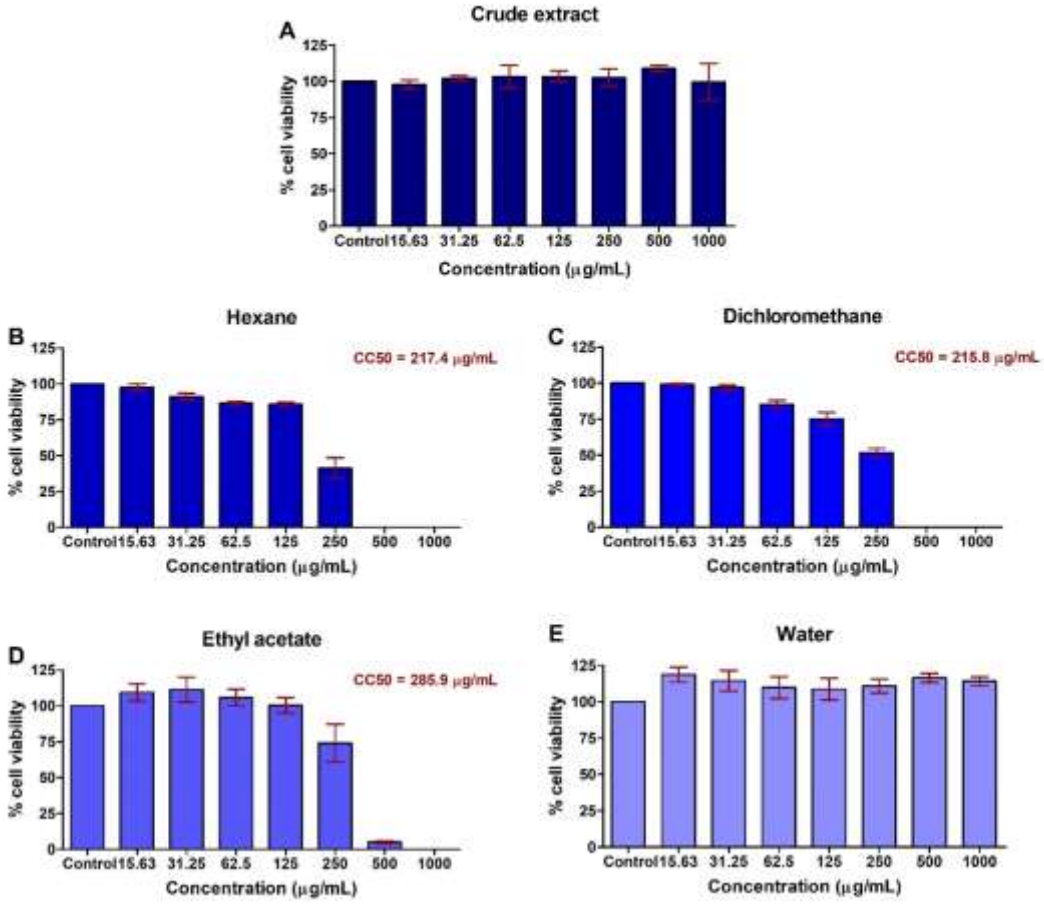

Figure 2 Cytotoxicity of *C. nutans* in CPAE cell lines for 24 hours. The cell viability of CPAE was measured after treatment with various concentrations (15.63-1000 µg/mL) of *C. nutans* crude extracts (A); hexane fractions (B); dichloromethane fractions (C); ethyl acetate fraction (D); water fractions (E)

The fractions which the most toxic to the cells were dichloromethane (CC50 of 215.8 µg/mL), hexane (CC50 of 217.4 µg/mL), and ethyl acetate fraction (CC50 of 285.9 µg/mL), respectively. The effect of extract fractions on anti-cell death was measured after 24 hours of LPS treatment (50 ng/mL) in the presence or absence of various concentrations of each fraction. The ethyl acetate fraction showed the most effective to reduce cell death followed by crude extract and water fraction. At the equal concentration (62.50 µg/mL), the ethyl acetate fraction, crude extract, and water fraction yielded 82.75%, 78.25%, and 81.75%, respectively (Figure 3). The results were concordant with the result of cell morphology in which the morphology of CPAE cell lines in LPS combined with *C. nutans* crude extracts and *C. nutans* fractions at the concentration of 125 µg/mL had fewer dead cells than LPS treated control (Figure 4).

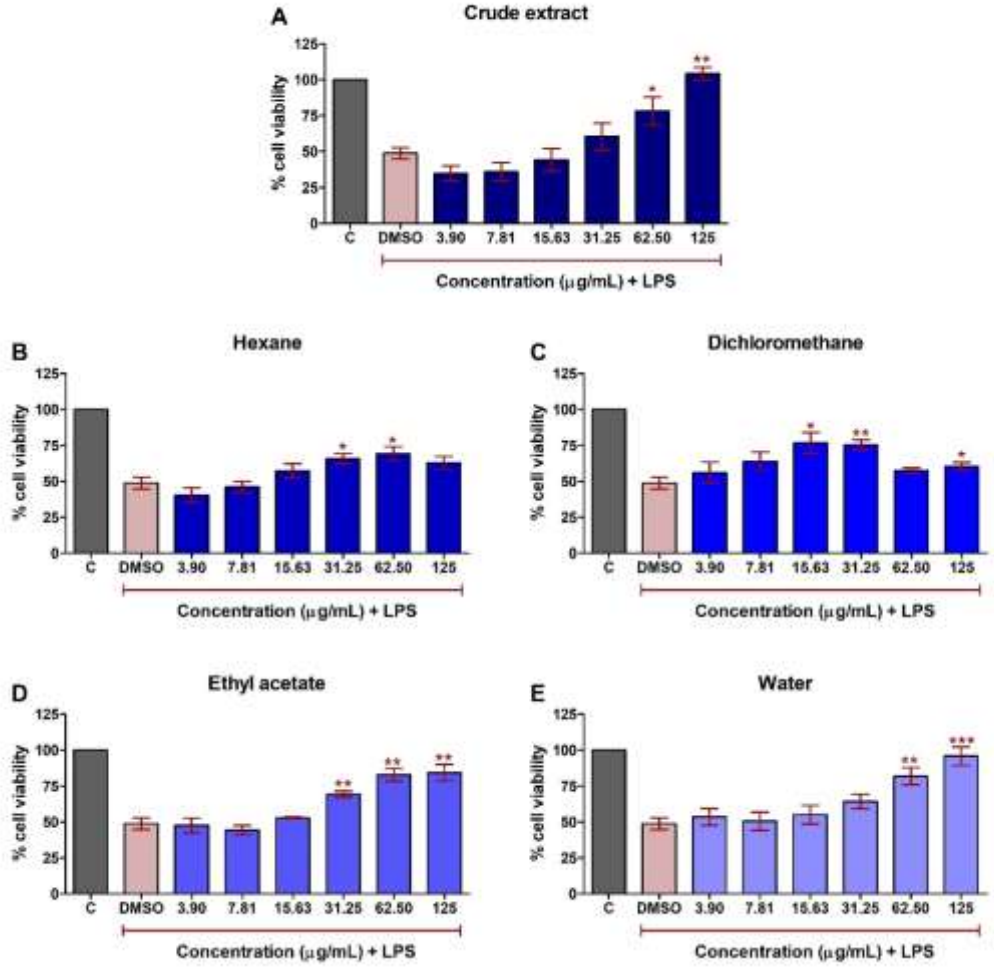

Figure 3 The anti-cell death effect of *C. nutans* extracts in CPAE cells after LPS treatment. The cell viability was measured after 24-hour treatment of LPS in the presence or absence of *C. nutans* extracts (A); hexane fractions (B); dichloromethane fractions (C); ethyl acetate fraction (D); water fractions (E)

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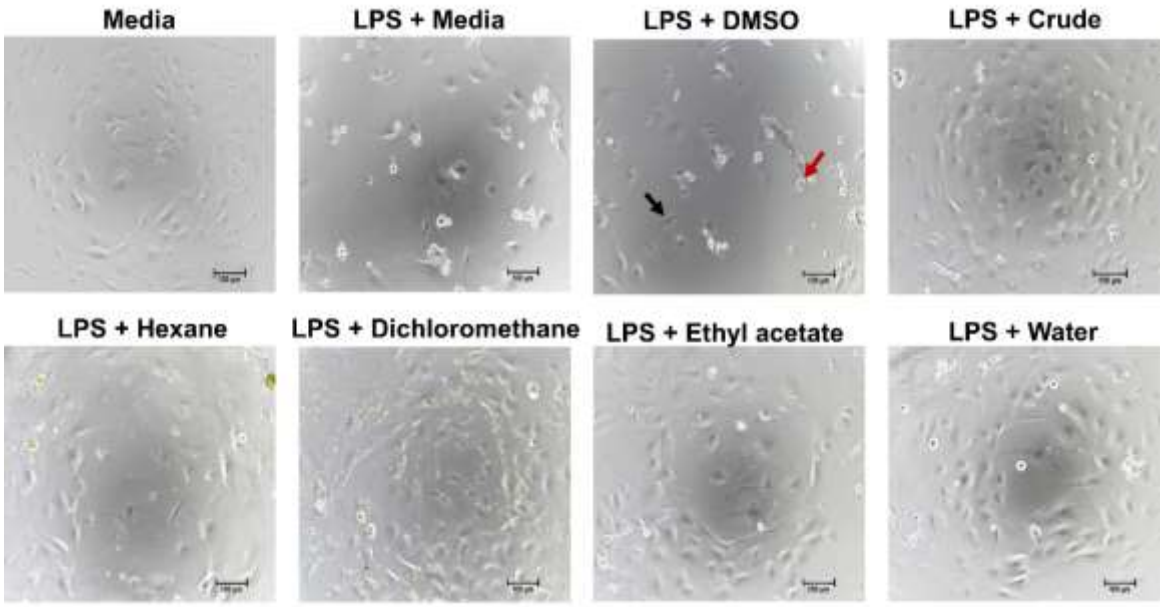

Figure 4 The changes in CPEA cell morphology upon LPS treatment with *C. nutans* fraction were observed under the light microscope, **in** which the black arrow showed the normal cell and the red arrow showed the death cell

The solvent used in plant extraction is one of the key successes since the variety of bioactive compounds contained in plants had different solubility properties in different solvents. In accompanied with the previous report, our study showed that ethyl acetate, ethanol, and water fraction were more effective to reduce cell death greater than hexane and dichloromethane fractions in CPAE cells. Thus, our study emphasized the polar solvent was optimal for *C. nutans* extraction at least for its anti-cell death activity. Currently, herbs and medicinal plants are alternative sources for drug development. Recently, the baicalin *cutellaria baicalensis*) extract, a traditional Chinese herbal medicine, was reported to reduce mRNA expression of proinflammatory cytokine *TNF-α* and *IL-1β* in the cow mammary epithelial cells (CMECs) after LPS treatment (10 μg/mL) (Yang et al., 2016). Notably, the inflammation caused by LPS induction is one of the significant hallmarks of bovine mastitis. According to our study that showed the *C. nutans* extract and its fractions could inhibit the effect of LPS thus, it might have diverse effects to inhibit the inflammation in bovine mastitis. However, further study is needed to investigate the anti-inflammation of *C. nutans* extract which would emphasize the benefit of bovine mastitis.

4.3 Identification of glyceryl 1,3 distearate in *C. nutans* extract by TLC analysis

The previous study reported glyceryl 1,3 distearate ($C_{39}H_{76}O_5$) as a bioactive compound in *C. nutans* leave extracts by LC-MS/MS analysis (Panya et al., 2020). To investigate whether the glyceryl 1,3 distearate was the bioactive compound that contributed to the biological activity, we determined the presence of glyceryl 1,3 distearate in *C. nutans* fractions by TLC analysis. As expected, the results showed that all fractions of *C. nutans* contained glyceryl 1,3 distearate ($C_{39}H_{76}O_5$) with a retention factor of 0.58 (Figure 5). Since glyceryl 1,3 distearate can be soluble in polar and nonpolar solvents; thus, it is possible to be detected in all fractions. However, its solubility would be varied in the different polarity solvents and influence the amount of glyceryl 1,3 distearate in the solvents while the TLC technique could not judge the concentrations of glyceryl 1,3 distearate.

The ethyl acetate fraction showed the most potential to reduce the LPS-induced cell death in our study. However, we did not exclude the possibility that the activity might be contributed by the effect of

the other minor substances or could be a synergistic effect of the compounds in the extract. Recently, the bioactive compound called vitexin has been reported in the ethyl acetate fraction of *C. nutans* extract (Febriansah et al., 2021). Interestingly, its activity in lowering inflammation has been revealed in human neutrophils (Nikafarjam et al., 2017). It is well documented that the overcontrolled inflammation might cause the cell-death, especially in the LPS-induced condition. Hence, there is the possibility that the most likely activity observed in ethyl acetate fraction might be contributed not only by glyceryl 1,3 distearate but through other substances such as vitexin. However, the association between inflammation and cell death, in addition to the effect of glyceryl 1,3 distearate in combination with vitexin, is needed to be investigated.

The anti-apoptosis effect of glyceryl 1,3 distearate has never been reported up to date. Previously, Cheng and colleagues (2017) reported the activity of the closely related compound called glyceryl 1,3-dipalmitate purified from *Lactobacillus paracasei* to prevent SH-SY5Y cells (human neuroblastoma cells) from injury after oxygen-glucose deprivation and reperfusion. This compound could effectively reduce the expression of the NF-$_K$B that causes inflammation and apoptosis cells. Considering the chemical structure, glyceryl 1,3 distearate possibly use this pathway to reduce the anti-cell death but needed concrete evidence to support this hypothesis in bovine endothelial cells.

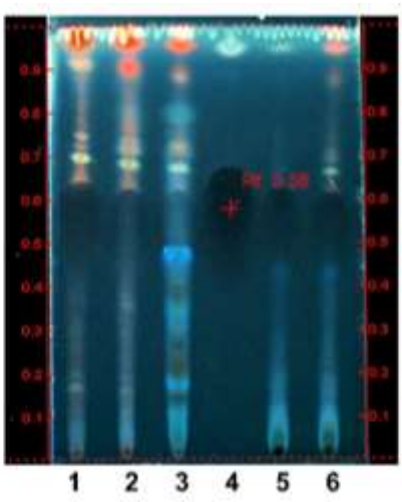

Figure 5. TLC chromatogram of *C. nutans* fractions (1); hexane fraction (2); dichloromethane fraction (3); glyceryl 1,3 distearate, Rf 0.58 (4); ethyl acetate fraction (5); and water fraction (6)

5. Conclusion

Our result demonstrated that LPS strongly induced cell death and the ethyl acetate fraction was the most effective to protected cell death. The glyceryl 1,3 distearate ($C_{39}H_{76}O_5$) was found in all *C. nutans* fractions and could contribute to anti-cell death activity. Our finding could lead to the development of *C. nutans* extract to treat inflammation during bovine mastitis. However, further studies especially clinical tests, should be carried out in the future.

6. Acknowledgements

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