International Food Research Journal 25(4): 1397-1402 (August 2018)
Journal homepage: http://www.ifrj.upm.edu.my

An evaluation of soyghurt potential on tumor necrosis factor-α and soluble endoglin levels in preclampsia maternal serum-induced placental trophoblast cell *in vitro*

[1*] Vioretti, R., [2]Khairani, A.F., [3]Fauziah, P. N. and [4]Hilmanto, D.

[1]*Department of Medical Nutrition, Faculty of Medicine, Universitas Padjadjaran, Bandung, 45363, Indonesia*
[2]*Department of Anatomy, Physiology and Cell Biology, Faculty of Medicine, Universitas Padjadjaran, Bandung, 45363, Indonesia*
[3]*Department of Medical Laboratory Technology, School of Health Sciences Jenderal Achmad Yani Cimahi, 40533, Indonesia*
[4]*Department of Pediatrics, Faculty of Medicine, Universitas Padjadjaran, Bandung, 45363, Indonesia*

Article history

Received: 10 April 2017
Received in revised form: 24 May 2017
Accepted: 25 May 2017

Keywords

Lactobacillus bulgaricus ATCC 11842
Preeclampsia
Soyghurt
sEng
TNF-α

Abstract

Soyghurt is soy milk that has been fermented by probiotic bacteria i.e *Lactobacillus bulgaricus* ATCC 11842, which can enhance the immune system (immunomodulator). *In vitro* laboratory analytical study has been conducted on soyghurt as immunosupressant to decrease of TNF-α and sEng levels as molecular marker in preeclampsia pregnancy. The study used experimental method with 4 stages (i.e making of soybean milk and soyghurt, making of growth and pH curve, trophoblast primary cell culture, and determination of TNF-α and sEng levels) and analyzed by ANOVA followed by Duncan's multiple range test (DMRT). Results showed that the level of TNF-α on preeclampsia trophoblast cell with soyghurt had decreased from 34.79 pg/ml to 25.87 pg/ml after incubation for 24 h, but more decreased from 36.16 pg/ml to 24.98 pg/ml after 48 h. The level of sEng on preeclampsia trophoblast cell with soyghurt had decreased from 31.23 pg/ml to 24.16 pg/ml after 24 h and more decreased from 31.76 pg/ml to 23.65 pg/ml after 48 h. Decreased levels of TNF-α and sEng were very significant, with $p < 0.001$. In conclusion, soyghurt has the ability as an immunomodulator that decrease the level of TNF-α and sEng in which he greatest result was obtained in concentration of 80% and 48 h incubation time.

Introduction

Maternal mortality was 321 per 100.000 living birth in 2007 according to Health Office, West Java. Preeclampsia and eclampsia are the second causes in maternal deaths after hemorrhage (Gurnadi *et al*., 2015). Maternal deaths due to hemorrhage was 40-60%, whilst preeclampsia and eeclampsia were 20-30%. For two years (2006-2007), preeclampsia occurence was 13.62% cases in Hasan Sadikin Hospital Bandung (Gunardi *et al*., 2016).

Etiology of preeclampsia remains unclear. This disease is therefore considered as disease of theory. Trophoblast invasion has been proposed as the major cause that promotes disturbance in spiral artery remodelling. Trophoblast invasion occurs due to immunology maladaptation. Poor trophoblast invasion causes endovascular trophoblast to layer decidual blood vessel, yet unable to reach myometrium that later diminish blood vessel. Diminished arteriol miometrium causes disturbance in placental blood circulation. Bad perfusion and hypoxia trigger release of placental debris, resulting in inflammation and other pathological events (Pramatirta *et al*., 2015).

Inflammatory mediators, angiogenics, antiangiogenics and placental metabolism are further present that lead to endothelial damage. Endothelial damage stimulates trombocyte to undergo adhesion, agegregation, and release. Both these events are terminated as indicated by decreased vasodilators, such as prostacyclin and nitrite oxide/ endothelium-derived relaxing factor and increased vasoconstrictors, such as tromboxan and endothelin, and TNF-α (Pramatirta *et al*., 2015).

There are angiogenic and antiangiogenic factors during placentation. In preeclampsia, angiogenic imbalance worsen hypoxia and placental implantation. There are two antiangiogenics excesscively produced in preeclamptic patients, soluble Endoglin (sEng) and soluble Fms-like tyrosine kinase (SFlt-1) (Gurnadi *et al*., 2015; Pramatirta *et al*., 2015; Gunardi *et al*., 2016).

*Corresponding author.
Email: reynavioretti@gmail.com

One of the promising strategies in balancing angiogenics and antiogenics, as well as act as immunomodulator, is by admistration of soyghurt. Previous study showed soyghurt increases immunity to pneumonia by decreasing the adhesion ability and number of *Klebsiella pneumonia* (Fauziah *et al.*, 2013). In that study, Hep-2 cell line were grown in medium supplemented with soyghurt 10-100%. Soyghurt is a synbiotic in fermented soymilk using *Lactobacillus* sp. and *Streptococcus thermophilus.* Lactic acid bacteria is a bacteria group that possess many beneficial effects for the hosts by enhancing the growth and immunity againts diseases. Soyghurt is also a health drink for those who suffer lactose intolerance by decreasing lactose to 25%. Other components such as saponin and soyprotein also have antioxidant properties. Yoghurt contain isoflavon known to reduce the risk of degenerative disease such as osteoporosis and cancer (Fauziah *et al*., 2015).

Trophoblast cells are derived from nine months placental trophoblast in the end of trimester. In previous in vitro study, trophoblast H8 cell line derived from first trimester, were disturbed after treated with serum which equal with common maternal disturbance (Neale *et al*., 2003). Serum is a blood plasma without fibrinogen composed of protein including electrolyte, antibody, antigen, hormone and exogenous substances. Antibody contained in serum is known to generate inflammation (Gunardi *et al*., 2016). This study aimed to observe effect of soyghurt on level of TNF-α and soluble Endoglin (sEng) in vitro on nine months trophoblast cell induced by maternal preeclampsia serum.

Materials and Methods

The research was conducted from October 2015 - June 2016 in Department of Obstetrics and Gynecology, Hasan Sadikin Hospital, Faculty of Medicine, Padjadjaran University, Laboratory of Cell Culture and Laboratory of Microbiology, Padjadjaran University. Blood samples were carried from normal and preeclamptic pregnant women. Informed consent were obtained from research subjects.

Preparation of soyghurt

Soymilk was made of yellow soybean. A 300 g soybean was washed and soaked in 5 L water mixed with sodium bicarbonate ($NaHCO_3$) of 0.25 – 0.5% for 12-24 h. Soybean was washed and peeled. Soybean was mixed with 2.5 L of hot water (80°C - 100°C) and crushed with a blender -for 7 min to obtain pasta form. Pasta was filtered, and added to 125 g sugar, sterilised at 121°C 1 atm (15 lbs) for 10 min. Soyghurt was made of soymilk using *L. bulgaricus* cultured in MRS. *L. bulgaricus* of 100 mL was inoculated in soymilk medium, then incubated in shaker bath incubator for 24 h at 37-40°C 125 rpm (Fauziah *et al.*, 2013).

Growth and pH curve of L. bulgaricus *ATCC 11842 in soyghurt*

Growth rate of *L. bulgaricus* was observed to obtain optimum age of inoculum to be suitable in assesment of proliferation and pH of cells. Starter of 10 mL was added into soymilk of 90 mL. Culture was then batch fermented in scale of 100 mL at 37°C for 24 min and the growth was measured every 2 hour. Bacteria was counted directly with total plate count. Growth curve was made by plotting colony towards time (Fauziah *et al*., 2015).

Measurement of IC_{50} (inhibitor concentration 50%) in various concentration of soyghurt on trophoblast cells

Samples in serial concentration (10%-100%) in trophoblast cells were used to determine cytotoxicity toward cells. Positive control was aquadest and DPPH, and negative control was aquadest™ and trophoblast without soyghurt. Absorbance was recorded at 517 nm wavelength at hour 24, and IC_{50} of each sample was then calculated. Negative control was used as standard in IC_{50} assesment (Gunardi *et al*., 2016).

Cell culture

Trophoblast cell was carried from maternal placenta of 34-42 weeks of pregnancy. AmnioMax was used as selective medium for placenta. Cells were passaged for 7 times using RPMI 1640. Primary culture of trophoblast was supplemented into new medium containing RPMI 1640 supplemented with 10% of normal or preeclampsia serum, antibiotic-antimikotic (1% Penicillin G-Streptomycin Solution Stabilised dan 1% Fungizone Amphotericin B). Cells were then incubated for 24 h at 37°C in atmosphere of 5% CO_2 (v/v) to reach confluence Viability was measured on its antibodi titer (Pramatirta *et al.*, 2016).

Measurement of TNF-α and sEng

Cells of $6x10^5$ cell/ml containing 10% of normal and preeclampsia serum, was placed into 96 well-plate, and incubated at 37°C 5% CO_2 (v/v) untill confluent (Figure 1). Wells were washed 3-4 times with PBS 37°C to remove the medium and unattached cells. Soyghurt in various concentration was added in accordance with growth rate and pH, incubated for

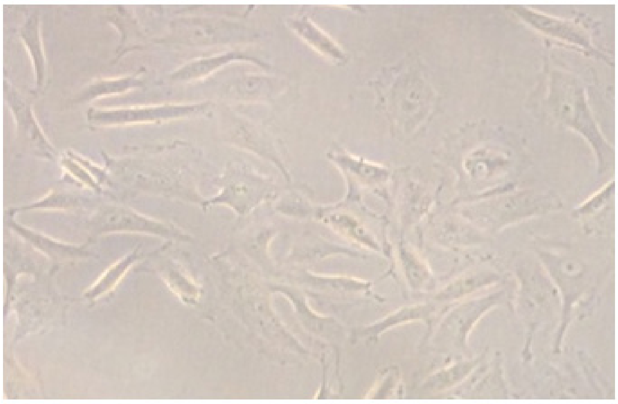

Figure 1. Trophoblast cell after 80% confluent

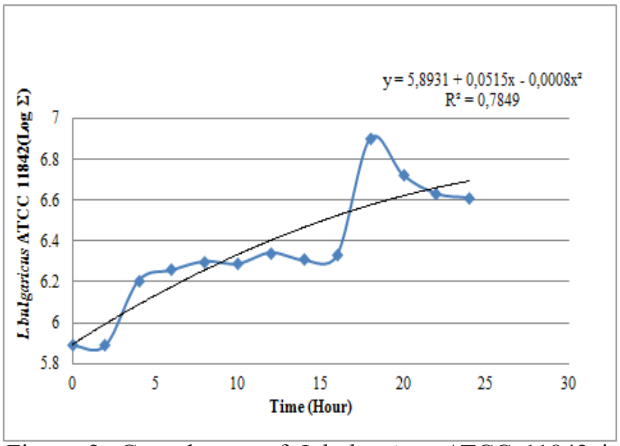

Figure 2. Growth rate of *L.bulgaricus* ATCC 11842 in soyghurt towards time

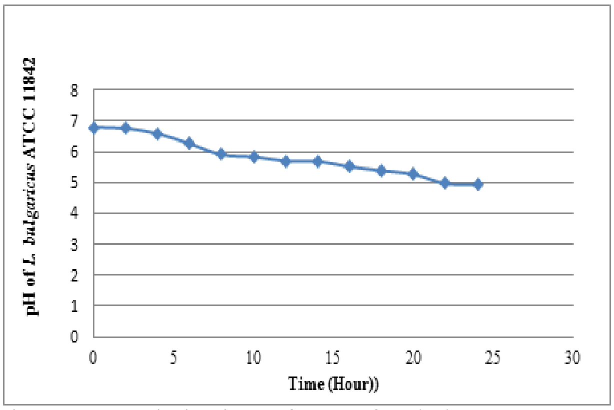

Figure 3. Optimization of pH of *L.bulgaricus* ATCC 11842 in soyghurt during growth period

24 h at 37°C atmosphere 5% CO_2 (v/v). Each well was washed with PBS pH 7.4 for 5 min. Level of TNF-α and sEng were measured with enzyme-linked immunosorbent assay (ELISA) (Gunardi *et al.*, 2016).

Data analysis

Data were analyzed with ANOVA and Kruskal Wallis test. Data analysis was performed with software SPSS 22.

Results

Growth rate and pH of Lactobacillus bulgaricus *ATCC 11842 in soyghurt*

Results showed growth rate of *L.bulgaricus* ATCC 11842 in soyghurt towards time can be seen in Figure 2. As shown in Figure 2, there was correlation between growth rate of *L.bulgaricus* ATCC 11842 and incubation time. *L.bulgaricus* ATCC 11842 showed highest growth at 18 h that reached seven-folds from 0 h, and decreased afterwards. These findings confirm that soyghurt is a good medium of *L.bulgaricus* ATCC 11842 growth. pH optimization of *L.bulgaricus* ATCC 11842 in soyghurt toward incubation time is presented in Figure 3. As shown in Figure 3, there was correlation between decreased pH *L.bulgaricus* ATCC 11842 and incubation time. Optimum growth of L.bulgaricus ATCC 11842 was on 18 h with pH of 5.39 which was considered not too acid that do not cause damage on mammalian cell. Mammalian cells are known to have tolerance for pH ±5.0 – 8.8 (Sayuti et al., 2012). LC_{50} values are presented in Figure 4. In this study, soyghurt were divided in 11 concentrations (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% dan 100%).

Measurement of IC_{50} of soyghurt in various concentrations on trophoblast cells

Figure 4 showed measurement of soyghurt in triplicate. At concentration of 81.6% (Amniomax), 90.7% (DMEM) and 87.2% (RPMI 1640), soyghurt showed 50% trophoblast cells. IC_{50} value less than 81% indicate non-toxicity towards cells.

Effects of soyghurt in various concentration, incubation time, and serums on level of TNF-α

Variables tested in this study were normally distributed both in normal and preeclampsia serum treated with soyghurt in various concentrations incubated for 24 h and 48 h (data are not shown). Effects of soyghurt in various concentration, incubation time, and serums on level of TNF-α is presented in Figure 5. As shown in Figure 4, level of TNF-α decreased with increasing incubation time and increased soyghurt concentration. Soyghurt decreased level of TNF-α ($p<0,001$). Level of TNF-α in preeclampsia-induced trophoblast cells decreased from 34.79 pg/ml to 25.87 pg/ml after treatment with soyghurt of 80% incubated for 24 h, and lowered after 48 h from 36.16 pg/ml to 24.98 pg/ml.

Effects of soyghurt in various concentration, incubation time, and serums on level of sEng

Effects of soyghurt in various concentration, incubation time, and serums on level of sEng, are presented in Figure 6. Based on Figure 6, soyghurt

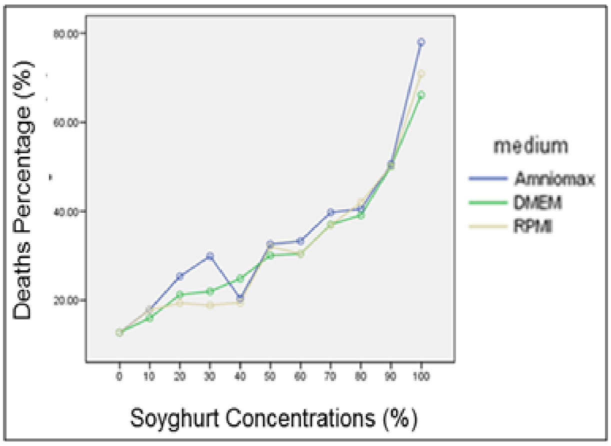

Figure 4. IC_{50} soyghurt on trophoblast cells

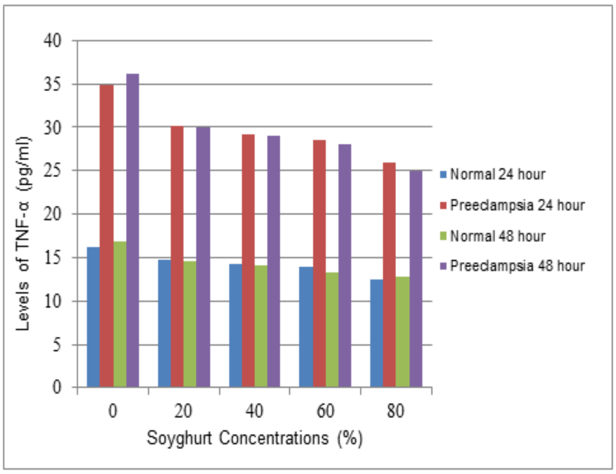

Figure 5. Effects of soyghurt in various concentrations and incubation time on level of TNF-α

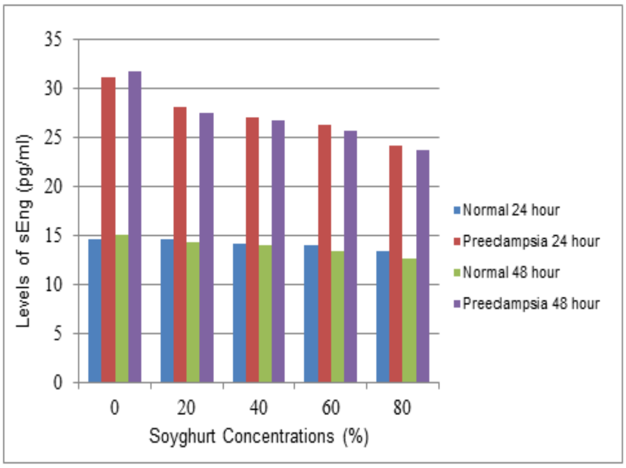

Figure 6. Effects of soyghurt in various concentrations and incubation time on level of sEng

significantly decreased sEng level ($p<0,001$) in concentration-dependent manner. Lower levels of sEng was associated with higher concentration of soyghurt. Level of sEng decreased from 31.23 pg/ml to 24.16 pg/ml at 80% soyghurt incubated for 24 h, and decreased from 31.76 pg/ml to 23.65 pg/ml after 48 h.

Discussion

In this study, soyghurt showed immunomodulatory and antioxidant activities. Level of TNF-α and sEng in preeclampsia-induced cells decreased which was comparable to that of normal cells. Trophoblast invasion occurred in preeclampsia due to immunology maladaptation, reduce uteroplacental perfusion that causes placental ischemia. Antiangiogenics such as soluble Endoglin (sEng) are produced excessively (Keogh *et al*., 2007; Pramatirta *et al*., 2015). Inflammatory mediators, angiogenics, antiangiogenics and placental metabolism leads to endothelial damage. Endothelial damage stimulates trombocyte to undergo adhesion, agegregation, and release. Both these events are terminated as indicated by decreased vasodilators, such as prostacyclin and nitrite oxide/endothelium-derived relaxing factor and increased vasoconstrictors, such as tromboxan and endothelin, and TNF-α (Yuan *et al*., 2005; Gurnadi *et al*., 2015).

To the best of our best knowledge, there is no studies that observe potential of probiotics and antioxidant in soyghurt on preeclampsia. Probiotics are orally ingested. Bacteria are usually killed by gastric acid, HCl, yet probiotics can be digested into colon. Probiotics play roles in producing essential nutrient and antibiotic that kills pathogens, and inhibits pathogen adhesion on colon receptor. Probiotics stimulate imunoglobulin E (IgE) release to againsts pathogens in digestive tract, and also neutralize toxin produced by pathogens that results in reduced free radicals in the body (Fauziah *et al*., 2015; Herawati *et al*., 2015).

Probiotics also plays role as antigen carrier, and bind to targeted tissue which activate macrophage to stimulate immune system. Probiotics stimulate cells to increase the producing IgA, IgM, and IgG, as well as spesific sIgA both in blood serum that transport nutrient to all tissues, and colon lumen, and also modulate immune response towards inflammation and balancing vasocontrictors (Herawati *et al*., 2015).

Lactobacillus bulgaricus was used in this study due to its ability to produce lactic acid with sufficient pH which is considered safe to the body. *L.bulgaricus* has high lipolitic among probiotics, as indicated by more favorable taste compared to other soymilks (De Preter *et al*., 2007). The presence of isoflavonoid in soybeans has been long known to possess antioxidant properties. Many health researches reported potential of soy-based products in treating degenerative diseases Isoflavones in soybeans has many beneficial effects on lipid profile repair, protection of LDL againts oxdation, enhance antioxidant activity in liver, anti-inflammatory, and kills pathogens. Anti-inflammatory mechanism of isoflavones underlies on inhibition of arakhidonat acid metabolism, prostaglandin synthesis, histamine release, or radical scavenging activity. Cells are therefore protected from negative effects, resulting in enhanced viability by balancing the molecules in the body. Beneficial effects of probiotics and plants are digested into cells through blood circulation (Kumar *et al.*, 2002; Lammersfeld *et al*., 2009).

Soyghurt is a fermented soymilk using probiotic, *Lactobacillus bulgaricus* (Fauziah *et al*., 2013). *L.bulgaricus* is the common probiotic used in fermentation, other than *Lactobacillus acidophillus* or *Bifidobacterium.* Soymilk can be a substrate for *Lactobacillus bulgaricus* that increases nutrient content in soymilk (Kaboosi, 2011). *Lactobacillus bulgaricus* produces secondary metabolite, lactic acid with higher pH than *L. acidophillus*, which is considered safe for cells (Isolauri *et al*., 2001; Pretzer *et al*., 2005; Fauziah *et al.*, 2015). Soyghurt possess ability to enhance immune system (immunomodulator) due to metabolite secreted by probiotics, strong antioxidant, and do not contain lactose (Sanjaya *et al*., 2007). Moreover, soybean is known to contain isoflavones that plays role in serum lipid repair, protection of LDL on oxidation, increase activity of certain antioxidant enzymes in liver and kills pathogens, and also prevent inflammation. Referring to previous study, *L.bulgaricus* has highest lipolitic content among *Lactobacillus* that can enhance nutrient content, mainly in dairy product (Tambekar and Bhutada, 2010; Fauziah *et al.,* 2013).

Conclusion

Soyghurt has the ability as an immunomodulator because can decrease the level of TNF-α and sEng. The highest treatment for decrease TNF-α and sEng was obtained in 80% concentration of soyghurt and 48 h incubated time.

References

DePreter, V., Vanhoutte, T., Huys, G., Swings, J. and DeVuyst, L. 2007. Effects of *Lactobacillus casei* Shirota, Bifidobacterium breve, and oligofructose-enriched inulin on colonic nitrogen-protein metabolism in healthy humans. American Journal of Physiology Gastrointestinal and Liver Physiology 292: 358-368.

Fauziah, P.N., Nurhajati J. and Chrysanti. 2015. Daya antibakteri filtrat asam laktat dan bakteriosin *Lactobacillus bulgaricus* KS1 dalam menghambat pertumbuhan *Klebsiella pneumoniae* strain ATCC 700603, CT1538, dan S941. Bandung Medical Journal 47(1):35-41. DOI: 10.15395/ mkb.v47n1.395.

Fauziah, P.N., Nurhajati, J. and Chrysanti. 2013. Daya antibakteri filtrat asam laktat dan bakteriosin *Lactobacillus bulgaricus* dalam soygurt terhadap pertumbuhan *Klebsiella pneumoniae.* New Bionatura 15(2): 2.

Gurnadi, J.I., Mose, J., Budi, H., Mieke, H.S., Anita, D.A., Prima, N.F., A. Yogi, P. and Dwi, D.R. 2015. Difference of concentration of placental soluble fms-like tyrosine kinase-1(sFlt-1), placental growth factor (PlGF), and sFlt-1/ PlGF ratio in severe preeclampsia and normal pregnancy. Journal of BMC Research Notes 8:534.

Gunardi, J.I., Mose, J., Mieke, H.S., Anita, D.A., Prima, N.F. and Triyuli. 2016. Effects of Papua Ant Nests *(Myrmecodia pendens)* on Level of sFlt-1, PIGF, MDA and NO in Preeclampsia-induced HUVEC Cell Line. Inter. Journal of PharmTech Research 9(6):424-435.

Herawati, I., Hilmi, D. and Fauziah, P.N. 2015. Effect of Lactic Acid Filtrate and Bacteriocins of *Lactobacillus acidophillus* on Phagocytosis Activity of Macrophages Cell againts Enteropathogen *Escherichia coli* (EPEC). Journal of Microbiology Indonesia 8(4):183.

Isolauri, E., Sutas, Y., Kanaanpaa, P., Arvilommi, J. and Salminen, S. 2001. Probiotics: Effects on Immunity. American Journal of Clinical Nutrition 73:444S–450S.

Kaboosi, H. 2011. Antibacterial effects of probiotics isolated from yoghurt againts some common bacterial pathogens. African Journal of Microbiology Research 5(25):4363–4367.

Keogh, R., Harris, J., Freeman, L.K., Baker, A., Aplin, P.N., Whitley, G.S. and Cartwright, J. E. 2007. Fetal-derived trophoblast use the apoptotic cytokine tumor necrosis factor-alpharelated apoptosis-inducing ligand to induce smooth muscle cell death. Journal of Circulation Research 100:834-841

Kumar, N. B., Cantor, A., Allen, K., Riccardi, D. and Cox, C. E. 2002. The specific role of isoflavones on estrogen metabolism in premenopausal women. Journal of Cancer 94 (4):1166-1174.

Lammersfeld, C. A., King, J., Walker, S., Vashi, P. G., Grutsch, J. F., Lis, C. G. and Gupta, D. 2009. Prevalence, sources, and predictors of soy consumption in breast cancer. Nutrition Journal 8:2.

Neale, D., Demadsio, K., Illuzi, J., Chaiworapongsa, Romero, R. and Mor, G. 2003. Maternal serum of women with preeclampsia reduces trophoblast cell viability: evidence for an increased sensitivity to Fas-mediated apoptosis. The Journal of Maternal-Fetal and Neonatal Medicine 13: 39-44.

Pramatirta, A.Y., Mose, J., Jusuf, S.E., Sofie, R.K., Anita, D.A., Prima, N.F., Jeffry, I.G. and Dwi, D.R. 2015. Correlation between cell-free mRNA expressions and PLGF protein level in severe preeclampsia. Journal of BMC Research Notes 8: 208.

Pramatirta, A.Y., Laksono, B., Prima, N.F., Anita, D.A., Sofie, R.K., Debbie, S.R., Ani, M.M. and Erlina, W. 2016. Effects of low dose aspirin on caspase 3, TNF-α and apoptotic index levels in preclampsia maternal serum-induced placental trophoblast cell line in vitro.

Inter. Journal of PharmTech Research 9(11): 47-53.

Sanjaya, I.G.N., Sudaryat, S. and Ketut, N.A. 2007. Effect of Probiotic Supplementation on Acute Diarrhoea in Infants: A Randomized Double Blind Clinical Trial. Journal of Paedriatrica Indonesiana 47: 172-177.

Sayuti, J.N., Chrysanti. and Syachroni. 2012. An in-vitro model for studying the adhesion of *Lactobacillus bulgaricus* in soyghurt and enteropathogenic Escherichia coli (EPEC) on HEp-2 Cells. African Journal of Microbiology Research 6(24): 5142-5146.

Tambekar, D.H. and Bhutada, S.A. 2010. An evaluation of probiotic potential of *Lactobacillus* sp. from milk of domestic animals and commercial available probiotic preparations in prevention of enteric bacterial infections. Journal of Recent Research Science and Technology 2(10): 82–88.

Yuan, H.T., Haig, D. and Karumanchi, S.A. 2005. Angigenic factors in the pathogenesis of preeclampsia. Journal of Current Topic Development Biology 71: 297–312.