

# SCREENING FOR PPAR $\gamma$ AGONIST FROM *MYRISTICA FRAGRANS* HOULT SEEDS FOR THE TREATMENT OF TYPE 2 DIABETES BY *IN VITRO* AND *IN VIVO*

In general, a type two diabetes mellitus (T2DM) patient suffers for hyperglycaemia and insulin resistance. Nowadays, there is an anti diabetes drug working as a PPAR $\gamma$ / $\alpha$  agonist and has a significant effect to improve the hyperglycemia and insulin resistance condition. However, this drug gives a negative effect on lipid circulation parameters. In this investigation, nutmeg (*Myristica fragrans*) extracts (NuSE) was tested against PPAR $\gamma$  using cell-based GAL4/PPAR chimera transactivation and transient transfection assay with three PPAR response element (PPREs) containing reporters. The results demonstrate that the NuSE has significant PPAR $\gamma$  agonist activity, although their potency was less than the standard PPAR $\gamma$  full agonist. An investigation of antidiabetic activity of the NuSE has been carried out on rats. Rats were treated orally every day for six days with the ethanol of NuSE in several doses. The result of the experiment showed that each dose of NuSE gave hypoglycemic activity ( $p=0.01$  and  $p=0.05$ ). The experiment also showed that an increase in dosage caused an increase in the hypoglycemic activity. Therefore, nutmeg (*Myristica fragrans*) seeds might be potential against antidiabetic agent for the treatment of type two diabetes with a PPAR  $\gamma$ / $\alpha$  agonistic mechanism.

**Keywords:** Nutmeg (*Myristica fragrans* Houtt.) seeds, antidiabetes, agonist, PPAR  $\gamma$ / $\alpha$

**UDC:** 616.379-008.64

KERI LESTARI<sup>1</sup>, JK HWANG<sup>2</sup>,  
SRI HARTINI KARIADI<sup>3</sup>, ANDI WIJAYA<sup>4</sup>,  
TRIHANGGONO AHMAD<sup>3</sup>,  
ANAS SUBARNAS<sup>1</sup>, SUPRIYATNA<sup>1</sup>,  
M. MUCHTARIDI<sup>1</sup>

<sup>1</sup> Faculty of Pharmacy, Universitas Padjadjaran, Indonesia

<sup>2</sup> Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Republic of Korea

<sup>3</sup> Faculty of Medicine, Universitas Padjadjaran, Indonesia

<sup>4</sup> Prodia Clinical Laboratory, Indonesia

## Introduction

WHO declares that Indonesia has become the fourth country that has the most diabetic patients in the world by following India, China and US (Sicree, Shaw, and Zimmet, 2010).

More than 80% of all diabetes is Type 2 Diabetes Mellitus (T2DM) patients<sup>2</sup>. Based on epidemiology research, there are increasing the incidence and prevalence of T2DM due to ageing population structures in developed countries and increasing obesity globally<sup>3</sup>. WHO predicts this pandemic of the prevalence of T2DM in Indonesia that increasing from 8.4 million in 2000 to rise over 21.3 million in 2030 (Sicree et al., 2010).

T2DM is the multi factorial and multi genetic disease which occurs as combination metabolic disorder; insulin resistance and beta pancreas cell insufficiency (Riddle, 2005). The both insulin resistance and beta pancreas cell insufficiency is caused by happening obesity and genetic factor (Abate and Chandalia, 2003; Gloyn, 2003). Sulfonylureas, metformin, acarbose, and thiazolidinones (TZDs) are current therapies for reducing plasma glucose (Hauer, 2002; Smith, 2001).

T2DM is the multi factorial and multi genetic disease, which occurs as combination metabolic disorder; insulin resistance and beta pancreas cell insufficiency<sup>4</sup>. The both insulin resistance and beta pancreas cell insufficiency is caused by happening obesity and genetic factor<sup>3, 5</sup>. Sulfonylureas, metformin, acarbose, and thiazolidinones (TZDs) are current therapies for reducing plasma glucose<sup>6, 7</sup>.

The antidiabetic effects of TZDs is due to the activation of the peroxisome proliferator-activated PPAR $\gamma$  receptors (Hauer, 2002). TZDs were high-affinity ligands for agonists of PPAR $\gamma$  and this phenomena was first reported by Lehman et al. (Lehmann, Moore,

Smith-Oliver, Wilkison et al., 1995). TZDs perform through PPAR $\gamma$  that facilitates the transcription of certain genes that are also responsive to insulin, enabling these agents to improve insulin action (Bailey and Day, 2001). However, this medicine gives side effect that may arise during treatment; TZDs also have side effects that increase the risk of heart attack and angina, fluid retention, weight gain, cardiac failure, thus TZDs use should be selective in diabetic patients who are not impaired liver and heart failure. For example, the treatment of TZD drugs such as rosiglitazone and pioglitazone, require monitoring to reduce the risk of adverse side effects, even troglitazone which are TZDs derivatives compounds have been withdrawn from the market because it showed an increased incidence of hepatitis induced by the drug (Belfort, Harrison, Brown, Darland, Finch, Hardies, Balas, Gastaldelli, Tio et al., 2006; Krentz and Friedmann, 2006).

Rosiglitazone gives effect to improve the condition of the patient hyperglycemia significantly T2DM, nevertheless, the medicine has side effects in lipid circulation parameters (Rangwala and Lazar, 2004). Based on the side effect story of TZDs, thus discovery of the other class drugs that selective into PPAR $\gamma$  and PPAR $\alpha$  agonist increases to reduce the risk of the fatal side effect of the drugs.

Recently, Fracchiolla et al. (Fracchiolla, Laghezza, Piemontese, Tortorella, Mazza, Montanari et al., 2009) reported the design and synthesis of a novel class of PPAR $\alpha/\gamma$  dual agonists, analog of 2-aryloxy-3-phenyl-propanoic acids compounds even they published the crystal (PDB id : 3HOD) that declares the compounds are active in nanomolar PPAR $\alpha/\gamma$  agonist. However, there are no compounds fully clinically report to date. The compounds explore to hydrophobic pocket in PPAR $\gamma$  binding site thus the compounds activate differential stabilization of helix 3 in partial agonist, when compared to full agonists (Montanari, Saccoccia, Scotti, Crestani, Godio, Gilardi et al., 2008).

Besides finding of novel synthetic compounds, the effort to explore alternative therapies using natural materials has been doing frequently in the community, because the materials relatively inexpensive and easily available as well as empirically shows efficacy for antidiabetic. However, the research that revealed the molecular mechanism of action of natural product antidiabetic has not much done, thus causing natural products potentially as antidiabetic cannot legally be used in treatment of diabetes.

Indonesia is a rich source of medicinal herbs and plants and is the world's oldest forest and the third most bio-diverse in Asia after India, and China. Many plants of Indonesian origin were used as antidiabetic such as *Momordica charantina* (Ooi, Yassin, and Hamid, 2010), *Snietenia macrophylla* (Mursiti, 2009).

We are encouraged to develop nutmeg seeds as PPAR $\gamma$  antagonist (Fracchiolla et al., 2009) such as lignin and neolignan derivatives even macelignan is established and patented by Jae-Kwang et al. (Han, Choi, Lee, Song et al., 2008; Hwang, Han, Sohn, Kim, Choo et al., 2007).

Here we screened nutmeg seeds extracts by using *in vitro* and *in vivo* by using MTT assay and anti hypoglycemic test activity, respectively. Nutmeg seeds have been used traditionally as a spice and for medicinal purposes in Indonesia and other asian countries (Muchtaridi, Subarnas et al., 2010; Olajide, Ajayi, Ekhelar, Awe et al., 1999; Ram, Lauria et al., 1996). In this study, evaluation of nutmeg seeds into PPAR $\gamma$  may have not published yet. However, one of the active compound of nutmeg seeds published by Jae-Kwang co-worker.

## Material and methods

### Plant material

Nutmeg seeds were taken from Wanayasa, Purwakarta, West Java. 1 kg nutmeg seeds were cleaned and dried at room temperature then crushed into powder.

The crushed seeds of nutmeg were extracted with MeOH for 3 x 24 hours. The MeOH extract was concentrated by vacuum evaporator at 40° C and 90-100 rpm. Concentrated extract was used for assay.

### **Toxicity test of nutmeg seeds**

Toxicity cellular of nutmeg seed extracts (NuSE) was tested into Cos-7 human cell lines by using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) methods<sup>16</sup>. The yellow tetrazolium MTT was reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan was solubilised and quantified by spectrophotometric mean.

Cos-7 cells (2 x 10<sup>4</sup> sel/well) was cultured on 96 wells microtiter plates, 100 µl each well. Twenty-four hours after plating, NuSE was added at concentrations ranging from 1 ppm, 5 ppm, and 10 ppm in DMSO. After 48h incubation, the medium was replaced with MTT<sup>17</sup> (SIGMA) dissolved at a final concentration of 1mg/ml in serum-free, phenol-red-free medium for further 4h incubation. Then, the MTT-formazan was solubilised in isopropanol and the absorbance was measured at a wavelength of 450 nm.

Cell death (%) = (A450 (control) - A450 (sample))/A450 (control) × 100%.

PPAR $\gamma$  activity of Nutmeg Seeds was Tested by Using GAL4/ PPAR chimera assay & reported gene (in vitro)

According to Han et al. (Han, Choi, Lee, Song, Joe, Jung et al., 2008), GAL-4/PPAR $\gamma$  transactivation assay was employed to measure PPAR $\gamma$  ligand-binding activity. COS-7 human cells were inoculated into a 96-well culture plate at 1.5 x 10<sup>4</sup> cells/well, and incubated in 5% CO<sub>2</sub>/air at 37°C for 24 h. The medium used DMEM (Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS) and 10 ml/l penicillin-streptomycin (5000 IU/ml and 5000 mg/ml) (Gibco, Grand Island, NY, U.S.A.). Cells were washed with Dulbecco's phosphate buffered saline (DPBS) (Sigma, St. Louis, MO, U.S.A.) and transfected with pFA-hPPAR $\gamma$ , pFR-Gal4 (UAS-Gal4-luciferase) and pFR-b-galactosidase (Stratagene, La Jolla, CA, U.S.A.) using Genejuice (Novagen, Madison, WI, U.S.A.). In an internal control, pFA and pFR-Gal4 (UAS-Gal4-luciferase) were transfected into COS-7 cells. 24 h after transfection, the medium was changed with DMEM containing 10% FBS and each sample, and the cells were further cultured for 24 h. Then, the cells were washed with DPBS, to which luciferase assay substrate (Promega, Madison, WI, U.S.A.) was added. The intensity of emitted luminescence was determined using a CytoFluor Series 4000 multiwell luminescence plate reader (PerSeptive Biosystems Inc., Framingham, MA, U.S.A.). PPAR $\gamma$  ligand-binding activity of the test sample was expressed as the relative luminescence intensity to that of control.

### **Hypoglycemic activity of NuSE (in Vivo)**

*In vivo* study has been done on male rat treated with hyperglycemia. Animals were divided into 6 groups (each groups consist three animal); negative control, positive control by adding drug 2,7 mg/kg, positive control by adding drug 10 mg/kg, and tested groups. A Blood level was measured before alloxan inducted. After animals were inducted by alloxan 70 mg/kg, the animals were fed with high carbohydrate content food provided by the Faculty of Veterinary laboratory, Universitas Padjadjaran. Animals were fasted for 8-10 hours and blood levels were measured before treated by with drugs.

Next step, animals were treated orally (volume 2 mL/kg) with adding drugs by following;

1. Negative control: *Pulvis Gom Arabicum* (PGA) 2%
2. Positive control I: PGA 2% + Pioglitazone 2,7 mg/kg
3. Positive control II: PGA 2% + Pioglitazone 10 mg/kg

4. Drug I: PGA 2% + nutmeg seeds extract 125 mg/kg
5. Drug II: PGA 2% + nutmeg seeds extract 250 mg/kg
6. Drug III: PGA 2% + nutmeg seeds extract 500 mg/kg

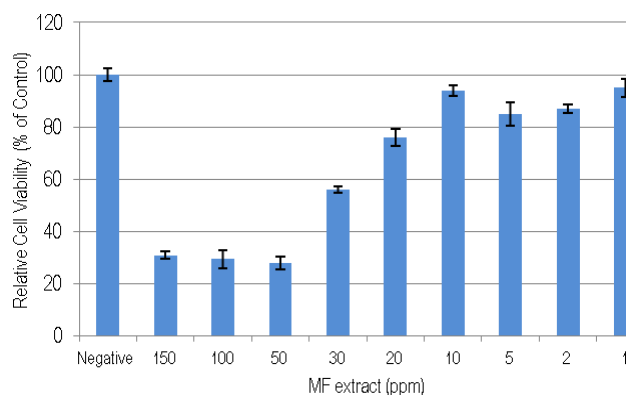
Blood was taken by cutting the tip of the mice tail, and blood level was calculated by glucometers. This procedure was repeated for 6 hours continuously and blood level was measured on the second, third and sixth day.

## Results and discussion

### Toxicity of nutmeg seeds

Toxicity of NuSE was displayed in Figure 1 using MTT assay methods. Nutmeg seeds extract in various concentration were tested into Cos-7 human cell lines. The results showed that NuSE at doses 20 ppm was not toxic into human cell lines Cos-7 with viability of cell line Cos-7 more than 60% as shown in Figure 1. This data concluded that NuSE could be used at concentration 20 ppm.

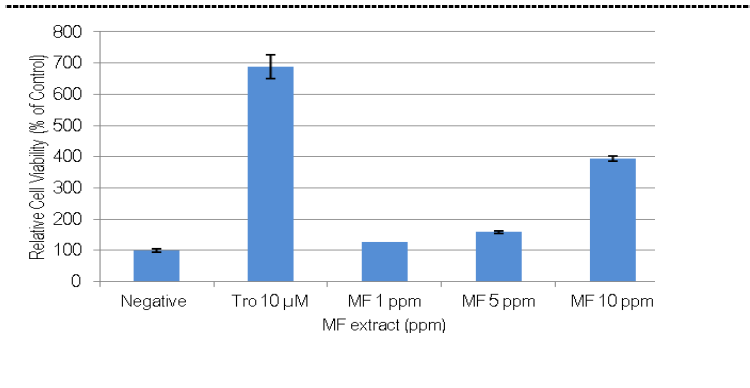
FIGURE 1. TOXICITY OF NUSE INTO HUMAN LINES COS-7



### ***In vitro* PPAR activity of nutmeg seeds was measured using a GAL4/PPAR chimera & reported gene**

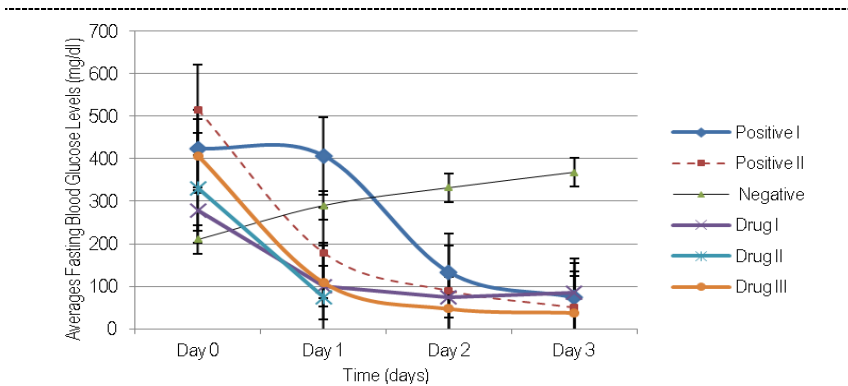
*In vitro* study, NuSE influenced into increasing *PPAR $\gamma$ -dependent luciferase* activity according to concentration of NuSE (Figure 2). In this research, NuSE was used at ranging concentration 1 ppm, 5 ppm, and 10 ppm, however NuSE was not toxic up to a concentration of 20 ppm (Figure 1) Viability of cell lines Cos-7 more than 90% at 10 ppm level, and whereas *PPAR $\gamma$ -dependent luciferase* activity less than 100% at 1 and 5 ppm that showed with RLA value (*relative luciferase activity*) were 110% and 170%, respectively (Figure 2). In addition, administration of NuSE at 10 ppm dose significantly increased *PPAR $\gamma$ -dependent luciferase* activity with RLA value 400% as shown in Figure 3. These results showed that NuSE gave as ‘insulin sensitizers’, providing a novel means to improve glycaemic control by reducing insulin resistance until a concentration of 10 ppm.

FIGURE 2. ACTIVITY OF NUSE INTO PPAR $\gamma$  IN VITRO



In addition, administration of NuSE at 10 ppm dose significantly increased PPAR $\gamma$ -dependent luciferase activity with RLA value 400% as shown in Figure 3. These results showed that NuSE gave as “insulin sensitizers”, providing a novel means to improve glycaemia control by reducing insulin resistance until a concentration of 10 ppm. Troglitazone (TGZ) was used as positive control, and showed that TGZ has RLA value 680% at concentration 10  $\mu$ mol (Figure3).

FIGURE 3. GRAPH OF AVERAGES FASTING BLOOD GLUCOSE LEVELS (MG/DL) OF EACH TREATMENT AFTER ADMINISTRATION NUSE



Note: Drug I: Tested treatment with administration PGA 2% + NuSE 125 mg/kg; Drug II: Tested treatment with administration PGA 2% + NuSE 250 mg/kg; Drug III: Tested treatment with administration PGA 2% + NuSE 500 mg/kg; Negative: control negative with administration PGA 2%; Positive I: Positive control with administration PGA 2% + Pioglitazone 2.7 mg/kg; Positive II: Positive control with administration PGA 2% + Pioglitazone 10 mg/kg

**In vivo of hypoglycemic activity of NuSE**

Evaluation of hypoglycemic activity of NuSE were carried out to determine the extract potential in lowering blood glucose levels effectively..

In this study, the animals were treated to become hyperglycemia by administrating alloxan 70 mg/kg. Alloxan is diabetogenic agent causing a condition of hyperglycaemic in normal

mice, because the compound has specific cytotoxic properties on pancreatic beta cells. The lack of beta cell lead to a decrease of insulin secretion thus the animals became hyperglycemic. The animals were hyperglycemic with blood levels of 284 mg/dl after 72 hours alloxan inducted.

Based on statistic results using Newman-Keuls with  $p=0.05$ , each treatment doses groups 125, 250, and 500 mg / kg of NuSE have significant differences compared to the negative control as shown in Table 1. This means that each dose group had influence on the decrease in blood glucose levels compared to negative controls. However, NuSE had no significant difference compare to the positive control (TZD).

TABLE 1. STATISTIC RESULTS OF NEWMAN-KEULS OF FASTING BLOOD LEVELS RELATIVE WITH CONFIDENCE LEVEL 95%

| Groups   | Averages | D III | C (+10) | C II  | D I   | C (+2.7) | C (-)   | F statistic |
|----------|----------|-------|---------|-------|-------|----------|---------|-------------|
|          |          | 36.96 | 40.40   | 41.34 | 48.49 | 61.25    | 142.76  |             |
| D III    | 36.96    | -     | 3.43    | 4.38  | 11.52 | 24.28    | 105.79* | 47.3386     |
| C (+10)  | 40.40    |       | -       | 0.94  | 8.09  | 20.85    | 102.36* | 57.7184     |
| D II     | 41.34    |       |         | -     | 7.15  | 19.91    | 101.42* | 64.1665     |
| D I      | 48.49    |       |         |       | -     | 12.76    | 94.27*  | 68.73       |
| C (+2.7) | 61.25    |       |         |       |       | -        | 81.51*  | 72.1874     |
| C (-)    | 142.76   |       |         |       |       |          | -       | -           |

Note: \*  $F_{6,35} = 3.15 P < 0.05$ , as compared with the control treatment. (by ANOVA followed by Newman-Keuls post hoc test); (C-) - control negative with administration PGA 2%; (C + 2.7) - Positive control with administration PGA 2% + Pioglitazone 2.7 mg/kg; (C +10) - Positive control with administration PGA 2% + Pioglitazone 10 mg/kg; (D I) - Tested treatment with administration PGA 2% + NuSE 125 mg/kg; (D II): Tested treatment with administration PGA 2% + NuSE 250 mg/kg; (D III) - Tested treatment with administration PGA 2% + NuSE 500 mg/kg

The others hand, Newman-Keuls analysis with  $p=0.01$  (confidence level 99%) produced NuSE with various doses had hyperglycaemic activity with significant difference compare to the negative control, whereas it has no significance compare to TZD as seen in Table 2.

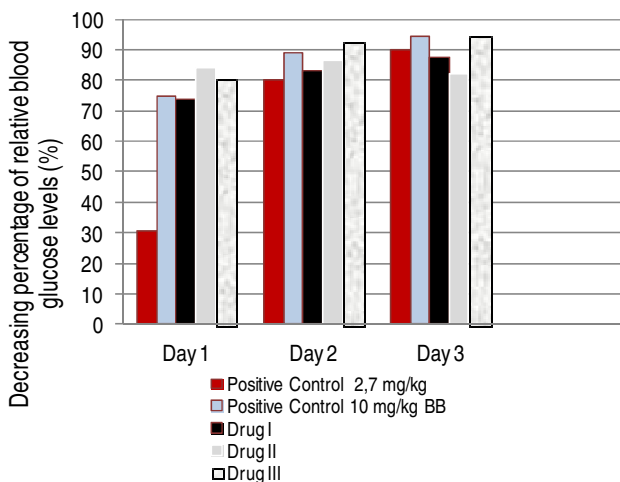
TABLE 2. STATISTIC RESULTS OF NEWMAN-KEULS OF FASTING BLOOD LEVELS RELATIVE WITH CONFIDENCE LEVEL 99%

| Groups   | Averages | D III | C (+10) | D II  | D I   | C (+2.7) | C (-)   | F Statistic |
|----------|----------|-------|---------|-------|-------|----------|---------|-------------|
|          |          | 36.96 | 40.40   | 41.34 | 48.49 | 61.25    | 142.76  |             |
| D III    | 36.96    | -     | 3.43    | 4.38  | 11.52 | 24.28    | 105.79* | 65.58       |
| K (+10)  | 40.40    |       | -       | 0.94  | 8.09  | 20.85    | 102.36* | 75.96       |
| D II     | 41.34    |       |         | -     | 7.15  | 19.91    | 101.42* | 82.57       |
| D I      | 48.49    |       |         |       | -     | 12.76    | 94.27*  | 87.44       |
| K (+2.7) | 61.25    |       |         |       |       | -        | 81.51   | 91.22       |
| K (-)    | 142.76   |       |         |       |       |          | -       | -           |

Note: \*  $F_{6,35} = 3.15 P < 0.01$ , as compared with the control treatment. (by ANOVA followed by Newman-Keuls post hoc test); (K-) - control negative with administration PGA 2%; (K + 2.7) - Positive control with administration PGA 2% + Pioglitazone 2.7 mg/kg; (K +10) - Positive control with administration PGA 2% + Pioglitazone 10 mg/kg; (D I) - Tested treatment with administration PGA 2% + NuSE 125 mg/kg; (D II) - Tested treatment with administration PGA 2% + NuSE 250 mg/kg; (D III) - Tested treatment with administration PGA 2% + NuSE 500 mg/kg

There are differences in the percentage reduction in blood glucose levels relative (%) of NuSE on each observation time as shown Figure 4. The highest decreasing percentage in fasting blood glucose levels of the rat hyperglycemia occurred on the 6th day after administration of NuSE. Administration of NuSE dose 500 and 250 mg/kg gave decreasing percentage in blood glucose levels 94.52% and 87.30%, respectively. Pioglitazone 2.7 mg/kg gave decreasing percentage in blood glucose levels 90%.

FIGURE 4. GRAPH OF DECREASING PERCENTAGE OF RELATIVE BLOOD GLUCOSE LEVELS (%) COMPARED THAN NEGATIVE CONTROL



## Further Discussion

Here we showed that nutmeg seeds extract (NuSE) might have potential as anti diabetic agent from natural product. Surprisingly, all concentration of NuSE gave increasing *PPAR* $\gamma$ -dependent luciferase activity even NuSE at 10 ppm dose significantly increased *PPAR* $\gamma$ -dependent luciferase activity with RLA value 400%. However this is not as good as TZD that enhanced the activity of *PPAR* $\gamma$ -dependent luciferase and improves glycaemic control in patient with Type 2 diabetes via improving insulin sensitivity through its action at *PPAR* gamma 1 and *PPAR* gamma 2 (Smith, 2001). Lignin derivatives compounds containing NuSE may play role in the activity to increase *PPAR* $\gamma$ -dependent luciferase. Macelignan is one of lignin derivatives have been published that has same mechanism (Han et al., 2008). Macelignan significantly improves glucose and insulin tolerance in mice, and without altering food intake, their body weights were slightly reduced while weights of troglitazone-treated mice increased (Han et al., 2008). Decreasing of blood glucose levels after administration NuSE orally also give evidence that NuSE is potent as antidiabetes. The greater NuSE dose, the higher reduction of blood glucose levels.

We noted that the study is not without limitations, including the relatively small size of the study's diabetic animal test. We hopes the research will be replicated in a larger sample size. Still, the findings present the immediate opportunity to explore NuSE as an antidiabetic agent.

## Conclusion

The study of *PPAR* $\gamma$ -dependent luciferase activity show that nutmeg seeds extract (NuSE) gave increasing *PPAR* $\gamma$ -dependent luciferase activity in all doses. The study of antihyperglycemic activity *in vivo* show that the greater NuSE dose can affected on the

greater reduction of blood glucose levels, Decreasing of blood glucose levels after administration NuSE orally also give evidence that NuSE is potent as antidiabetes NuSE NuSE has potential activity as anti diabetes agent with mechanism of action as a PPAR $\gamma$  agonist and has a significant effect to improve the hyperglycemia and insulin resistance condition.

Further research on toxicity study and formulation of NuSE are required to provide NuSE as a new antidiabetic agent in Diabetic management therapy

## Acknowledgements

We would gratefully acknowledge Indonesian Ministry of National education for funding this project through STRATEGI NASIONAL grant project 2004.

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