**Research Article**

# Effect of *Nigella Sativa* Encapsulated in Poly (Lactide-Co-Glycolide) Nanoparticles on Plasma Malondialdehyde and Ischemia-Modified Albumin in A Diabetes Rat Model

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**ABSTRACT**

**Introduction**: An imbalance between pro-oxidant and endogenous antioxidant levels in diabetes mellitus (DM) leads to elevated levels of oxidative stress which triggers accumulation of lipid peroxidation products (e.g.,malondialdehyde/MDA) and ischemia markers(e.g.,ischemia-modified albumin/IMA). *Nigella sativa* (NS), a herb with antioxidant activity, has poor therapeutic bioavailability. Therefore, modification using biodegradable nanoparticles delivery system [e.g.,poly (lactide-co-glycolide)(PLGA)] offers enhancing in antioxidant properties. This study was designed to analyze the effect of NS-loaded PLGA nanoparticles on plasma MDA and IMA levels in type 2 DM model rats. **Methods**: Twenty-four Sprague-Dawley rats were fed a high-fat diet (HFD) for 26 days, injected intraperitoneally of 35 mg/kgBW streptozotocin then separated on to glibenclamide [0.45 mg/(kgBW.day)], NS extract [48 mg/(kgBW.day)],NS-loaded PLGA nanoparticles [48 mg/(kgBW.day)], and untreated control groups. Plasma MDA and IMA levels were measured using a TBA colorimetric assay and ELISA, respectively. **Results**: MDA levels before and after treatment were observed to be suppressed in the glibenclamide group (0.187 ± 0.472 ng/mL) but the gap in all groups is not significant (p=0.345). In addition, the sharpest decrease in IMA levels before and after treatment was found in NS-loaded PLGA nanoparticle group (264.918 ± 3.411 ng/mL) which is significantly different compared to other groups (p=0.001). **Conclusion**: The NS-loaded PLGA nanoparticles is proved to have remarkable reduction in plasma IMA levels compared to other groups but not in plasma MDA levels of type 2 DM model rats.

**Keywords:** Diabetes mellitus, ischemia-modified albumin, malondialdehyde, PLGA

Received 04 Oct 2016 Received in revised form 02 Nov 2016 Accepted 10 Nov 2016

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## INTRODUCTION

The prevalence of diabetes mellitus (DM) is increasing in line with the trends of other global metabolic diseases such as obesity and aging, making it a major health burden worldwide. The estimated prevalence of DM in 2010was 6.4%(285 million adults), with 90% of the cases being classified as type II DM[1,2]. Reactive oxygen species (ROS) formation and hyperglycemia contribute to the pathophysiology of DM by worsening its progression and complications [3]. The former factor exacerbates cell death through DNA damage and lipid peroxidation, while the later induces cell death through advanced glycation end-product (AGE) formation, and both processes are implicated in the development of diabetic cardiovascular and nephropathy complications. Moreover, impaired ROS scavenging caused by prolonged activation of NAD(P)H oxidase contributes to DM complications [4].

Malondialdehyde (MDA), a widely used indicator of free radical formation, is a product of lipid degradation, and acts as an important marker for analyzing oxidative stress in diabetic conditions [3]. MDA levels were found to be significantly increased in male smokers with a history of DM and concurrent microvascular and macrovascular complications lasting more than five years [5]. Moreover, the levels of ischemia modified albumin (IMA), a potential biomarker of myocardial ischemia and type II DM, were found to be increased by ROS and free radicals [6,7]. Previous

studies have shown that IMA levels are higher in diabetic patients than in healthy subjects. Other study by Muhtaroglu *et al.*, 2016, indicated that IMA levels were greater in patients with diabetic foot than the diabetes group, as the consequence of the peripheral ischemia [8]. Moreover, IMA can be used as a biomarker for reperfusion ischemia that occurs in other tissues and involves oxidative stress-related processes. Formation of IMA is attributed to the reduced binding affinity of albumin for transition metal ions such as Co (II), Ni (II), and Cu (II) via change in the ability of the first three amino acids of albumin (N-Asp-Ala-His)to interact with the free metal ion [9].

*Nigella sativa*(*N. sativa* or NS), also known as the black seed, has been used as an antidiabetic agent in most Asian countries[10]. The main active ingredient of *N. sativa* is thymoquinone, which is a major constituent of the seed oil. Inhibition of lipid peroxidase activity by thymoquinone can decrease glucose levels through the reduction of oxidative stress [11]. However, many traditional medicines including thymoquinone have relatively low bioavailability, thus leading to failures in clinical trials[12]. Nanoparticle-based formulations of thymoquinone have shown improved efficacy when used as antimicrobials, anti-oxidants, anti-cancer, and anti-inflammatory agents[10, 13, 14].

Poly lactic-glycolic acid (PLGA), a drug carrier with high loading capacity has been proven to increase the efficacy of herbal products and also to act as an efficient carrier for curcumin, coumarin, estradiol, and several proteins [15]. The present study was designed to analyze whether nanoparticle-based formulations can increase the bioavailability of *N. sativa* seed extracts.

## MATERIALS AND METHODS

### Research design

This research used a true experimental design with pre-test/post-test control group design.

### Animal treatment

All experiments on animals were conducted with ethical approval from the Faculty of Medicine, Brawijaya University Ethical Committee (No. 528/EC/KEPK/09/2014). Inclusion criteria for the24 male Sprague-Dawley rats used in this study were 18 months of age and 250-400 g body weight. They were divided into 4 groups, namely negative control (DM-induced rats without treatment), positive control (DM-induced rats treated with glibenclamide [0.45mg/(kg BW.day), po)], P1 (DM-induced rats treated with crude *N. sativa* seed extract [48mg/(kg BW.day), po)], and P2 (DM-induced rats treated with *N. sativa* seed extract formulated in PLGA nanoparticles[48mg/(kg BW.day), po)]. All rats were feda high-fat diet (HFD), containing 50% PARS, 25% wheat flour, 1% cholesterol, 0.1% cholic acid, and 2.5% pig fat for 26 days. On day27, positive control, P1, and P2 were injected with streptozotoc in (STZ) (35mg/kg BW) intraperitoneally. After 26 days of normal diet and different treatments for each group, rats were anesthetized and sacrificed. Fasting blood glucose (FBG) was measured three times, i.e., before administrating HFD, 3 days after the STZ injection, and 26 days after treatment.

### Extract preparation

NS seeds (150 g) were added to 900 mL of 95% ethanol and extracted using the soxhlet extraction method for 3 h with the heater set at 90%.The solvent was then evaporated using a water bath rotary evaporator. The semisolid extract thus obtained was qualitatively analyzed using thin layer chromatography (TLC) method with thymoquinone standard as reference.

### PLGA nanoparticle formulation

The formulation of PLGA nanoparticles was prepared by mixing 10 mg of NS extract and 50 mg of PLGA and dissolving the mixture in 3mL of propylene carbonate (PC) using a magnetic stirrer at 500 rpm for 30 min. Subsequently, 1% (w/v) of polyoxyethylene-polyoxypropylene (Pluronic F-68) was dissolved in aqua bidest using a magnetic stirrer at 1250 rpm for 30 min. The mixture containing NS extract, PLGA, and PC was then added to Pluronic F-68 solution (at 0.5 mL/min) and centrifuged at 10,000 *g* for 30 min at 4°C to produce a pellet [16]. The pellet was then dissolved in aqua bidest before being administered to rats.

**MDA Measurement**

Blood samples (0.5 mL) mixed with 2 mL of $H_2SO_4$, 1 mL of 20% TCA (w/v), and 2.5 mL thiobarbituric acid (TBA) were centrifugedat1000 rpm for 10 min and placed in a boiling water bath for 15 min and centrifuged again. The centrifugation and heating steps were repeated after the addition of 2 mLTBA. The concentration of MDA was measured using a spectrophotometer at a wavelength of 523 nm [17].

**IMA Measurement**

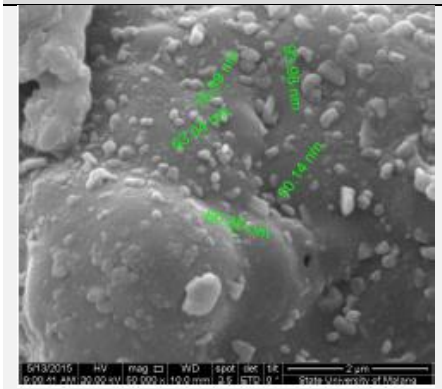
Two-hundred microliters of plasma mixed with 50 µL of 0.1% cobalt chloride was incubated for 10 min at 37 °C. Subsequently, 50 µL of dithiothreitol was added as a colorizing agent. After 2 min, the reaction was quenched by adding 1 mL of 0.9% NaCl. The absorbance was determined using a spectrophotometer at a wavelength of 470 nm witha mixture of plasma pluscobalt chloride solution as a blank [18].

**RESULTS**

In this study, a qualitative phytochemical assay was conducted to determine the profile of chemical compounds contained in NS extract. The extract was found to contain volatile oils, alkaloids, saponins, flavonoids, tannins, and terpenoids. Additionally, TLC was performed using n-hexane:ethyl acetate (9:1) as an eluent, and thymoquinone contained in NS extract was matched with standard thymoquinone using the Rf value. The result showed that both samples had an Rf value of 0.765.

In addition to the phytochemical assay, PLGA nanoparticles were characterized using scanning electron microscopy (SEM)(FEI S50).The particles in the nanoparticle preparation were found to be rough and amorphous in appearance and varied in size from 50.14 to 93.98 nm (**Table 1**).

**Table 1: SEM test results on NS extract encapsulated in PLGA nanoparticles**

| Figures | Magnification | Diameters (nm) | Diameter Mean (nm) |
|---|---|---|---|
|  | 50,000 times | 50.14<br>60.85<br>63.04<br>75.99<br>93.98 | 68.80 |

With respect to plasma MDA levels, the results showed that there was no significant difference among treatment groups, with a p-value of 0.076 for the baseline and 0.508 for post-treatment MDA levels. In addition, Wilcoxon test was conducted to determine the difference between baseline and post-treatment MDA levels for all treatment groups. The results revealed that there was no significant difference between pre- and post-treatment plasma MDA levels in all groups with p-values > 0.05 (**Table 2**). Plasma IMA levels were also examined as a marker of ischemia. Mean levels of plasma IMA pre- and post-treatment were significantly different for all groups (p-value = 0.028) which were P2 compared to Positive control, negative control, and P1. These results indicated that there were at least two differences among the groups with respect to plasma IMA levels. However, pre- and post-treatment test showed no significant difference in plasma IMA levels among all treatment groups. The results obtained through Wilcoxon test showed no significant difference in plasma IMA levels

in the negative control group before and after treatment. However, for the other groups, paired t-tests were used to evaluate the differences between pre- and post-treatment plasma IMA levels because the data were normal.

**Table 2: Mean levels of plasma MDA before and after treatment**

| Treatment Groups | Mean Levels of Plasma MDA ± SD (ng/mL) | | Mean Levels of Plasma IMA ± SD (ng/mL) | |
|---|---|---|---|---|
| | Before-Treatment | After-Treatment | Before-Treatment | After-Treatment |
| Negative control | 0.3564 ± 0.5230 | 0.3537 ± 0.0731 | 13.5331 ± 3.4370 | 9.9418 ± 1.7155 |
| Positive control | 0.6103 ± 0.2205 | 0.4233 ± 0.2796 | 18.1482 ± 7.6761 | 10.4440 ± 3.5679 |
| P1 | 0.9121 ± 1.2250 | 0.7483 ± 0.7658 | 13.8509 ± 7.2073 | 9.1478 ± 0.8484 |
| P2 | 0.3705 ± 0.1075 | 0.3264 ± 0.1069 | 33.9283 ± 3.5341 | 7.4364 ± 0.9198 |

**Notes:**
Negative Control: type 2 DM rats without any therapy
Positive Control: type 2 DM rats treated with glibenclamide (0.45mg/(kg BW.day))
P1: type 2 DM rats treated with NS extract (48mg/(kg BW.day))
P2: type 2 DM rats treated with PLGA loaded NS extract (48mg/(kg BW.day))

## DISCUSSION

### Effect of *N. sativa* seed extract encapsulated in PLGA nanoparticles on MDA levels of diabetic model rats

NS is considered to possess the ability to minimize oxidative stress-mediated tissue damage encountered in DM condition. This property is attributed to the active compound thymoquinone, which has been proven to be effective in scavenging free radicals generated via the lipid peroxidation process. Additionally, essential oils contained in NS could normalize endogenous antioxidant supplies, such as glutathione, superoxide dismutase, and catalase, or improve antioxidant defense mechanisms by reducing the levels of MDA and diene conjugates. Therefore, the activity of NS is associated with an increase in antioxidant capacity and suppression of free radical formation [19]. Thymoquinone could scavenge free radicals by a mechanism similar to that of superoxide dismutase (catching superoxide anions and free radicals) [20]. In addition, NS has hypoglycemic effects that improve glucose level maintenance in type 2 DM [21]. In type 2 DM, lipid peroxidation, which triggers excessive oxidative stress, stimulates cell membrane damage leading to cell death. Excessive oxidative stress may be countered by defense mechanisms that block the production of free radicals provided there are sufficient amounts of hepatic enzymes. Imbalance between free radicals production and defense mechanisms results in the formation of lipid peroxidation products such as MDA [22].Oxygen free radicals, which are abundant in type 2 DM, contribute to the breakdown of phospholipids and formation of MDA, which could in turn modify low-density lipoproteins (LDL) and facilitate pathophysiological changes through non-enzymatic and auto-oxidative glycosylation. These processes interfere with the pro-oxidant/antioxidant equilibrium, thus leading to microvascular and macrovascular complications of type 2 DM [5].

Based on the data on pre-treatment average of MDA levels, the sequence of groups from the highest to the lowest was [P1], [P+], [P2], and [P-]. These pre-treatment average values were not significantly different ($p = 0.076$); however, those sequences were considered for the whole data analysis. Alternatively, post-treatment average values showed a different trend, i.e.,[P1], [P+], [P-], and [P2],implying that the NS-loaded PLGA nanoparticles treatment group had the lowest post-treatment MDA levels. Transformation of both data into deviation revealed that the largest decrease in MDA levels was in [P+] group while the least decrease was in [P-] group, and the order

was [P+], [P1], [P2], and [P-]. However, the null hypothesis test could not prove that the deviation in MDA levels among groups was statistically significant (p = 0.508), although the data indicated that the treatments had a stronger effect on MDA levels than that observed in the control group.

NS extract given to diabetic rats possesses the beneficial effect of decreasing MDA levels because it contains thymoquinone, which scavenges superoxide, hydroxyl, and oxygen radicals. This antioxidant activity is associated with the quinone moiety of thymoquinone, which easily enters the subcellular compartment and catches free radicals. Thymoquinone exhibits a dose-dependent antioxidant activity, i.e., low doses possess good antioxidant activity, whereas the higher the dose (above 20 µmol/L), the more it switches to pro-oxidant and pro-apoptotic roles through the up regulation of p53 and down regulation of Bcl-2 [23].

Treatment with NS-loaded PLGA nanoparticles showed a smaller decrease in MDA levels. This may be explained by the fact that the release of essential oils from NS is influenced by surface modification of the nanoparticle delivery system. Nanoparticle formulation plays an important role in the release of NS extract because the NS essential oils are composed of highly lipophilic natural fatty acids. Therefore, better release of NS essential oils can be achieved when the extract is delivered through a more hydrophilic-base formulation [21]. However, PLGA encapsulates lipophilic substances within the hydrophobic core of the nanoparticle system. Consequently, PLGA could limit the release rate of the highly lipophilic natural fatty acids of NS. Thus, encapsulation of NS extract by PLGA could impede the quinone moiety of thymoquinone, hindering its reaction with free radicals and resulting in the relatively smaller decrease in MDA levels than that in NS extract group.

**Effect of *N. sativa* seed extract encapsulated in PLGA nanoparticles on IMA levels of diabetic model rats**

IMA is typically formed under conditions such as brain-ischemia, renal disease, liver disease, certain neoplasms, and vascular disease. Several studies have shown that IMA formation occurs as a result of hypoxia, reduced oxygen tension, and ROS generation [24]. In DM, a prolonged hyperglycemic condition leads to ROS formation and impairs free radical defense mechanisms. Moreover, increasing ROS levels generate a modified human plasma albumin known as IMA, which is regarded as an important biomarker of the DM condition [9, 24].

The seeds of NS exhibit potent anti-diabetic activity by reducing appetite, glucose absorption, and hepatic gluconeogenesis and stimulating insulin secretion [15]. Therefore, NS extract is hypothesized to reduce the levels of lipid peroxidation products such as MDA and hydroperoxide, and increase anti-oxidant activity via up regulation of enzymes such as super oxide dismutase (SOD) and catalase [10].

As seen from the data, group P2, which received NS-loaded PLGA nanoparticles, had higher IMA levels than other groups (p=0.028), which correlates with a higher level of oxidative stress and thus, possibly, the most severe diabetic condition of all four groups. Studies conducted by Piwowar et al. (2008) and Kaeffer et al. (2010) revealed that diabetic patients had significantly higher IMA levels than healthy (control) subjects did. These studies also indicated that patients with uncontrolled glucose levels had higher IMA levels than those with controlled glucose levels [9,24]. Other study by Muhtaroglu *et al.*, 2016, also exposed that the IMA levels in diabetic foot patients were higher than diabetic patients. IMA level were declined in post-amputation patients, showed that the infection and necrotic had correlation with the IMA levels [8].

Results of our study showed that NS extract formulated in PLGA nanoparticles significantly decreased IMA levels in diabetic rats before and after treatment (p=0.001),while the decrease was not statistically significant in other groups including the one which received non-PLGA formulated NS extract. Thus, the PLGA nanoparticle formulation was proven to have greater activity than glibenclamide did and to increase the efficacy of NS extract in reducing plasma IMA levels.

Surprisingly, the group that received glibenclamide recorded the highest IMA levels among other treatment groups. This could be explained based on the finding that glibenclamide acts as an insulin stimulator, thus increasing the glucose uptake. Previous studies have reported that increased glucose uptake could reduce protein glycation and inhibit AGEs synthesis, which in turn reduces the oxidative stress [25]. However, glibenclamide by itself has lower antioxidant activity than that of other antidiabetic agents, such as gliclazide [26]. Therefore, glibenclamide has to be used in combination with other active compounds to increase anti-oxidant activity [27]. Hence, it can be concluded that NS extract has higher anti-oxidant activity than glibenclamide based on the IMA level reduction.

Nanoparticle encapsulated bioactive compound formulations have greater activity than conventional formulations do because of their small size and biodegradable characteristic. Other advantages of nanoparticle formulations include rapid entry into the target cell, greater bioavailability and requirement of lesser amount of the bioactive compound than with conventional formulations. Previous studies showed that nanoparticle-encapsulated forms of *Syzygium jambolanum* and *Gymnema sylvestre* have greater anti-hyperglycemic activity than the non-encapsulated forms do [15]. A study by Nallamuthu et al., (2012) proved that nanoparticle-encapsulated thymoquinone had better anti-oxidant activity than that of the non-encapsulated form [13]. However, the present study is the first to demonstrate that NS-loaded PLGA nanoparticles have better anti-diabetic activity than conventional formulations, through the effect of reducing plasma IMA levels.

## CONCLUSION

Thus, results of the present study indicate that the drug delivery system of NS-loaded PLGA nanoparticles has no superiority over NS extract and glibenclamide with respect to reduction of plasma MDA levels. However, the nanoparticles seem to have marked potential with respect to reducing plasma IMA levels when compared to NS extract and glibenclamide. Further investigation is needed to prove the advantages of NS-loaded PLGA nanoparticles with respect to other biomarkers and tissues.

## ACKNOWLEDGEMENT

We thank the study program of pharmacy, Faculty of Medicine, Universitas Brawijaya, for providing funding and laboratory facilities. We also thank to Editage, BPPM FKUB, and Reza Nugraha for their suggestions.

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