

## Evaluation of *In vitro* anti-diabetic and anti-inflammatory effects of *Nigella sativa* seed extracts in five different solvent systems

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### Abstract

**Objective:** The objective of this study was to screen the phytochemical constituents of the seeds of the *Nigella sativa* and to obtain preliminary *in vitro* data about its anti-diabetic and anti-inflammatory effects.

**Methods:** First extracts were prepared in five solvents i.e. Methanol, ethanol, n-hexane, petroleum ether and chloroform through cold extraction procedure. Determinations of the phytochemical constituents of these extracts were then carried out by employing standard procedures. In order to determine the *in vitro* anti-diabetic activity, amylase inhibition studies were carried out. *In vitro* anti-inflammatory test were carried out using protein denaturation inhibition principle.

**Results:** With the exception of the chloroform extract, all the other extracts were found to be rich in phytochemicals. The methanol extract contained the maximum number of phytochemicals. The alpha amylase inhibition and protein denaturation inhibition were all found to be highest for the methanol extract.

**Conclusion:** The present study proves that the methanol extract of *N. sativa* a potent anti-diabetic and anti-inflammatory agent. It can prove to be a valuable source of drugs targeting these diseases.

**Keywords:** anti-diabetic, anti-inflammatory, phytochemical, protein denaturation inhibition, methanol extract

### Introduction

Medicinal plants are the richest bio-resource of drugs for traditional systems of medicine, nutraceuticals, food supplements, modern medicines, pharmaceutical intermediates, folk medicines and chemical entities for synthetic drugs. It has been reported that up to 80% of the people depends on traditional medicinal plants for their medicines [1]. Plant secondary metabolites used as aliments derived from barks, flowers, roots, leaves, seeds, fruits are the part of phytomedicines [2]. Phytochemical components such as tannins, carbohydrates, alkaloids, terpenoids, phenolic compounds, steroids and flavonoids are responsible for various pharmacological activities of the plants [3,4]. These phytochemical compounds are synthesized by primary or secondary metabolism of living organisms. They are extensively used in agriculture, human therapy, veterinary and related scientific research etc [5].

*N. sativa* is an indigenous herbaceous plant belongs to the Ranunculaceae family that is more commonly found in Middle East and Southeast Asian countries. In south Asia it is called kalonji, its Arabic name is Hbat-ul-sauda, its English name is Black cumin and in bangla it is known as kalojira [6]. NS has long been used as a natural medicine for the treatment of many acute diseases. This plant has been great focus of research for centuries and has several traditional uses and consequently has been extensively studied for its chemical constituents and biological activities. It has been traditionally used for thousands of years as folk medicine and some of its active compounds were reported against many ailments [7]. Different pharmacological effects such as gastric ulcer healing, anti-microbial effect anti-cancer activity, tremendous effects on cardiovascular disorders, gastroprotective, antioxidant activity, immunomodulatory, antitumor effects, anti-anxiety effect, anti-

asthmatic effect, anti-helicobacter activity, tumor growth suppression, anti-viral activity against cytomegalovirus hepatoprotective activity have been previously reported for this medicinal plant [8].

The present study was carried out to find phytochemicals present in *Nigella sativa* extracts in different solvent. *In vitro* anti-diabetic and anti-inflammatory test were also carried out to evaluate its potential role against diabetic and inflammatory diseases and make a conclusion based on analytical results which extract might has been used as pharmaceutical supplement.

### 2. Methods

#### 2.1 Preparation of extracts

##### 2.1.1 Drying and Pulverization

The NS seeds were purchased from a local market in Rajshahi, Bangladesh. The *N. sativa* seeds were washed with water in such a way that adhering dirty materials were completely removed. The seeds were sun dried for 7-10 days and finally kept in an electric oven for 72s hours at 40 °C. After complete drying, the seeds were then pulverized into a coarse powder with the help of a grinding machine (FFC-15, China) and were stored in an airtight container for further use.

##### 2.1.2 Cold extraction of powdered plant materials

After pulverization, the weight of total powdered plant material was 1000 gram. For extraction, the 200 gm. of powdered material was kept immersed in 500 ml of different solvents including Methanol (Methanol extract of Kalojira, MK), Ethanol (Ethanol extract of Kalojira, EK), Chloroform (Chloroform extract of Kalojira, CK), Petroleum ether (Petroleum extract of Kalojira, PK) and n-hexane (n-hexane extract of Kalojira, NK) in an aspirator bottle at room

temperature for 7 days with occasional shaking and stirring. Then the contents were pressed through the Tincture Press (Karlkolb, Scientific-Technical Suppliers, Frankfurt / M-Germany) in Pharmaceutical Laboratory of the Department of Applied Chemistry and Chemical Engineering, Rajshahi University, to get maximum amount of extract. The extract was then filtered through a filter paper (Whatman No.1) and concentrated with a rotary evaporator under reduced pressure at 40 °C to obtain different extracts of extract of *Nigella sativa* seeds. Then the extracts were stored at 4°C for further analysis.

## 2.2 Phytochemical analysis of *Nigella sativa* seed extracts

### 2.2.1 Alkaloids test<sup>[9]</sup>

#### (a) Dragendorff's test

To 2 mg of the extracts were dissolved in 5 ml of distilled water, 2M Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendorff's reagent was added. Formation of orange or orange red precipitate indicates the presence of alkaloids.

#### (b) Hager's test

To 2 mg of the extracts were taken in a test tube, a few drops of Hager's reagent was added. Formation of yellow ppt confirms the presence of alkaloids.

#### (c) Wagner's test

2 mg of extracts were acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent were added. A yellow or brown ppt. indicates the presence of alkaloids.

#### (d) Mayer's test

To a few drops of the Mayer's reagent, 2 mg of each ethanol extract was added. Formation of white or pale yellow precipitate indicates the presence of alkaloids.

### 2.2.2 Carbohydrate test

#### (a) Anthrone test

2 mg of each extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 2ml of anthrone reagent solution was added. Formation of green or blue colour indicates the presence of carbohydrates.

#### (b) Benedict's test

2 mg of each extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 5 ml of Benedict's solution was added and boiled for 5 minutes. Formation of brick red coloured ppt. indicates the presence of carbohydrates.

#### (c) Fehling's test

2 mg of each extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 1 ml mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes. Formation of red or brick red coloured precipitate indicates the presence of reducing sugar.

#### (d) Molisch's test

2 mg each of extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 2 drops of freshly prepared 20% alcoholic solution of  $\alpha$ - naphthol was added. 2 ml of conc. sulphuric acid was added so as to form a layer below the mixture. Red violet ring appear, indicating the presence of

carbohydrates which disappear on the addition of excess of alkali.

### 2.2.3 Flavonoid test<sup>[10]</sup>

2 mg of each extract was dissolved in 5ml of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicates the presence of flavonoids.

### 2.2.4 Glycosides<sup>[9]</sup>

#### Molisch's test

2 mg of each extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To these 2-3 drops of Molisch's reagent was added, mixed and 2ml of concentrated sulfuric acid was added carefully through the side of the test tube. Reddish violet ring appear, indicating the presence of glycosides.

### 2.2.5 Triterpenoids<sup>[9]</sup>

#### Liebermann - Burchard's test

2 mg of each dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of a pink colour indicates the presence of triterpenoids.

### 2.2.6 Resins

1 ml of each extract was dissolved in acetone and the solution was poured in distilled water. Turbidity indicates the presence of resins.

### 2.2.7 Saponins<sup>[11]</sup>

In a test tube containing about.5 mg of each extract were dissolved in 5 ml of water, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicates the presence of saponins.

### 2.2.8 Steroids

#### (a) Liebermann-Burchard's test

2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicates the presence of steroids.

#### (b) Salkowski reaction

2 mg of dry extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

### 2.2.9 Tannins<sup>[12]</sup>

To 1-2 ml of extract, few drops of 5% w/v FeCl<sub>3</sub> solution was added. A green colour indicated the presence of gallotannins, while brown colour indicates the presence of pseudotannins.

### 2.2.10 Coumarins test

A small amount of sample was placed in a test tube. The tube was covered with filter paper moistened with dilute sodium hydroxide solution. The covered test tube was then placed in boiling water for several minutes. The paper was removed &

exposed to ultraviolet light. A yellow-green color appearing with in a few minutes.

**2.2.11 Cardio glycoside test**

**Kedde’s test**

1.0 ml extract was mixed with 1.0 ml 2% 3,5-dinitrobenzoic acid in methanol & 1.0 ml aqueous NAOH. A violet color precipitate indicates lacton ring present in cardenolide.

**2.2.12 Anthracenosides test**

4ml extract was concentrated to 2ml, and then ammonium solution (25%, 1-2 ml) was added by shaking. A cherish red color of the alkaline solution indicates the presence of emedol (aglycanes of anthracenosides) in an oxidized form.

**2.3 In vitro anti-diabetic activity test of Nigella sativa seed extracts**

**2.3.1 Dinitrosalicylic acid (DNSA) method**

The inhibition assay was performed according to Miller [13] using DNSA method. 500µl of extracts at different concentration were added to 500 µL of 0.02 M sodium phosphate buffer (pH-6.9 containing 6 mM sodium chloride) containing .5mg/ml of α-amylase solution and were incubated at 37°C for 10 min, followed by addition of 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH-6.9) all the test tubes. The reaction was stopped with 1.0 ml of 3, 5 DNSA reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. The control samples were also prepared accordingly without any plant extracts and were compared with the test samples containing various concentrations of extracts prepared. The results were expressed as % inhibition calculated using the formula:

$$\alpha\text{-Amylase inhibition activity} = [1 - \{(A_1 - A_2) / A_0\}] \times 100$$

Where A<sub>1</sub> is the absorbance of test sample, A<sub>2</sub> is the absorbance of product control (sample without α-Amylase solution) and A<sub>0</sub> is the absorbance of negative control (α-Amylase without extract).

**2.3.2 Starch-iodine Color Assay**

Screenings for α-amylase inhibition by extracts were carried out according to Xiao et al. [14] with slight modification based on the starch-iodine test. 500µl Of extracts at different concentration were added to 500 µL of 0.02 M sodium phosphate buffer (pH6.9 containing 6 mM sodium chloride) containing .5mg/ml of α-amylase solution and were incubated at 37 °C for 10 min. then 500 µL soluble starch (1%, w/v) was added to each reaction test tube and incubated at 37 °C for 15 min. 1 M HCl (20 µL) was added to stop the enzymatic reaction, followed by the addition of 100 µL of iodine reagent (5 mM I<sub>2</sub> and 5 mM KI). The color change was noted and the absorbance was read at 620 nm. The results were expressed as % inhibition calculated using the formula:

$$\alpha\text{-Amylase inhibition activity} = [1 - \{(A_1 - A_2) / A_0\}] \times 100$$

Where A<sub>1</sub> is the absorbance of test sample, A<sub>2</sub> is the absorbance of product control (sample without α-Amylase solution) and A<sub>0</sub> is the absorbance of negative control (α-Amylase without extract).

**2.4 In vitro anti-inflammatory activity test of Nigella sativa seed extracts (Inhibition of protein denaturation method)**

**2.4.1 Egg Albumin denaturation inhibition method**

According to previously reported protocol, [15] The reaction mixture consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (pH 6.4) and 2 ml of varying concentrations of the test extract, by which the concentrations (µg/ml) became 100, 200, 500 and 1000. Similar volume of double-distilled water served as control. Then the mixtures were incubated at 37°C ± 2°C in a biological oxygen demand incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm) by using vehicle as blank. Aspirin at the final concentrations (µg/ml) of 100, 200, 500 and 1000 were used as reference drugs and treated similarly for determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times ([Vt/Vc] - 1).$$

Where, Vt = absorbance of test sample, Vc = absorbance of control.

**2.4.2 Inhibition of Bovine serum albumin (BSA) denaturation method**

Inhibition of protein denaturation was evaluated by the method of Sakat et al. [16] with slight modification. 500 µL of 1% bovine serum albumin was added to 100 µL of plant extracts. This mixture was kept at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetyl salicylic acid was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

$$\% \text{ Inhibition} = 100 - [(A_1 - A_2) / A_0 \times 100]$$

Where A<sub>1</sub> is the absorbance of the sample, A<sub>2</sub> is the absorbance of the product control and A<sub>0</sub> is the absorbance of the positive control.

**3. Results and Discussion**

**3.1 Phytochemical composition of NS seed extract in different solvent system**

*Nigella sativa* seeds contain most of the phytoconstituent that are responsible for their biological activities. Alkaloids, Flavonoids, steroids, resins, tenins are major components were found in different extracts of *nigella*

**Table 1:** Phytochemical screening for the determination of the presence of phyto-constituent in different extracts of *Nigella sativa* seed

	Name of test compound	Name of sample extract				
		MK	EK	PK	NK	CK
Alkaloids	Dragendorff’s test	+	-	+	+	-
	Hager’s test	+	+	-	-	-
	Wagner’s test	+	+	+	-	-
	Mayer’s test	+	-	-	+	+

Carbohydrate test	Benedict's test	+	+	+	-	-
	Fehling's test	+	+	-	+	-
	Molisch's test	-	+	-	+	-
Flavonoid test		+	+	+	+	+
Glycosides		-	+	+	-	+
Triterpenoids		-	-	-	-	-
Resins		+	+	+	+	+
Saponins		+	+	-	+	+
Steroids	Liebermann-Burchard's test	+	+	+	+	+
	Salkowski reaction	+	-	+	+	-
Tannins		-	+	-	+	-
Coumarins test		+	+	-	-	-
Cardio glycoside test		+	+	-	+	-
Anthracenosides test		+	+	-	-	-

(+) indicate the presence and (-) indicate the absence of phyto-constituents. MK; methanol extract of kalojira, EK; ethanol extract of kalojira, NK; n-hexane extract of kalojira, PK; petroleum ether extract of kalojira, CK; chloroform extract of kalojira,

*sativa* seeds. Table-1 shows the phytochemical composition of *Nigella sativa* seeds. The finding is in line with previously demonstrated analytical result from Ishtiaq *et al.* [8]. Previous studies indicate that phytoconstituents present in NS extract have potential pharmaceutical benefits against bacterial growth [17].

The presence of tannins in our tested extracts suggests the probable biological activities. Tannins facilitate to promote tissue regeneration in case of superficial burn injury and also have antibacterial, antiviral, antifungal, and antioxidant effects [18]. The presence of flavonoids in the extracts indicates their potentiality to reduce *in vitro* cholesterol agents and to induce an antifungal activity [18, 19]. Flavonoids are known to inhibit  $\alpha$ -

amylase activity which regulates the amount of glucose in the blood; therefore the extracts of *N. sativa* can be used as an antidiabetic.

### 3.2 In vitro anti-diabetic activity of NS seed extract in different solvent system

Alpha-amylase inhibitory activities of *Nigella sativa* seed extracts illustrated in Fig-1 and 2. Acarbose, the alpha-amylase inhibitor can reduce the digestion of carbohydrate in the upper part of the small intestine, thus ameliorating postprandial hyperglycemia and enabling the beta-cells to make up the first phase insulin secretory defect in Type 2 diabetes mellitus.

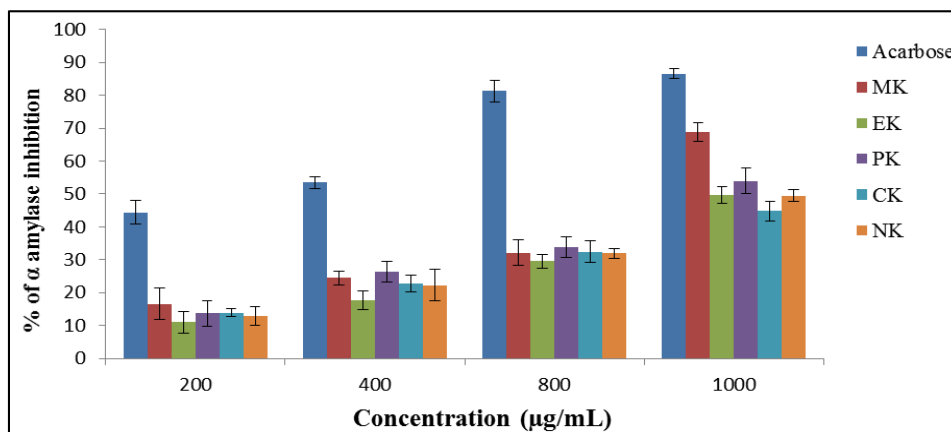


Fig 1:  $\alpha$ -amylase inhibition activity of different extracts of NS in 3,5-dinitrosalicylic acid (DNSA) method

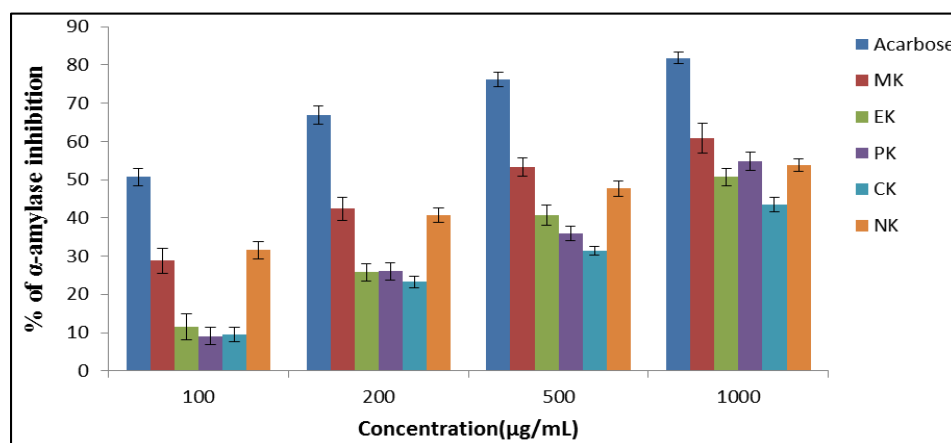


Fig 2:  $\alpha$ -amylase inhibition activity of different extracts of NS in Starch Iodine color assay method

According to Fig-1, it was demonstrated that each extract showed alpha-amylase inhibitory activity, which was in a dose-dependent manner. Moreover when treated with the concentration of 1000µg/ml, methanol extract had the highest inhibitory activity (68.5%) among five extracts. Nevertheless, compared with the commercial drug acarbose, no precedence of extracts possessed higher inhibitory activities than acarbose in this test. Although the consequence that lowers activities of both extracts from *N. sativa* seed were obtained. It could be implicated that a high potential of medicine utilization and a novel alpha-amylase indicator instead of acarbose extracted from *N. sativa* seed after purification was completed feasibility. Methanol extract causes highest % of alpha-amylase inhibition at 1000µg/mL concentration as compare with another extract. At 1000µg/mL concentration 68.34±2.90, 49.63±2.59, 55.63±1.81, 53.95±3.81, 44.84±3.00 percentage of α-Amylase inhibition were observed in 3,5-Dinitrosalicylic acid (DNSA) method by MK, EK, NK, PK and CK respectively, where standard Acarbose exhibit 86.57±1.49 percentage inhibition. In starch Iodine colour assay method following order of alpha-amylase inhibition were observed in experimental test; Acarbose>MK>PK>NK>EK>CK. Diabetes mellitus is a chronic, systemic, metabolic disease defined by hyperglycemia and characterized by alterations in the metabolism of carbohydrate, protein and lipid. When the regulation of

metabolism is disrupted due to the imbalance of hormonal regulation, diabetic arises and cause to malfunctioning of body. But NS has potential benefit in the management of diabetic mellitus through the inhibition of Alpha-amylase and thus its facilitating body to regulate glucose on its normal level. Furthermore, NS contains many bioactive constituents such as thymoquinone, p-cymene, pinene, dithymoquinone and thymohydroquinone which have potential effect on diabetic [20].

**3.3 In vitro anti-inflammatory activity of NS seed extract in different solvent system**

The method of anti-denaturation of egg albumin and bovine serum albumin were chosen to evaluate anti-inflammatory property of the all extracts. In anti-denaturation assay the denaturation of egg albumin and BSA induced by heat treatment. The denatured protein expresses antigens associated to Type III hyper-sensitive reaction which are related to diseases such as serum sickness, glomerulo-nephritis etc. [21]. Moreover, it was already proved that conventional NSAID’s like phenylbutazone and indomethazine do not act only by the inhibition of endogenous prostaglandins production by blocking COX enzyme but also by prevention of denaturation of proteins [22].

**Table 2:** *In vitro* anti-inflammatory effects of Different NS extracts in Egg albumin denaturation method

CONC. (µg/ml)	% of inhibition					
	Egg Albumin denaturation method					
	MK	EK	NK	PK	CK	ASPIRIN
100	15.55±2.59	21.94±0.52	14.44±1.38	18.24±1.93	9.25±0.39	41.98±1.74
200	32.73±2.64	33.01±2.78	27.26±2.15	30.52±2.11	22.92±1.69	57.28±1.03
500	50.15±0.78	44.74±0.49	37.63±0.55	40.38±1.55	32.24±2.29	66.68±0.96
1000	58.18±0.75	53.84±0.97	43.61±1.16	44.06±0.82	37.84±0.80	73.69±0.58

Result was expressed as mean ± S.D. (n=3). MK; methanol extract of kalojira, EK; ethanol extract of kalojira, NK; n-hexane extract of kalojira, PK; petroleum ether extract of kalojira, CK; chloroform extract of kalojira, Thus anti-denaturation assay is the convenient method to check the anti-inflammatory activity. In the present study for *in vitro* anti-inflammatory test, MK extract in egg albumin denaturation method showed mean inhibition of protein denaturation of 55.55, 32.73, 50.15 and 58.18% at doses of 100, 200, 500 and 1000 µg/mL respectively, whereas, for Aspirin it was found to be 41.98, 57.28, 66.68 and 73.69% for same doses respectively (Table-2). On the other hand EK showed second highest inhibition activity to compare with MK and which was

significantly similar with the activity of MK. CK showed lowest inhibition activity and significantly different from aspirin activity. The MK extracts showed good anti-inflammatory activity with a linear response in BSA method. Maximum inhibition of 60.85% and 81.82% was observed at 1000 µg/mL by MK and standard anti-inflammatory drug (Aspirin) respectively 56.92% and 43.43% inhibition were showed at the concentration of 1000 µg/mL by NK and CK (table-3). From the result of the present study, the extracts had shown considerable anti-inflammatory activity. The secondary metabolites like phenolic compounds and tannins which were found in preliminary phytochemical screening might be responsible for this activity.

**Table 3:** *In vitro* anti-inflammatory effects of Different NS extracts in BSA denaturation method

CONC. (µg/ml)	% of inhibition					
	BSA denaturation method					
	MK	EK	NK	PK	CK	ASPIRIN
100	28.78±3.30	11.61±3.42	31.56±2.32	9.09±2.28	9.59±1.00	50.75±2.28
200	42.42±3.03	25.75±2.28	40.65±1.91	26.01±2.32	23.23±1.58	66.92±2.32
500	53.28±2.32	40.65±2.66	53.79±2.01	35.85±1.90	31.31±1.16	76.26±1.91
1000	60.85±3.82	50.75±2.28	56.92±1.58	54.79±2.43	43.43±1.90	81.82±1.52

Result was expressed as mean ± S.D. (n=3). MK; methanol extract of kalojira, EK; ethanol extract of kalojira, NK; n-

hexane extract of kalojira, PK; petroleum ether extract of kalojira, CK; chloroform extract of kalojira,

#### 4. Conclusion

Based on the results, we concluded that the methanol extract of *N. sativa* is both a potent anti-inflammatory and anti-diabetic. This study provides scientific proof for the anti-diabetic and anti-inflammatory properties of the *N. sativa*. This plant can definitely be used for developing drugs targeting both diabetes and inflammatory diseases. Before usage of the extract by humans is a possibility, clinical studies to test its safety and elucidate its mechanism of action are needed.

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