The Thrombolytic and Cytotoxic Effects of *Nigella sativa* (L.) Seeds: The Prophetic Medicine


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1. Introduction

*Nigella sativa* (*N. sativa*) is an annual flowering herb of the Ranunculaceae family and genus *Nigella* which has 18 species, native to South and Southwest Asia, Southern Europe, and North Africa. Since 1880, numerous studies had been conducted on its bioactivities including thrombolytic and fibrinolytic properties (Awad and Binder, 2005; Asgary et al., 2012; Lebda et al., 2012). Since time immemorial, *N. sativa* (*L.* ) seeds had been used traditionally in Unani, Tibb, Ayurveda, Siddha, and Arabic medicines (Ahmad et al., 2013). Primarily, it is composed of fixed...
oil (≤38%), essential oil (≤1.5%), dietary protein (≤26.7%), fat (≤28.5%), and carbohydrate (≤40%). In its essential oil, thymoquinone (TQ), the so-called most bioactive compound was reportedly found up to 48% whilst other bioactives include t-anethole (4%), 4-terpineol (7%), sesquiterpene longifolene (8%), carvacrol (12%), p-cymene (15%), thymol, dihydrothymoquinone (DTQ) and thymohydroquinone (THQ). In fixed oil, it was reported to contain mainly linoleic acid (65%), oleic acid (24%), palmitic acid (20%), dihomoolinoleic acid (10%) and eicosadienoic acid (3%) and many fatty acids including sterol esters and glycosides (Ahmad et al., 2013; Al-Jassir, 1992; Houghton et al., 1995; Cheikh-Rouhou et al., 2007; Gholamnezhad et al., 2016).

The WSE was found to be highly polar by nature with solubility in both 5% NaOH and HCl solutions which might be an indication of a wide range of solubility or therapeutic index with active δ+ and δ– sites. It has also shown more than two-fold stronger antioxidant capacity than its fixed oil (Babar et al., 2019a).

Thrombolytic or fibrinolytic drugs are being used to treat coronary artery disease, deep vein thrombosis, myocardial infarction, strokes, and pulmonary embolism, etc. Most of the thrombolytic drugs target the fibrin, one of the main constituents of a blood clot to lyse the clot (Babar et al., 2019a; Dewan and Das, 2013). Fibrin, thrombin, and plasmin play vital roles individually and/or collectively in blood coagulation, thrombosis, and hemostasis while various enzymes, cofactors, and receptors, such as serine protease, cofactor V and VIII and protease-activated receptors (PARs) are also involved respectively (Tanaka et al., 2009; Ali et al., 2014; Chapin and Hajjar, 2015).

Cytotoxicity of thymoquinone, essential oil, and different fractions of *N. sativa* (L.) seeds has been studied extensively but there was no such report about WSE. A recent study conducted on the cytotoxicity of various fractions (i.e., petroleum ether, ether, methanol, chloroform, and water) and essential oil of *N. sativa* on brine shrimp through the paper disc and solution methods found petroleum ether and chloroform to be the most cytotoxic with LC50 of 7 and 21 µg/mL respectively whilst the aqueous fraction has shown insignificant cytotoxicity for the above two methods (Mahmoudvand et al., 2017). Therefore, the current study was aimed to investigate the thrombolytic (i.e., % clot lysis) and cytotoxic (i.e., % mortality) effects of WSE using human blood clot lysis and brine shrimp lethality (BSL) assays respectively.

2. **Materials and methods**

2.1 Extraction and yield of WSE

The detailed process of extraction and yield of WSE has been discussed in our earlier report (Babar et al., 2019a). This is the preliminary study of our recent study (Babar et al., 2019b) on WSE, which was preserved at 3 ±1°C in a Falcon® tube (50 mL) wrapped with aluminium foil. It was noted that 1 µL contains ≈0.88 mg (w/v) of WSE. A significant amount (≈15 g) of WSE was sent safely to the Department of Pharmacy, International Islamic University Chittagong (IIUC), Bangladesh from Kuala Lumpur. Upon arrival at the mentioned institution, the sample was kept at 4 °C until use.

2.2 Ethical consideration

As the current study is a part and preliminary experiment of our recent study (Babar et al., 2019b), thus, this study protocol was also approved by the Institutional Ethics Committee (IEC) of the Department of Pharmacy, IIUC under the same reference (Pharm/PND/138/13#2019).

2.3 Guidelines

The current study has strictly followed and maintained the recommendations (v 1.1, June 2018) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) on the best practices of venous blood sampling (Simundic et al., 2018). For hatching the brine shrimp nauplii, the notes and instructions of ‘Ocean Nutrition Americas’, was followed.
2.4 Materials
The stabilized pure streptokinase (STK® Inj.; 1.5 million IU) vial in powder form produced by Incepta Pharmaceuticals Ltd., Sodium Chloride BP 0.9% w/v and Dextrose 5% USP w/v solution (Solodex™ IV Infusion) manufactured by Square Pharmaceuticals Ltd., Bangladesh, and two vials of vincristine sulfate (Criston 2, 2mg/2mL; Beacon Pharmaceutical Ltd., Bangladesh) were bought from a local pharmacy shop in Chittagong. The 50 g of brine shrimp of ‘S.K Artemia Cysts’ brand imported from Thailand was purchased from a local supplier in Cox’s Bazar. The portable aeration pump (Brand: Amplab Bangladesh) with a single air-flow was purchased from the online shopping site (www.daraz.com.bd).

2.5 WSE stock and working solutions preparation
A stock solution of WSE (10,000 µg/mL) was prepared by adding 125 µL of WSE into 11 mL of normal saline (NS) water for both thrombolytic and cytotoxic studies, and 12 random 5x concentrations of aliquots (i.e., 5x/mL w/v; 3 mL/ aliquot) for 44.14, 66.21, 88.28, 132.42, 176.56, 264.84, 353.13, 529.69, 706.25, 1059.38, 1,412.50 and 2,000.00 µg/mL of WSE were prepared by adding the corresponding volume of 66.21 µL, 99.32 µL, 132.42 µL, 198.63 µL, 264.84 µL, 397.26 µL, 529.70 µL, 794.54 µL, 1,059.38 µL, 1,589.07 µL, 2,118.75 µL and 3,000 µL of WSE from the stock respectively into ≤3.00 mL (i.e., 2,933.79 to 0.00 µL) of the artificial seawater (3.8% NaCl/L w/v, pH 8.0). For thrombolytic assay, 166.67 µL of stock was directly added into the tubes (n=6×3) containing 0.5 mL blood and filled up to 1.0 mL using NS (≈333.33).

2.6 Preparation of streptokinase (STK) stock
The powder form of STK vial (1.5 million IU) was reconstituted and diluted by adding 5.0 mL of Solodex™ IV Infusion at the sidewall of the vial just immediately before the use. It was tilted, rolled gently until fully reconstituted. The stock concentration was 300,000 IU/mL.

2.7 Preparation of vincristine sulfate (VCF) solution
The two VCF vials (Criston 2; 1mg/mL; 2mL/vial) were further diluted by adding 2 mL of normal saline (NS;0.9% NaCl) to each vial to get a 4 mL vial of 500 µg/mL of two stocks so that six consecutive concentrations (500-15.625 µg/mL) of aliquots (4 mL/tube) could be prepared and the stocks also left with 3 mL/vial after dilution. The details of the dilution process have been described graphically in Fig. 4.

2.8 Blood samples collection
After having the prior consent, the whole fasting blood (WFB) sample (5.0 mL/subject) of adult male (23-26 years old) was aseptically withdrawn in the morning (i.e., 8:00-8:15 am) after at least 12 hr of fasting using a 5.0 mL syringe with a needle via venipuncture method (Simundic et al., 2018).

2.9 Brine shrimp nauplii hatching & collection
The brine shrimp nauplii were hatched in 1 L of artificial seawater (i.e., 3.8% NaCl/L w/v) prepared by adding 38 gram of unrefined sea salt into 1 L of distilled water in a beaker to which 2.5 g of ‘S.K Artemia Cysts’ was added. A 60 W bulb above the beaker (8°), single air-flow aeration pump and glass thermometer was fitted to maintain the light, the temperature at 28 °C and oxygen supply while the pH was 8. The brine shrimp nauplii hatched after 24 hr were immediate transfer to a freshly prepared media before drug exposure.

4.10 Thrombolytic assay
The in-vitro thrombolytic activity of WSE was performed using the methods described by (Prasad et al., 2006) with some minor modifications followed by the EFLM recommendations (Simundic et al., 2018). 0.5 mL of WFB sample from each subject in a triplicate manner for three different tests (i.e., NS, STK, and WSE) was transferred into the corresponding pre-weighed (0.80±0.01 g/empty tube) sterile collection tubes (1.5 mL) and were again weighed before incubation for 45 minutes at 37 °C to allow clot formation. After clot formation, the serum was gently aspirated and the weight of each of the 54 tubes (n=6×3×3) containing blood clot was taken at room temperature (24 ±2 °C) before NS, STK and WSE was added. Subsequently, 166.7 µL of WSE from the stock was added into 18 respective tubes (n=6×3). Similarly, 100 µL of STK (300,000 IU/mL) and 500 µL of NS (0.9% NaCl) were added before incubation at 37 °C for 90 minutes and observed closely for clot lysis. After incubation, the lysate (i.e., upper phase) was discarded carefully and the weight of the tube containing the blood clot was recorded. The difference in clot weight before and after lysis was expressed as the percent of clot lysis (Fig. 1) using the following equation (1).

\[
\text{Clot lysis (\%)} = \frac{(\text{Weight of lysate})}{(\text{Weight of clot before lysis})} \times 100
\]

Eq. 1

The weight of blood clot was calculated using the equation (2) as followed:

\[
\text{Weight of clot (g)} = \text{Weight of tube containing blood clot} - \text{Weight of empty tube}
\]

Eq. 2

2.11 Brine shrimp lethality (BSL) bioassay

The BSL bioassay was carried based on the methods of (Meyer et al., 1982; Houghton et al., 1995; Cheikh-Rouhou et al., 2007; Gholamnezhad et al., 2016), with few modifications. 10 healthy-looking immobilized nauplii (Babar et al., 2019a) along with 1 mL of media were transferred into each of the 57 glass test tubes labeled and pre-filled with 1 mL of fresh media in advance. Then, 1 mL of WSE from each of the 12 aliquots, 1 mL of VCS from each of the 6 aliquots were added in triplicate manner. The volume was filled up to 5 mL using the seawater. The negative control contained only 5 mL seawater. The final log concentrations of VCS were from 100 to 3.125 µg/mL (Fig. 4). Finally, all the treated and control groups (5 mL/test/tube) were incubated at room temperature (26 ±1 °C) for 24 hr (12/12; light/dark cycle), the survived and died nauplii population was screened and counted using a handheld magnifying glass. The median lethal concentration (LC50) of the test samples was calculated from the linear regression equation of the scatter graph whilst the % of mortality was calculated by using the following equation (3):

\[
\% \text{ Mortality} = \frac{(\text{Total no of nauplii died/treatment})}{(\text{Total no of nauplii used/treatment})} \times 100
\]

Eq. 3

2.12 Data analysis

The results are expressed as mean ± SD and compared between treated and control groups, employing a Student’s unpaired two-tailed \(t\)-test using GraphPad Prism 8 online. A \(p\)-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Thrombolytic effect of WSE

The exposure of a single, randomly selected WSE (1666.67 µg/mL) to healthy adult male human blood (0.5 mL×3) against streptokinase (STK:72.41 ±0.01%) of 30,000 IU/mL and normal saline (NS:3.76 ±0.49%) has shown statistically significant (\(p <0.0063\) & \(p <0.0001\)) % clot lysis by WSE (90.00 ±0.02%) as shown in Fig. 1.
Fig 1. The mean % clot lysis ± SD of WSE (90 ±0.02%) after 24 hr of exposure vs. normal saline, NS (3.76 ±0.49%) and streptokinase, STK (72.41 ±0.01%). * Indicates the % clot lysis of WSE to be extremely statistically significant (p<0.0001) vs. NS and (p<0.0063) vs. STK.

3.2. Cytotoxic effect of WSE

The exposure of twelve randomly selected concentrations of WSE (44.14, 66.21, 88.28, 132.42, 176.56, 264.84, 353.13, 529.69, 706.25, 1059.38, 1,412.50 and 2,000.00 µg/mL) to brine shrimp nauplii for 24 hours against the vincristine sulfate, VCS (LC_{50}=39.25 µg/mL) in artificial seawater (3.8% NaCl/L) has exhibited very mild cytotoxicity bearing LC_{50}=1795.90 µg/mL as shown in Fig. 2. The current result of WSE is about 46 times less potent than VCS, Fig. 3. It is also found that the current LC50 of VCS is more than 8 times potent than a previous report (LC_{50}=0.33 ±0.055 mg/mL) made by (Rahmatullah et al., 2010).

Fig 2. The mean % mortality of brine shrimp nauplii (n=10×12×3) caused by WSE of 44.14-2,000 µg/mL in triplicate manner, after 24 hr of exposure at room temperature showing LC_{50}=1795.90 µg/mL.
Fig 3. The mean % mortality of brine shrimp nauplii (n=10×6×3) caused by the VCS of 100-3.125 µg/mL in triplicate manner after 24 hr of exposure at room temperature having LC$_{50}$=39.25 µg/mL.

Fig 4. The preparation of six vincrestine sulfate (VCS) aliquots (500-15.625 µg/mL) (4 mL/ aliquot) via serial dilution using normal saline (NS), and treatment (5 mL) concentrations (100-3.125 µg/mL) in triplicate manner.

4. Conclusion

It could be concluded that WSE has a potent thrombolytic effect with mild dose-dependent cytotoxicity towards brine shrimp nauplii (Artemia salina). It is also proposed that WSE might have a vital enzymatic role on thrombin, fibrin, and plasmin of blood which may involve serine proteases and protease-activated receptors (PARs). This pharmacological action of WSE is might
be due to its antioxidant property, short-chain fatty acids, and/or amino acids. Further studies are highly recommended on the enzymatic role(s) and bioactive phytoconstituents of WSE.

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References


