The effect of fixed oil and water extracts of *Nigella sativa* on sickle cells: an *in vitro* study

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ABSTRACT

Introduction: Various drugs have been investigated in the treatment of sickle cell disease (SCD), such as hydroxyurea, piracetam and calcium antagonists. Most of these drugs are potentially toxic and are not suitable for long-term therapy. Recently, *Nigella sativa* (NS) has been reported to have calcium antagonist and antioxidant activities, both of which play a role in the management of the disease. This study aimed to investigate the *in vitro* antisickling effect of extracts from NS.

Methods: Thirty-two patients with SCD, aged 7–47 years old, were recruited for the study. A total of 3 ml of venous blood was collected from each patient and divided into six tubes with heparin. The blood was mixed with 0.5 ml of either 0.1 percent, 0.05 percent or 0.01 percent v/v of the oil extract of NS. A slide was prepared by spreading a drop of treated blood, covered with a cover slide to ensure the complete deoxygenation condition. The separation of irreversibly sickled cells (ISCs) was performed on eight patients by a density gradient (Percoll-Renografin) centrifugation method.

Results: The 0.1 percent v/v concentration of the oil extract of NS resulted in an approximately 80 percent reduction in the formation of sickle cells. The 0.05 percent v/v concentration of NS produced an intermediate effect, while the 0.01 percent v/v concentration had no effect on the formation of sickle cells. The 0.1 percent v/v concentration of the fixed oil of NS led to a considerable reduction in the formation of ISCs.

Conclusion: The fixed oil extracted from NS seeds has an *in vitro* antisickling activity.

Keywords: antioxidant, antisickling, fixed oil extract, *Nigella sativa*

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INTRODUCTION

Sickle cell disease is a social problem in the southern part of Iraq. A clear estimate of the prevalence of the disease is not well documented. However, in a recent study in Basrah, it was estimated that the frequency of the sickle cell trait was 6.5%.[1] The disease varies in severity and usually presents as vaso-oclusive crises with severe pain and disability, or as haemolytic crises with persistent anaemia. As a hereditary disease, no specific drugs are yet available; however, the use of various drugs such as hydroxyurea, piracetam, calcium antagonists and others has been tested against this disease.[2,3] The oral administration of hydroxyurea has been attempted *in vivo* and showed promising results. This effect is either due to an increase in the content of foetal haemoglobin (HbF),[4] or due to its antioxidant effect,[5] but as a cytotoxic drug, it has potentially toxic reactions. Therefore, long-term administration, especially in children, should be carefully monitored. The calcium antagonist drugs, verapamil and nifedipine, have been tested as well, but for these drugs to attain antisickling plasma levels, high doses need to be administered. Such levels cannot be attained without severe side effects. The present study originated from the increasing evidence that the oil of *Nigella sativa* contains antioxidants,[6,7] as well as calcium antagonist activities,[8] both of which are known to play a role in sickle cell disease.

Thus, this *in vitro* study was undertaken to investigate the effect of extracts from *Nigella sativa* on the blood obtained from patients with sickle cell disease.

METHODS

A total of 32 patients with sickle cell disease were recruited for the study during their consultation at the outpatient department at Basrah Teaching Hospital, Iraq, for minor illnesses as well as for inpatients who had been admitted for a painful crisis. The study was explained to the patients, and written informed consent was obtained. The age of the study population ranged from 7–47 years.

Table I. Summary of patient characteristics with sickle cell disease.

Parameter	No.
Hb-electrophoreses pattern	
Hb SS	22
Hb SF	5
Hb AS	5
Total	32
No. of patients in stable condition	20
No. of patients in painful crisis	12
Mean age ± SD (years)	25.2 ± 11
Mean Hb ± SD (gm/dl)	9.2 ± 0.98

Hb: haemoglobin; Hb SS: homozygous sickle haemoglobin; Hb SF: sickle-foetal haemoglobin; Hb AS: heterozygous sickle haemoglobin; SD: standard deviation

Table II. The *in vitro* effect of the fixed oil extract of *Nigella sativa* on sickle red blood cell. (n = 32)

Concentration of the oil extract of *Nigella sativa*	Percentage of normal red blood cells in a high power field (Mean ± SD)
Control (normal saline)	0
0.01% V/V	0
0.05% V/V	32.8% ± 5.5
0.1% V/V	80.4% ± 3.5*

*The mean difference between the 0.05% and 0.1% concentrations = 47.9; 95% confidence interval of the mean difference = 43.4–52.4, *t*-value = 22.51, P < 0.0001.
SD: standard deviation

The type of haemoglobin was determined by cellulose acetate electrophoresis.

Plant seeds were purchased from a local market in the city of Basrah. They were identified and authenticated by a local pharmacist. Voucher specimens were kept in the Department of Pharmacology.

The seeds of *Nigella sativa* were powdered mechanically using a blender for six minutes. A total of 80 g of the powder was dissolved in petroleum ether and the fixed oil of *Nigella sativa* was extracted by soxhlet at 60°C–90°C for 18 hours. The extract was then concentrated using a rotary evaporator for 30 minutes. In order to eliminate any remnants of petroleum ether, the extract was left in an open dish for 24 hours. Approximately 20 ml of the fixed oil was obtained. A volume of 0.1 ml of the oil was dissolved in 9.9 ml of absolute ethanol to give a concentration of 1% v/v of the extract (stock solution). The following concentrations of 0.1% v/v, 0.05 v/v and 0.01 v/v of the oil were obtained by diluting the stock solution in normal saline. In order to investigate the effect of ethanol on the shape of red blood cells, we had demonstrated in a pilot *in vitro* study that diluted ethanol (1:4 in normal saline) had no effect on the shape of red blood cells in a blood sample obtained from a patient with sickle cell disease.

The 80 g powder of *Nigella sativa* was placed in an ordinary beaker containing 100 ml of distilled water. A magnetic stirrer was used to continuously mix the suspension for 18 hours. The mixture was then filtered using Whatman filter paper number 41. The filtrate was left to dry in an open dish for 24 hours and then freeze-dried to obtain a dry powder. A total of 4 g of the freeze-dried powder was obtained. Of that, 0.5 g was dissolved in 100 ml normal saline to obtain a stock solution of 0.5% w/v of the extract. Concentrations of 0.05% w/v, 0.025% w/v and 0.005% w/v were prepared from the stock solution by adding normal saline.

Slides were prepared for all patients with sickle cell disease (n = 32). A total of 1.5 ml of venous blood was taken from each patient and delivered into three heparinised plastic tubes, with 0.5 ml of blood in each tube. To these tubes, 0.5 ml from each of the following concentrations of *Nigella sativa* fixed oil, 0.01% v/v, 0.05% v/v and 0.1% v/v, were added. The contents of the tubes were mixed immediately, and a drop of the mixture from each tube was spread on a slide and covered with a cover slide. Canada balsam was then threaded onto the edges of the cover slide to ensure complete deoxygenating conditions. The slide was kept in an incubator at 37°C and examined after 24 hours using an ordinary microscope. Three randomly selected locations were chosen for the examination of red blood cells in a high power field. Cell morphology was identified and the average count of normal and sickle cells was taken. Normal saline was used instead of *Nigella sativa* solution as a control. An additional 1.5 ml of venous blood was taken from five patients and the same procedure was repeated using a cold water extract at concentrations of 0.05% w/v, 0.005% w/v, 0.025% w/v and 0.5% w/v.

The preparation of irreversibly sickled cells (ISCs) was performed on eight patients in painful sickle cell crises from the study population. An additional 7 ml of heparinised venous blood was centrifuged at 1,500 gm (3,000 rpm) for five minutes. The plasma and buffy coat were removed by aspiration. The packed cells were washed and centrifuged twice in a suspending medium containing 110 mM NaCl (Evans, North West, England), 5 mM KCl (Merck, Serono, France), 27 mM sodium bicarbonate (Hopkins & Williams, Nottinghamshire, England), 30 mM glucose (Hopkins & Williams, Nottinghamshire, England), 2.4 mM sodium phosphate (Hopkins & Williams, Nottinghamshire, England), 1 mM $MgCl_2$ (Hopkins & Williams, Nottinghamshire, England) and 2% bovine serum albumin (Fluka, Geneva,

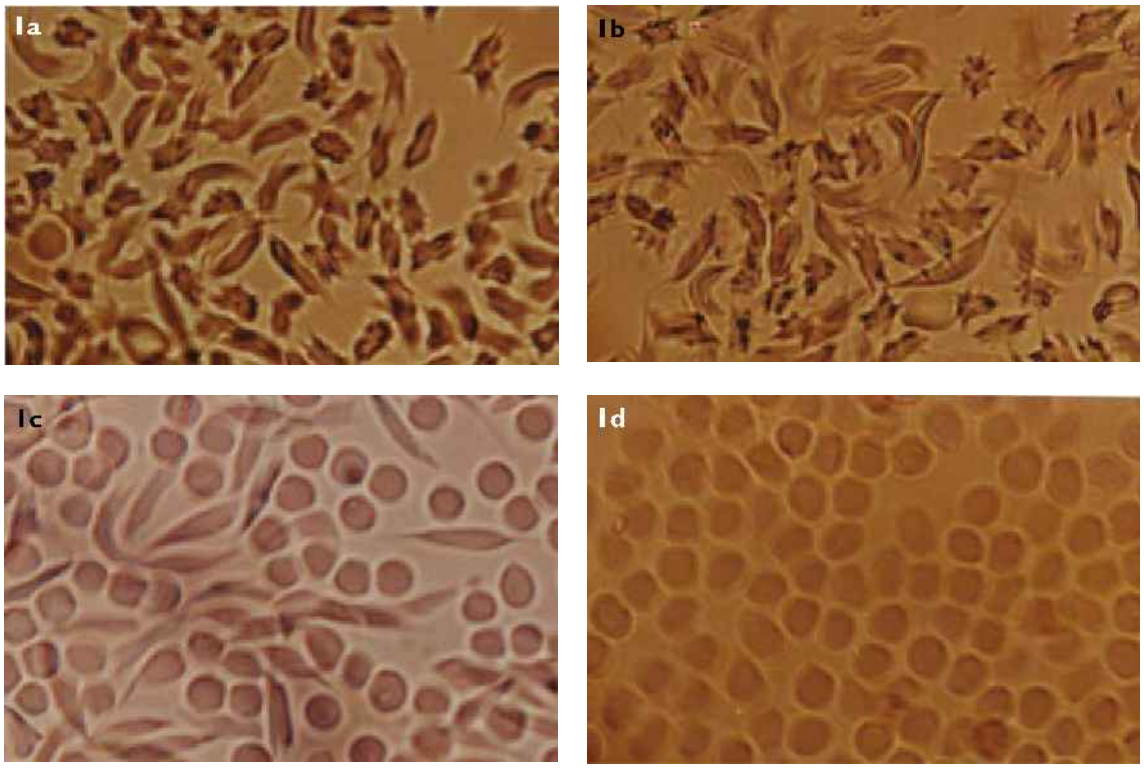

Fig. 1 A representative slide which shows the effect of increasing concentrations of fixed oil extract of *Nigella sativa* on the inhibition of sickling under a deoxygenation condition for 24 hours. (a) Typical sickled cells (control); (b) The effect of 0.01% v/v concentration; (c) The effect of 0.05% v/v; and (d) The effect of 0.1% v/v concentration (Leishman's stain, × 400).

Switzerland). The red blood cells were then suspended in the incubation medium (suspending medium plus 2 mM $CaCl_2$, penicillin G 200 IU/ml (SDI, Baghdad, Iraq) and 0.2 mg/ml streptomycin (SDI, Baghdad, Iraq). The deoxygenation process was accomplished by transferring 2 ml samples of red blood cell suspension in an incubation medium into three 2 ml closed plastic tubes. These were incubated with or without drugs in a water bath at 37°C with slight shaking for two hours. In this experiment, diltiazem (Sigma-Aldrich, Taufkirchen, Germany), a calcium antagonist drug at 7.4 uM and *Nigella sativa* oil at 0.1% v/v were tested. Normal saline was used as a control. After incubation, cell suspensions were oxygenated by room air for 30 minutes to obtain ISCs.[9] The cells were examined microscopically to ensure that they became sickled.

The separation of ISCs was performed according to the method of Ohnishi.[9] A 5 ml density gradient solution, which contained 53% v/v Percoll (Pharmacia, Stockholm, Sweden), 18% v/v meglumine iothalamate (Conray 280, May & Baker, Dagenham, England), 27 mmol/l sodium bicarbonate, 1 mmol/l $MgCl_2$, 1 mmol/l glucose and 0.5% w/v bovine serum albumin at pH 7.4, was centrifuged at 15,000 rpm at 4°C for 15 minutes in an angel rotor (HI-Spin 21 refrigerated centrifuge, MSE, Lancashire, England) in a 10 ml plastic centrifuge tube to form the density gradient. A 0.4 ml aliquot of incubated red blood cells suspension (after oxygenation by room air for 30 minutes) was over-layered on top of the preformed density gradient, and the tube was then centrifuged again in a swing-rotor (MSE, Lancashire, England) at 3,000 rpm for 20 minutes. Both the upper and lower layers were collected separately by aspiration and then washed and centrifuged twice in normal saline (0.9% sodium chloride solution). The shape of the cells in both layers was studied under a phase contrast microscope.

RESULTS

A total of 32 patients with sickle cell anaemia completed the study. 12 patients had vaso-occlusive (painful) crises, and their mean age was 25 ± 11 years and the mean haemoglobin level was 9.2 ± 0.98 gm/dl. This data is presented in Table I.

The antisickling effect of various concentrations of *Nigella sativa* was investigated using the simple slide method under the deoxygenation condition. Microscopic examination revealed that the lower concentration of the fixed oil extract of *Nigella sativa* (0.01% v/v) had no effect on the sickling process of red blood cells. This was observed in all the examined samples and did not differ from that of the control group.

The antisickling effect began to appear when the

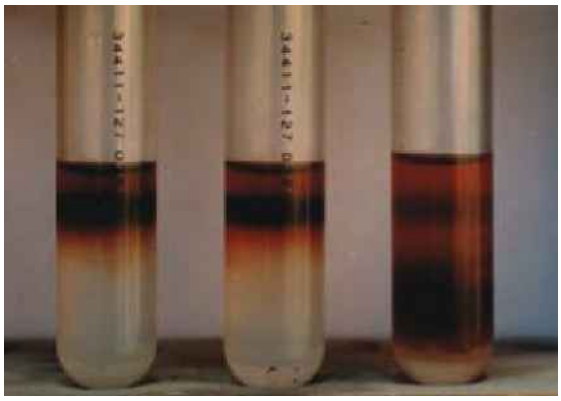

Fig. 2 A representative photograph of the Percoll-Renografin density gradient centrifugation. The left and middle tubes show the effect of *Nigella sativa* and diltiazem, respectively, which resulted in complete inhibition of the bottom layer (dense cells), as compared to the control (normal saline, right tube), which shows a bottom layer consisting of dense cells formed after 24 hours of deoxygenation followed by 30 minutes of reoxygenation.

concentration of *Nigella sativa* was increased by five times to 0.05% v/v. Microscopic examination showed that the mean percentage of red blood cells that maintained a normal shape was 32.8% ± 5.5%. The use of 0.1% v/v concentration of the fixed oil of *Nigella sativa* resulted in a considerable antisickling effect. Microscopic examination revealed that around 80% of the red blood cells were normal in shape. This effect was noted in all the studied samples. The data is presented in Table II and Fig. 1.

The use of cold water extract of *Nigella sativa* at concentrations of 0.005% w/v, 0.025% w/v and 0.05% w/v produced no antisickling effect. A higher concentration of *Nigella sativa* at 0.5% w/v was tested on five specimens of blood and was found to have a minimal antisickling effect. At this concentration level, around 15% of the cells maintained a normal shape.

The separation of HbSS red blood cells by a Percoll-Renografin density gradient yielded a top layer containing less dense reversible sickle cells and a bottom layer containing more dense ICSs. In this model, the shapes of the cells in the top and bottom layers were examined microscopically, and this revealed normally shaped cells in the top layer, while the cells in the bottom layer were all sickle-shaped.

Diltiazem at a concentration of 7.4 uM resulted in complete inhibition of the formation of dense cells (bottom layer). Similar to diltiazem, the fixed oil extract of *Nigella sativa* at a concentration of 0.1% v/v also resulted in complete inhibition of the formation of dense cells. This result was found in all the studied blood samples (n = 8), and is presented in Fig. 2.

DISCUSSION

As sickle cell anaemia is a genetic disease, medications have to be taken for life. Medications with minimal side effects have not yet been made available. Thus, this study was undertaken to investigate the effects of various extracts of *Nigella sativa* on sickle cell anaemia. In this preliminary study, two methods were used for the evaluation of the antisickling effect of *Nigella sativa:* the slide method and the density gradient separation method. The slide method has been described as being reliable, simple and cheap.[10] Both fixed oil and water extracts have been used in this test. The observed antisickling effect of the fixed oil extract and to a lesser extent, of the water extract, might possibly be related to an active ingredient, thymoquinone, which is present in the fixed oil extract.[11] Such an active substance may be missing or present in small amounts in the water extract. It was also noticed that the antisickling effect increased as the concentration of the fixed oil extract increased. Although the data suggests that the concentrations of the fixed oil extract were small and not sufficient to construct a proper dose- or concentration-response relationship, the observed increase in antisickling effect could be related to an increased concentration of an active substance. The second method used was the formation and separation of dense sickle cells. This model is suitable for evaluating the effect of drugs on the formation of dense sickle cells.

The fixed oil of *Nigella sativa* at a concentration of 0.1% resulted in the complete inhibition of the formation of dense cells. The same effect was observed with the calcium antagonist drug diltiazem at a concentration of 7.4 uM, which is ten times its therapeutic concentration. It was reported by using this method that several membrane acting drugs, such as the calcium antagonist drug, diltiazem, can inhibit the formation of dense sickle cells.[2,12] Thus, it cannot be ruled out that the inhibitory effect of *Nigella sativa* on dense cell formation involves an effect on the red blood cell membrane, possibly through a calcium antagonist activity.[13,14] It is worth mentioning that the minimum concentration of diltiazem that shows an inhibitory effect on the formation of dense sickle cells is too high.[2,12] Such levels cannot be achieved without severe side effects. This places the use of calcium antagonist drugs in the treatment of sickle cell disease in jeopardy and lays the basis for the use of other alternatives such as *Nigella sativa.* Moreover, *Nigella sativa* has antioxidant activity.[6,15,16] The fixed oil extract of *Nigella sativa* has greater antioxidant activity levels than those of thymoquinone, the active ingredient in the volatile oil of *Nigella sativa.*[15] It is likely that

the antioxidant activity of *Nigella sativa* is involved in its inhibitory effect on the formation of dense cells. This result is in agreement with that of Ohnishi,[17] who attributed the inhibition of dense sickle cell formation by aged garlic to its antioxidant activity. *Nigella sativa* has many trace elements including zinc.[11] Zinc deficiency in adults with sickle cell disease has previously been reported.[18] Further confirmation of the beneficial effects of zinc in sickle cell disease has been reported by Gupta and Chaubey,[19] who found that a zinc sulphate supplement at a dosage of 220 mg three times daily resulted in a significant reduction in the mean number of infective episodes in patients with sickle cell anaemia. Although a controversial finding, it does seem that zinc has antioxidant activity,[20,21] and therefore, this effect of zinc may be added to other effects of *Nigella sativa*. In conclusion, *Nigella sativa* has an *in vitro* antisickling effect. As a highly consumable herb, many reports have confirmed its safety for use.[22,23] Therefore, a clinical trial to investigate the antisickling effect *in vivo* is recommended.

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