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Evaluation of *Nigella sativa* Seed Constituents for Their in vivo Toxicity in Mice

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Abstract – This study was designed to investigate the effects of main constituents of *Nigella sativa* (NS) seed on the survival and CNS responses in experimental animals. The toxicological investigations were conducted for the determination of median lethal doses (LD_{50}) of NS seed constituents [i.e. aqueous extract (AE), fixed oil (FO), volatile oil (VO)] and main components of its VO [i.e. thymoquinone (TQ), α-pinene (AP) and *p*-cymene (PC)]. A part of this study includes evaluation of NS constituents in the induction of minimal neurological deficit (MND) as a parameter for neurotoxicity using chimney test. In this study, the i.p. LD_{50} values of AE, FO, VO, TQ (suspended in 0.5%CMC), TQ (dissolved in corn oil), AP and PC, were 3020, 3371, 1853, 616.6, 90.3, 1726 and 1523 mg/kg, respectively. All the NS constituents can be considered moderately toxic (LD_{50} ranged from 616.6 to 3371 mg/kg), except the oily solution of TQ, which was very toxic (LD_{50} was 90.3 mg/kg). It appeared that the toxicity of the whole VO is mainly due to its content of TQ and to some extent PC. All the NS constituents induced different degrees of MND at certain dose levels. The median neurotoxic (or sedating) doses (TD_{50}) of AE, FO, VO, TQ (suspended in CMC) and AP and PC, were 950, 1403, 306, 88.1, <173 and 368 mg/kg, respectively. TQ was the most potent component in inducing MND, whereas the FO and AE were the least. Neurotoxicity induced by the VO in the chimney test may refer basically to its contents of TQ and to some extent PC and AP.
Key words – *Nigella sativa*, thymoquinone, fixed oil, volatile oil, α-pinene, *p*-cymene, LD_{50}

Introduction

Nigella sativa Linn. is an annual herbaceous plant from *Ranunculaceae* family (Mahfouz and El-Dakhakhny, 1960) producing small black seeds with aromatic odor and taste (Al-Jassir, 1992; Hag *et al.*, 1995; El-Kadi and Kandil, 1986; El Tahir, 1998; Nergiz and Ötles, 1993). It is used as a flavoring agent for cheese (Babayan *et al.*, 1978) and during baking of bread (Hashim and El-Kiey, 1962). In some parts of Germany, France and Asia, it is used as a condiment and a spice (Al-Hader *et al.*, 1993).

Total oil from *Nigella sativa* (NS) seeds constitute two types of oils, i.e. fixed oil (30-36% w/w) and volatile oil (0.43-0.72% w/w) based on the seed weight (Hashim and El-Kiey, 1962; El-Alfy *et al.*, 1975; Rathee *et al.*, 1982; El Tahir, 1998). Chemical analysis revealed that the volatile oil (VO) of NS seed is composed mainly of thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) and monoterpenes (El-Dakhakhny, 1963; Canonica *et al.*, 1963; Aboutabl *et al.*, 1986). Thymoquinone (TQ) contents range from 18.4 (Canonica *et al.*, 1963) to 24% w/w of the volatile oil (El-Dakhakhny, 1963). The monoterpenes in the volatile oil amount to 46% w/w. The major components of these monoterpenes are *p*-cymene (isopropyl toluene, MW 134.22), which comprises 31.7% of the volatile oil, and α-pinene (2,6,6-tri-methyl-bicyclo [3.1.1.] hepta-2-ene, MW136.24) [Merck index, 1996], which comprises 9.3% of the oil. Other components include phenols (1.7%) and some esters (16%), (Aboutabl *et al.*, 1986; El Tahir, 1998), thymol, dithymoquinone (El-Alfy *et al.*, 1975) and thymohydroquinone (El-Fatary, 1975; Ghosheh *et al.*, 1999).

Besides being used as a spice and a condiment, NS seeds have been used for medicinal purposes in many Middle Eastern and Far Eastern countries for more than two thousand years (Hag *et al*, 1995). It has a long history of folkloric medicinal use in Arabian countries for the treatment of various ailments (Abou Basha *et al.*, 1995). The Arabian authors like Ibn Elbitar, Ibn Sina and Dawood El Antaki described the preparations containg NS seeds to be an excellent remedy for headache, respiratory depression, asthma, calculus of bladder, kidney and as a diuretic (Hashim and El-Kiey, 1962). Its expressed oil is useful in asthma and cough (Hashim and El-Kiey, 1962) and as a

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Abbreviations: *Nigella sativa* = NS; aqueous extract = AE; fixed oil = FO; volatile oil = VO; thymoquinone = TQ; α-pinene = AP; *p*-cymene = PC; minimal neurological deficit = MND

topical treatment for pain and stiffness of joints (Sayed, 1980). Other traditional uses of NS and its derived products included rheumatism and related inflammatory disorders (Houghton *et al.*, 1995). Extracts prepared from NS seeds were widely used as native remedies for diabetes mellitus (Al-Awadi *et al.*, 1991), as carminative, for delayed menses and toothaches (Hashim and El-Kiey, 1962), as a stomachic (Babayan *et al.*, 1978) and digestive stimulants (Al-Jassir, 1992).

The seeds of NS have been subjected to a range of pharmacological investigations in recent years. Clinical and animal studies involved selected constituents of NS seed. Some work on toxicological assessment was also carried out. Total oil of NS seeds (1 ml/kg, p.o.) exhibited potent effects in mice against chemical and thermal noxious stimuli, potentiated pentobarbital hypnosis, reduced ambulatory activity in open field test and enhanced immobility in swimming stress test (Khana *et al.*, 1993).

Total oil of NS (up to 10 ml/kg, p.o.) showed no visual signs of toxicity or mortality during the observation period of 48 hours (Khana *et al.*, 1993). Badary *et al.* (1998) claimed that after acute oral administration the LD_{50} value of TQ (dissolved in corn oil) was 2.4 g/kg in mice. In subacute part of their study, mice were given TQ in drinking water at doses up to 90 mg/kg/day for 90 days with no resultant mortality or signs of toxicity. Their results indicated that the acute oral toxicity of TQ in mice is of a low order and it is generally tolerated when given subchronically at the doses used.

Some of NS constituents (i.e. the total oil) are known to have analgesic, antidepressant and central nervous system (CNS) sedative activity in experimental animals (Khana *et al.*, 1993). Literature survey has shown that there is lack of toxicological investigations. Therefore, determination of acute toxicity of major constituents of NS seeds (i.p.) would be of interest. It was aimed to explore this aspect precisely by i.p. route of administration with other oil constituents of NS. Toxicological screening of aqueous extract of NS seeds and some of its constituents (i.e. FO, VO, TQ, AP and PC) was done by determining

1. The i.p. acute toxicity (LD_{50}) and
2. Minimal neurological deficit (Neurotoxicity) using chimney test.

Experimental

Animals – Male Swiss albino (SWR) mice, weighing 25-30 g were used. The animals were housed in groups, under controlled conditions of temperature (22±1°C) and relative humidity (~50%) as well as 12 hour light/dark cycle; with light between 7:0 a.m. to 7:0 p.m. Animals were allowed food (standard laboratory rodentís chow) and water *ad libitum*.

Isolation of *Nigella sativa* seed constituents

The aqueous extract (AE) of *Nigella sativa* seed: The authenticated *Nigella sativa* seeds of Ethiopian origin were procured from local markets of Riyadh, Saudi Arabia. The identity of the seeds was verified by our laboratory and voucher specimen was kept on record. These seeds were finely crushed, washed with *n*-hexane and macerated in water for 48 hours with occasional shaking. After filtration, the solvent (water) was evaporated under vacuum (Akhtar *et al.*, 1996). The yield of the AE was 116 g per 1 kg of crushed NS seeds.

The fixed oil (FO) – Total oil was extracted from the NS seeds using hexane extraction. The yield was 240 ml per 1 kg crushed NS seeds. The accompanying volatile oil was removed using steam-distillation. The remaining fixed oil was then purified using silica gel column chromatography (El Tahir *et al.*, 1999). The yield of purified fixed oil was 152 ml per 240 ml total oil, i.e. 152 ml fixed oil per 1 kg crushed NS seeds.

The volatile oil (VO) – *Nigella sativa* seeds were crushed and their volatile oil was extracted using steam distillation. The oil was separated from water using diethyl ether. The latter was completely removed from oil via distillation under reduced pressure (400 mbar) at 40°C, (El Tahir *et al.*, 1993) and identified in this laboratory by TLC method. The yield of the volatile oil was 6.2 ml per 1 kg crushed NS seeds.

Chemicals – Thymoquinone (Sigma Chemical Co., St. Louis, MO, USA), α-Pinene (Hopkins and Williams, England), *p*-Cymene (Riedel De Haens, Germany), sodium carboxymethyl cellulose (Winlab, Edgware, Middlesex, UK) and corn oil (Afia®, 100% natural pure corn oil, produced by Savola Edible Oils, Jeddah, Saudi Arabia). Thymoquinone (used in neurotoxicity studies) was suspended in 0.5% carboxy methylcellulose Na (CMC-Na). While doing survival studies TQ dissolved in corn oil was also used for comparison.

Determination of LD_{50} – Preliminary experiments were carried out for each of the NS constituents to determine the approximate doses which produced 0-100% lethality. Doses were chosen based on preliminary data and were administered randomly. Male mice were divided randomly into groups of 5 to 6 mice each. Three to five doses in a dose/response manner were tried for each NS constituent. The test agents were injected intraperitoneally to each group of mice. Death in each group was observed over the next 24 hours. Percent mortalities in all groups were then calculated. The LD_{50} value (95% CL) was calculated using the method of Litchfield and

Wilcoxon (1949).

Induction of minimal neurological deficit (MND) (Neurotoxicity) – Evaluation of the NS constituents for MND inducing activity was done by using chimney test introduced by Boissier *et al.*, (1960), a simple test for the assessment of tranquilizing and muscle relaxant activity in mice. A 30 cm high Pyrex® glass cylinder was used. The internal diameter varies with animal's weight. For mice weighing 25~30 g a cylinder with 30 mm internal diameter was used.

Initially the tube was held in a horizontal position. A mouse was introduced with the head forward. When it reached the other end of the tube, the tube was moved to a vertical position. Immediately the mouse tries to climb backwards. The time required by the mouse to climb backwards out at the top of the cylinder was recorded. Before going for the real experiment the naïve animals were tested to the chimney model as a criterion for their selection. Only those mice that succeeded to climb up within 30 seconds were selected to use in evaluation of test agents. Groups of male mice were given NS constituents intraperitoneally and tested for minimal neurological deficit 45 min post-treatment using chimney model. Several doses of constituents were used to construct a dose-response curve. Minimum of 5 animals per dose level were used. After treatment, the inability of mice to climb up backwards in the tube within 30 seconds was taken as a measure of neurological deficits induced by the test agent.

Statistical analysis – From dose/response analysis of acute toxicity (mortality), neurotoxic (in chimney test) and median lethal doses (LD_{50}) and median MND-inducing doses (TD_{50}) were calculated according to the method of Litchfield and Wilcoxon (1949).

Results

Effects of acute doses of *N. sativa* constituents on survival of mice – The results of LD_{50} (95% CL) values of NS constituents, (i.p.) are shown in Table 1. Variation in LD_{50} values of TQ dissolved in corn oil and that suspended in 0.5% CMC indicated that the bioavailability of suspended TQ was low, while the oily solution of TQ showed high degree of toxicity, which was a result of high absorption into systemic circulation. Large doses of NS constituents induced marked difficulty in respiration, lethargy and writhing in animals. Low LD_{50} values indicated high toxicity. LD_{50} values (mg/kg) as in table 1 can help to arrange degree of toxicities of NS constituents, starting from highly toxic to least toxic constituent, are follows:

1) Oily solution of TQ 90.3 [0.6 mmole/kg]; 2) TQ in CMC 616.6 [3.8 mmole/kg]; 3) PC 1523 [11.3 mmole/kg]; 4) AP 1726 [12.7 mmole/kg]; 5) VO 1853; 6) AE (3020) and 7) FO (3371). It is clear that oily solution of TQ was very toxic when administered intraperitoneally.

Effect of *N. sativa* constituents on the MND (Neurotoxicity) in chimney test – Table 2 shows that NS constituents have varying degrees of MND-inducing potential in chimney test. TQ was most potent (as indicated by low TD_{50}; 88.1 mg/kg), followed by AP (<173 mg/kg), VO (306 mg/kg) and PC (368 mg/kg). The FO and AE were the least effective in the induction of MND. However, the AE (950 mg/kg) was more potent than the fixed oil (1403 mg/kg) in inducing MND. TQ being the most potent in this test showed a dose relationship, was least effective at 10 mg/kg and having 60% failure in chimney test at 50 mg/kg (Table 3).

Table 1. Determination of LD_{50} for *N. sativa* constituents in mice

Treatment	AE	FO	VO	TQ (in CMC)	TQ (in corn oil)	AP	PC
LD_{50} (mg/kg),	3020	3371	1853	616.6	90.3	1726	1523
(95% CL.)	(2530-3610)	(2676-5109)	(1634-2106)	(451.3-842.4)	(77.9-104.7)	(1571-1899)	(1181-1960)
	–	–	–	[3.8 mmoles/kg]	[0.6 mmole/kg]	[12.7 mmoles/kg]	[11.3 mmoles/kg]

AE = Aqueous extract, FO = Fixed oil, VO = Volatile oil, TQ = Thymoquinone, AP = α-pinene, PC = *p*-cymene. 3 to 5 dose levels were used to calculate LD_{50}. 5 to 6 male mice were used at each dose level. Litchfield and Wilcoxon test.

Table 2. TD_{50} of *N. sativa* constituents for the induction of minimal neurological deficit (Neurotoxicity) in mice

Treatment	AE	FO	VO	TQ (CMC)	AP	PC
TD_{50} (mg/kg)	950	1403	306	88.1	<173	368
(95% C.L.)	(430-2100)	(584-3374)	(192-463)	(54.3- 143.1)	–	(271-508)
(Range)	–	–	–	[0.5 mmoles /kg]	[<1.3 mmoles/kg]	[2.7 mmoles/kg]

AE = Aqueous extract, FO = Fixed oil, VO = Volatile oil, TQ = Thymoquinone, AP = α-pinene, PC = *p*-cymene. 3 to 5 dose levels were used to calculate TD_{50} (in mg/kg). 5 to 6 male mice were used at each dose level. Litchfield and Wilcoxon test.

Table 3. Dose-dependent effect of TQ treatment on the induction of minimal neurological deficit (Neurotoxicity) in mice

Treatment	Number of animals	Dose (mg/kg)	% Failure in chimney test
TQ	5	10	–
TQ	5	25	20
TQ	5	50	60
TQ	5	100	80

TQ was administered intraperitoneally.

Discussion

The aim of this study was to assess the safety of NS constituents when injected acutely intraperitoneally to mice. The median lethal dose (LD_{50}) studies can provide valuable information on the toxicity of various compounds. Applying Klaassen and Doull (1980) toxicity rating to results obtained in this study, it appeared that all NS constituents (except the oily solution of TQ) could be considered as moderately toxic (LD_{50} ranged from 616.6 to 3371 mg/kg), whereas the oily solution of TQ was very toxic (LD_{50} was 90.3 mg/kg). The dose of TQ required to cause death of a 25 g mouse (2.26 mg) was far less than its expected amount in the volatile oil dose used. TQ constituted 21.2% of the VO (18.4% to 24%). The amount of TQ by percentage equals to 9.82 mg of TQ in the dose of the VO administered per mouse. Thus failure of whole volatile oil to show higher degree of lethality compared to TQ alone may be due to presence of other constituents, which counter balance the toxicity of TQ. Concerning *p*-cymene, its lethal dose per 25 g mouse was 38.1 mg. *p*-Cymene constitutes 31.7% of the VO contents, so its amount in lethal dose of VO per mouse equals 16.8 mg. This amount is only 53% of the lethal dose of AP. Lethal dose of α-pinene per mouse was 43.2 mg. α-pinene concentration in the VO equals to 9.3 % (meaning that its amount in the lethal dose of VO per mouse is 4.3 mg), which can be considered negligible and may not have a true role in the toxicity of the VO. It can be concluded that lethality of the VO is mainly due to its content of TQ and to some extent *p*-cymene.

In this study, LD_{50} of TQ was 90.3 mg/kg (77.9-104.7, 95%CL) after i.p. administration. In contrast, Badary *et al.* (1998) reported that LD_{50} of TQ was 2.4 g/kg (1.52-3.77, 95%CL) by oral route of administration. The differences in these results indicated that bioavailability of TQ after oral administration is very low, while after i.p. administration toxicity is increased as a result of maximum absorption of TQ into the systemic circulation. Oral TQ may be biotransformed in gastrointestinal tract and/ or liver into less toxic metabolites, like dihydro-thymoquinone. This metabolism may be achieved by the widely distributed enzyme, DT-diaphorase, which is well known to catalyze reduction of a wide variety of quinones (Nagi *et al.*, 1999). This catalysis has generally been considered to be detoxification pathway since the resulting hydroquinone may be conjugated and excreted. This fact may explain the low toxicity of oral TQ (Nagi *et al.*, 1999). High toxicity of TQ after i.p. administration compared to oral TQ may be due to high activity of DT-diaphorase in the GIT as compared to liver and other tissues in the body.

Abdel-Fattah *et al.* (2000) investigated antinociceptive effect of oral and i.p. TQ administration in hot-plate, tail-pinch test, acetic acid-induced writhing, the early and late phase of the formalin test in mice. Only 50% of the oral TQ dose was required to get equipotent effects when i.p. route was used. These results can explain differences in oral and i.p. routes in TQ-induced lethality. On the other hand Nigellone (the carbonyl polymer of TQ) was found to be far less toxic than TQ (Chakravarty, 1993).

Similarly, Khana *et al.* (1993) claimed that total oil in the doses up to 10 ml/kg, p.o. showed no mortality up to 48 hours in rats. The total oil of NS constituents is composed of fixed oil and volatile oil. Our findings were at variance from the claims of Khana and colleagues. In the present study both the fixed oil (3371 mg/kg, i.p.) and volatile oil (1853 mg/kg, i.p.) were lethal to 50% of mice within 24-hour period. The main reason for this discrepancy might be the difference in the route of administration. Or it may be due to animal species sensitivity difference. However, in the present study it was found that, the AE of NS caused death to 50 % of SWR mice at a dose of 3020 mg /kg, i.p.

There is no report in the literature on the LD_{50} of *p*-cymene by i.p. route in the experimental animals. Oral LD_{50} of *p*-cymene in rats is 4750 mg/kg, (Merck index, 1996). In the present study we found that the LD_{50} by i.p. route in mice was far less than this dose. It was calculated to be about 1523 mg/kg. This variation seemed to be attributed mainly to difference in routes of administration. Oral *p*-cymene may be poorly absorbed or biotransformed in the GIT to less toxic metabolites. Difference in animal species could be another reason for this variation.

Concerning neurotoxicity inducing potential of NS constituents in the chimney test, administration of all the constituents showed varying degrees of MND. The median toxic (i.e. MND-inducing) doses of AE, FO, VO, TQ (suspended in 0.5%CMC), α-pinene and *p*-cymene were 950, 1403, 306, 88.1, <173 and 368 mg/kg, respectively. TQ was the most potent agent causing MND. Whereas, FO was the least neurotoxic constituent. TD_{50} of VO, TQ, α-pinene and *p*-cymene per mouse were 7.65 mg, 2.2 mg, 4.3 mg and 9.2 mg, respectively. MND induced by the

VO can refer mainly to its contents of TQ, and to some extent *p*-cymene and α-pinene. In these experiments, the effects of different doses (10, 25, 50 and 100 mg/kg) of TQ on the dose-response were investigated for its MND-inducing effects. TQ at 50 and 100 mg/kg doses significantly induced the neurotoxicity as seen by a failure in the chimney test. It seems that the neurotoxicity induced by VO basically refers to its contents of TQ and to some extent to PC and AP.

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