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Evaluation on Cytotoxicity of Ethanolic Extract of *Nigella sativa* Seed

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ABSTRACT

Introduction: Adverse effects of herbal products have often been ignored apparently due to its common use and acceptance over allopathic. Diverse studies on the medicinal properties of *Nigella sativa* are easily accessible, nevertheless, very few concerns for its toxicity.
Objective: The present study was designed to evaluate cytotoxicity of ethanolic extract of *Nigella sativa* seed (NSEte) at various dose levels.
Methods: Three doses of NSEte i.e. 5, 50 and 500 mg/kg bd. wt/day, administered orally for 90 days in Wistar albino rats. Chromosomal aberration and micronucleus test were estimated in bone marrow. Mutagenicity was evaluated through percentage of cellular survival for colony forming units following induction with NSEte in *Salmonella typhimurium* strains - TA100, TA98 and TA97a at concentrations 0.1, 1 and 10 g/plate.
Results: The mitotic indices revealed no statistically significant variation. There were no changes in number of micronuclei when compared to control. The mutagenic indices were found insignificantly variable when compared to control (MuI<2).
Conclusion: It was concluded therefore, that the doses of ethanol extract of *Nigella sativa* seed used in this study are safe and are highly unlikely to have any adverse effect.

Keywords- *Nigella sativa*, Alternative medicine, AMES test, Cytotoxicity.

INTRODUCTION

The plant *Nigella sativa* is well known for its therapeutic effect against various diseases/conditions such as, Parkinson, urease, infertility, overweight, memory impairment, lung cancer, diabetes etc[1-7]. Thymoquinone, dithymquinone, thymohydroquinone, and thymol are the main active compounds responsible for the therapeutic effects of *Nigella sativa* seeds. Variable doses of extracts of *Nigella sativa* are being recommended by the medical practitioner around South Asian, Middle Eastern and European countries for a range of medical conditions. However, limited

attention is given to the related dose dependent toxicity.

Few earlier studies have reported adversity of uses of *Nigella sativa* as a therapeutic agent. A recent study evaluated the potential role of *Nigella sativa* seed oil on oxidative stress and revealed that it was able to elevate the concentration of glutathione, lactate dehydrogenase, hepatic succinate dehydrogenase and attenuate the level of malondialdehyde, superoxide dismutase, etc[8]. Another study reported that cultured rat cortical neurons when exposed to different times and concentrations of *Nigella sativa* dry methanolic extract, it did not induce any toxicity. However, the same study also advocates evidently that it modulated amino acid release in cultured neurons, for example, secretion of glutamate, aspartate, and glycine were decreased[9]. In addition, few studies have noticed increased phagocytic activity of peritoneal macrophages, hepatotoxicity and nephrotoxicity following *Nigella sativa* administration[10-13]. Thymoquinone, which has been shown to exert anti-inflammatory, anti-oxidant and anti-neoplastic effects both *in vitro* and *in vivo* appear to have multiple physiological side-effects, including cell death[14]. From the above studies it is apparent that despite its medicinal properties, it also has potential for serious side-effects if taken at higher doses.

We assume that prolonged traditional herbal usage cannot be guaranteed for its safety, because, it is difficult to monitor or to detect the collective multifactorial delayed effects arising from long-term use. In this study we have attempted to estimate cytotoxicity of ethanolic extract of *Nigella sativa* seed (NSEte) in Wistar albino rats and through reverse mutation test in strains of *Salmonella typhimurium*.

MATERIALS AND METHODS

Test materials

Fresh seeds of *Nigella sativa*, were procured commercially (voucher no.21, dated 04.02.2012), authenticated in the Department of Botany, University of Ranchi, Ranchi, India. The seeds were shaded, dried and powdered. The powdered seeds (500 g) was extracted with petroleum ether (60-80°C) to remove lipids. It was then filtered and the residue was extracted with 95% ethanol by soxhlet extraction. The extract obtained was evaporated and concentrated on a water bath at atmospheric pressure to a semisolid condition, which was further dried in an oven at 30°C on a shallow dish to constant weight to remove the solvent completely (yield, 22.78%).

Animals

Adult Wistar albino rats, approximately three months old, weighing 180–200 g, were used in this investigation. The animals were maintained in individual polypropylene cages with a 12:12-h light: dark schedule. The temperature in the animal house during the study period was maintained at 23 ± 2ºC, and the relative humidity ranged between 32-70%. The feeding schedule consisted of two rat pellet meals a day, and water was provided *ad libitum*. Daily intake of food and water were quantified precisely. The animals were maintained under veterinary supervision in accordance with the Guidelines for Care and Use of Animals in Scientific Research[15]. The experimental protocol has the approval of the Institutional Animal Ethical Committee (IAEC). (Z/23/3.12.2011).

Experimental design

The animals were divided into four groups, ten animals in each, Group I, served as control, were treated orally with olive oil at 10 ml/kg body weight each day for 90 days, Group II, Group III and Group IV

were treated orally with the NSEte, at a standardized dose of 5, 50 and 500 mg/kg body weight, respectively, each day for 90 days.

Euthanization

After the last scheduled dose of extract was administered animals were euthanized by cardiac puncture under ether anesthesia.

Chromosomal aberration

Animals were injected with 0.2 ml of 0.5% colchicines to arrest the cells at metaphase. Bone marrow was aspirated from femur in a test tube and cell suspension was prepared. Cells were then treated with KCl solution (0.56%) and fixed in Carnoy's fixative. Cells were then stained with 5% Giemsa and observed under 100x oil immersion. A total of 500 metaphase arrested cells were evaluated for aberrant chromosomes. Likewise, 500 bone marrow cells were also examined for mitotic activity by scoring the number of cell in mitosis. Cytotoxic activity was estimated by the mitotic index, calculated by dividing cells to the total numbers of cell counted. For positive control ten Wistar albino rats were used. Positive control animals were orally administered with 50 mg/kg body weight of acrylamide (Himedia Laboratories, Pvt. Ltd., Mumbai) for 5 days.

Micronucleus test

Bone marrow cells were collected in fetal bovine serum (FBS) and after washing smeared on to glass slide. Further, slides were stained with May-Gruenwald and Giemsa stain according to Schimid[16]. A total of 500 erythrocytes were examined for micronuclei. For positive control ten Wistar albino rats were used. Mitomycine C (3 µg/g body weight) was injected before 48 h of euthanization for positive control.

Reverse mutation assay

The test strains used in this study were *Salmonella typhimurium* TA100, TA98 and TA97a (Bruce Ames Laboratory, Molecular and Cell Biology, University of California) (Table 1). Each test strain was incubated overnight in 2.5% Nutrient broth at 37°C with shaking at 100 strokes/min, in dark. Rat liver was used to prepare S9, treated with Phenobarbital (Sigma-Aldrich, MO, USA) and 5, 6-benzoflavone (Sigma-Aldrich, MO, USA). The S9 metabolic activation mixture (S9 mix) was used for the bacterial reverse mutation test and survival test as described by Maron and Ames[17] with slight modification. S9 mix was prepared by adding 0.1 ml of S9, 8 mmol of $MgCl_2$, 33 mmol of KCl, 5 mmol of D-glucose-6-phosphate, 4 mmol of NADPH, 4 mmol of NADH and 100 mmol of sodium phosphate (pH 7.4). Top agar consisting of 0.05 mmol histidine, 0.6% NaCl and 0.6% agar (Himedia Laboratories, Pvt. Ltd., Mumbai) was sterilized by an autoclave.

Reverse mutation test was conducted according to the standard protocol (with slight modification) as described by Yahagi *et al.*[18] and Mortelmans and Zeiger[19]. Briefly, separate test tubes containing 0, 0.1, 1 and 10 g of NSEte, 500 µl of buffer (0.1 M sodium phosphate buffer, pH 7.4) with or without S9 mix and 100 µl of test strain suspension was incubated for 20 min at 37°C with shaking at 120 strokes/min in dark. Further, 2 ml of top agar was added to the test tube and mixed, the mixture was then poured onto the surface of a plate containing minimal glucose agar medium (VBE medium (Vogel Bonner E), 0.5% Glucose, 1.5% Agar). Plates containing 20 ml of minimal glucose agar medium was incubated for 48 h at 37°C and the reverted colonies were counted. Experiments were repeated thrice for each dose to calculate an average value. The positive controls for the culture without S9 mix was Sodium Azide (20 µg/plate) (Sigma-Aldrich, MO, USA)

and for cultures with S9 mix was 2-aminoanthracene (20 µg/plate) (Sigma-Aldrich, MO, USA) for all test strains.

Survival experiments

To determine the cytotoxic effects of NSEte, the pre-incubation assay mixture in the bacterial reverse mutation test was diluted in 0.9% NaCl. This suspension contained 2.0×10^3 cells/ml. An aliquot (100 µl) of this suspension was plated on nutrient agar (0.8% nutrient broth, 0.5% NaCl, and 1.5% agar). The plates were then incubated at 37^oC for 24 h and survival fractions were calculated as percentage survival compared to the negative group. All experiments were performed in triplicate.

Statistical analysis

Significant differences between treatment and the control group in the micronucleus assay were assessed by Dunnet's multiple comparison tests. One-way ANOVA and Tukey's HSD post hoc analysis was used for positive results of the assay. Values are expressed as mean ± standard deviation (sd), and $P<0.05$ was considered statistically significant.

RESULTS

Chromosomal aberration in bone marrow cells

Chromosomal aberration per 500 dividing cells was recorded. The incidences of structural abnormalities of chromosomes, in the single chromatid and double chromatid were minimal. The variation was recorded insignificant in all test groups, however Group II and Group IV showed more than one type of aberration such as fragments, rings, and variably damaged chromosomes. The mitotic indices revealed no statistically significant variation. The variation in number of aberration between test groups and positive controls were found highly significant, as shown in Table 2.

Formation of polychromatic and normochromatic erythrocytes

The incidences of micronuclei in polychromatic red blood cells (RBC) of Group I animals were found close to 0.2%, whereas, ratio of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were about 6%. There were no significant variations in number of micronuclei, observed in Group II, Group III and Group IV when compared to control (Table 3). Animals treated with Mitomycine C showed significantly higher percentage of PCEs, NCEs and their respective ratios.

Salmonella reverse mutation assay

Average number of revertant colonies found in negative control showed almost three folds increase for TA100 and TA97a in both the presence and absence of S9 mix when compared to TA98. Incorporation of NSEte showed insignificant mutagenic activity for the applied range of doses in both absence and presence of S9 mix. No significant variation in mutagenic indices were observed for any applied doses and found comparable to negative control. Percentage of survival fractions were mostly recorded 100%, however, TA100 show slight variation in survival rate but considered insignificant for any dose related response (Table 4). Sodium Azide and 2-aminoanthracene incorporated plates were observed with more than 500 colonies, the mutagenic indices were found significantly higher when compared to control and the test groups (Not shown in table).

DISCUSSION

An earlier study reported that *Nigella sativa* fixed oil has low toxicity which was evident by its high value of LD_{50} and almost no morphological changes in the histopathological examination of vital organs[20]. There are wide ranges of studies which proved its hepato-protective effect as

well as its mild hepatotoxicity[21-25]. Overall, there are mixed conclusion over the toxicity of *Nigella sativa* in the scientific world, it falls in both positive and negative categories. We presume that it is a commercially available supplement, and the consumption in raw form is still quite popular. People usually consume it in very small quantity, therefore, unaware to the unknown effect of its constituents. In the present study we observed no clear sign of cytotoxicity caused by NSEte regardless of the concentration of doses.

The result of chromosomal aberration in this study showed normal chromosomal morphology despite varying doses. Similarly, micronucleus test revealed no adverse activity induced by doses applied in this investigation. In addition, no growth inhibition of bone marrow cell was observed in any of the experimental groups, since no differences were found in the PCE: NCE ratio among the groups.

Lower nontoxic concentrations are important to be preliminarily determined in order to show the real mutagenic effects of the tested compounds[26,27]. Therefore, in this study lower doses were also taken in to account. A measurable increase at one or more concentration in the number of revertant colonies per plate in at least one strain with or without a metabolic activation system, and/or a statistical analysis of an increase over background response can determine the positive result. Our results clearly show that none of the *Salmonella* strains used in this experiment indicated NSEte induce mutagenesis with or without metabolic activation, thus, revealing the doses under examination are less likely to induce genetic interference.

CONCLUSION

We estimate from the study that the ethanolic extract of *Nigella sativa* seeds is highly unlikely to produce any cytotoxic activity at doses investigated. However, further studies are required to ascertain its safety at higher doses.

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Table 1. *Salmonella typhimurium* strains used in this experiment

Salmonella strain	Reversion	Genotype
TA97a	Frameshift	*his* D6610 rfa*
TA98	Frameshift	*his* D3052 rfa
TA100	Base-pair substitution	*his* G46 rfa

*Genes for biosynthesis of the lipopolysaccharide core are termed rfa.

Table 2. Frequency of aberrant cells and mitotic indices (MI) of bone marrow following treatment with NSEte (Values are in mean ± SD) (n=10)

Groups	Cells with Single aberration	Cells with more than one Aberration	Average number of Aberrant Cells	No. of Cells in Mitosis (MI)[a]
Group I	20.45±1.76	-	20.45	72.12±11.25 (14.42)
Group II	19.35±0.75	0.31±0.05	19.66	65.25±7.01 (13.05)
Group III	20.33±0.30	-	22.33	85.39±3.74 (17.08)
Group IV	21.20±1.30	0.80±0.44	22.00	68.66±10.80 (13.73)
Positive Control[b]	46.40±3.64	23.80±2.86	70.20*	18.05±5.28 (3.61)*

Group I: Control (Olive oil 10 ml/kg bd. Wt.); Group II: Oral administration of NSEte 5 mg/kg bd. wt.; Group III: Oral administration of NSEte 50 mg/kg bd. wt.; Group IV: Oral administration of NSEte 500 mg/kg bd. wt. All groups received treatment of 90 days.
[a]Mitotic index (MI): Ratio of the total 500 cells counted from induced samples/spontaneous number of cells in mitosis.
[b]Acrylamide (50 mg/kg bd. Wt.) was orally administered for 5 days.
*P>0.05

Table 3. Micronucleus record of rats following treatment with NSEte. (A total of 500 erythrocytes were counted) (Values are in percentage) (n=10)

Groups	Micronuclei in Polychromatic RBC	Micronuclei in Normochromatic RBC	Polychromatic RBC/Normochromatic RBC
Group I	0.17	0.03	5.66
Group II	0.21	0.04	5.50
Group III	0.20	0.05	4.00
Group IV	0.19	0.04	4.75
Positive Control[a]	3.98*	0.47*	8.46*

Group I: Control (Olive oil 10 ml/kg bd. Wt.); Group II: Oral administration of NSEte 5 mg/kg bd. wt.; Group III: Oral administration of NSEte 50 mg/kg bd. wt.; Group IV: Oral administration of NSEte 500 mg/kg bd. wt.
[a]Mitomycine C (3 µg/g bd. Wt.) was injected before 48 h of euthanization.
*P>0.05

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Table 4. Mutagenicity in *Salmonella typhimurium* strains with and without metabolic activation following incorporation of NSEte. (Values are in mean ± SD)

Strain	NSEte (g/plate)	-S9 Mean ± SD (MuI*)	-S9 Survival	+S9 Mean ± SD (MuI*)	+S9 Survival
TA98	0	38.25±6.21 (1.00)	100	40.77±3.33 (1.00)	100
	0.1	40.33±4.50 (1.05)	100	42.33±4.50 (1.03)	100
	1	43.67±3.25 (1.14)	95	47.00±3.00 (1.15)	100
	10	42.67±5.03 (1.12)	90	45.67±6.02 (1.12)	100
TA100	0	124.29±15.38 (1.00)	100	121.37±30.01 (1.00)	100
	0.1	122.00±39.94 (0.98)	100	130.33±43.15 (1.07)	100
	1	148.67±19.73 (1.20)	91	159.33±18.58 (1.31)	85
	10	134.33±28.74 (1.08)	80	148.00±30.78 (1.21)	95
TA97a	0	125.09±28.36 (1.00)	100	129.17±33.49 (1.00)	100
	0.1	133.33±12.70 (1.07)	100	142.00±12.48 (1.10)	100
	1	157.67±15.04 (1.26)	100	164.00±19.07 (1.26)	100
	10	154.67±8.32 (1.24)	100	166.00±9.16 (1.29)	100

*Mutagenic index (MuI): ratio of the *his*+ induced with samples/spontaneous *his*+ in the negative control. Positive responses considered significant when MuI ≥ 2.