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# Phytochemical Screening and *In vivo* Antileukemic Activity of Methanolic Extract of Seeds of *Nigella Sativa* L. on Benzene-Induced Leukemia in Wistar Rats

M. D. Imaduddin[1]*, N. Saba[2], A. B. Eman[2], K. Saba[2], A. Kashfiya[2]

## Abstract

*Nigella sativa* is exquisite bounty of nature to humankind. It is an annual flowering plant of family Ranunculaceae majorly found in Southwest Asia, seeds are used as flavor food. The present work was directed to evaluate antileukemic effect of *N. sativa* seeds extract in benzene-induced leukemia in female Wistar rats. Seed powder was extracted using petroleum ether, chloroform, ethyl acetate, and methanol. OECD guidelines 423 were used to study acute oral toxicity study and intravenous injection of benzene was given on every 2nd day up to 21 days to induce leukemia. To assess burden of leukemia, various parameters analyzed are body weight (B.W), organ weight, complete blood picture, serum potassium, phosphate, uric acid, and glutathione. The presence of alkaloids, carbohydrates, cardiac glycosides, tannins, phytosterols, and flavonoids is confirmed by phytochemical screening. Acute oral toxicity study found 2000 mg/kg as a safe dose of *N. sativa* seed extract. Significant decrease in potassium, phosphate, uric acid, WBC, and increase in B.W, glutathione demonstrated therapeutic efficacy of extract in leukemia. Our results confirmed significant antileukemic effect of *N. sativa* seed extract, suggesting role of this herb for the treatment of malignancies such as leukemia.

**Keywords:** Benzene, Glutathione, Hematological parameters, Leukemia, *Nigella sativa*

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## Introduction

Certain mutations trigger leukemia by activating oncogene, deactivating tumor suppressor genes, and causing disruption of regulation of cell death.[1] According to the study of Center for Research in Epidemiology and Population Health in France, mothers who use fertility drugs to induce ovulation bear a child who is more than twice as likely to develop leukemia during their childhoods as other children.[2] Herbalism is genre of medical science in which traditional herbs are studied for their use as an alternative to existing ones. Herbal medicines consolidate cure of chronic diseases such as cancer, asthma,[3] diabetes,[4] and end-stage renal disease,[5] with very less or null adverse effects. It was only then that scientists prioritized herbal medicines as their major asset and imminently developed herbal drugs.

*Nigella sativa*, discovered by Egyptians, was found to have admanting should be replaced with efficient curing capacity of ailments, be it chronic or acute, malignant, or benign by magnifying immune system. It was found to be one of the items to be entombed with a pharaoh to assist him in his afterlife.[6] Alpha-hederin and thymoquinone, phenolic content, and flavonoids are principal known anticancer constituents of *N. sativa*,[7] thymoquinone exhibits antiproliferative effect in human myeloblastic leukemia HL-60 cells.[8] $\alpha$-hederin also induced death of murine leukemia P388 cells by a dose- and time-dependent increase in apoptosis.[9] Several such activities are exhibited by *N. sativa* which include antioxidant and anti-inflammatory,[10] gastroprotective,[11] hepatoprotective,[12] cardioprotective,[13] nephrotoxicity,[14] anti-asthmatic,[15] anti-infertility,[16] antimicrobial,[17] antidiabetic,[18] immunomodulatory,[19] and anti-tumor.[20] Hence, based on above assumptions and widely accepted pharmacological efficiency of *N. sativa*, this study was conducted to evaluate claimed anticancer activity in benzene-induced leukemia (BIL) in female Wistar rats.

[1]Department of Pharmacology, Pulla Reddy Institute of Pharmacy, Sangareddy, Telangana, India

[2]Department of Pharmacology, MAK College of Pharmacy, Hyderabad, Telangana, India

**Corresponding Author:** M. D. Imad Uddin, Department of Pharmacology, Pulla Reddy Institute of Pharmacy, Sangareddy - 502 313, Telangana, India. Mobile: +91 837 417 5556. E-mail: imadpharma111@gmail.com

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## Materials and Methods

### Chemicals and Instruments

All chemicals and reagents, namely, petroleum ether, chloroform, methanol, benzene, glutathione, (5,5-dithio-bis-(2-nitrobenzoic acid) DTNB, trisodium citrate, disodium hydrogen phosphate, and dihydrogen sodium phosphate are of analytical grade. Cyclophosphamide obtained as a gift sample from Biochem Pharmaceuticals Limited. UV–Visible Spectrophotometer (Agilent technologies, G6860A-cary 60 UV-Vis), Centrifuge (LAFCO Pvt. Ltd.), Incubator (SISCO Pvt. Ltd) are the various instruments used in the study.

### Animals

All experiments were performed in accordance with the direction of Institutional Animal Ethics Committee (1970/PO/Re/S/17/

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CPCSEA) on female Wistar rats weighing about 130–150 g obtained from the Central Animal House of Sanzyme Pvt. Ltd. Hyderabad, India. Rats were housed under 12 h dark and 12 h light cycle at 22–28°C and relative humidity of 65 ± 10% in standard propylene cages with free access to water and food.

### Collection, Authentication, Extraction, and Phytochemical screening of *N. sativa*

*N. sativa* seeds were collected from local market and authenticated by Botanical Survey of India, Hyderabad region, Telangana, India, and deposited with a voucher specimen no. of COL/14/012. These fresh seeds are bowdlerized into small pieces, later they are dried under shade for 13 days and finally fed through blender to obtain coarse powder of seeds. One hundred and twenty grams of the dried powder were successively extracted by hot continuous extraction method for 36 h by Soxhlet apparatus, using different organic solvents such as petroleum ether (40–60°C), chloroform (61.15°C), ethyl acetate (56°C), methanol (64.7°C), and water (100°C). All extracts evaporated in a rotary evaporator at 60°C and dried in desiccator for 1 h. Percentage yield of each extract was determined by formula % yield = weight of extract in grams/weight of sample in grams ×100. Preliminary phytochemical assessment was carried out using methanol for further for further study because methanolic extract showed the presence of alkaloids, flavonoids, carbohydrates, cardiac glycoside, and phytosterols.[21-23]

### Acute Oral Toxicity Test

Acute oral toxicity study of the methanolic extract of seeds of *N. sativa* (MENS) was performed on female Swiss albino mice (18–28 g) according to the OECD guideline No. 423. Animals were divided into two groups with three animals in each, all animals were fasted for 4 h and to the first and second group, saline (1 ml/kg) and MENS 2000 mg/kg were administered orally, respectively. After oral administration animals were monitored for the first 4 h for autonomic and neurobehavioral indications, death, and then for 14 days for weight loss and mortality. The absence or presence of compound-related mortality of the animals dosed at one step will determine next step. All observations are systematically recorded with individual records being maintained for each animal.

### Antileukemic Activity of MENS in BIL in Wistar Rats

Twenty healthy female Wistar rats are used for the present study and divided into five groups of four in each as follows, Group I: Normal control, rats treated with vehicle (Tween 80, 10% v/v, 1 ml/kg), Group II: Negative control, effect of vehicle (Tween 80, 10% v/v, 1 ml/kg) on BIL in Wistar rats, Group III: Positive control, effect of standard drug (cyclophosphamide 10 mg/kg, body weight [B.W], P.O.) on BIL in Wistar rats, Group IV: Effect of lower dose (MENS 500 mg/kg, B.W, P.O.) on BIL in Wistar rats, and Group V: Effect of higher dose (MENS 1000 mg/kg, B.W, P.O.) on BIL in Wistar rats. Leukemia was induced by intravenous injection of 0.2 ml of a 1:10 diluted benzene solution (Chromasolv, in water/2-propanol [50/50] v/v). Intravenous injection was given on every 2nd day for 3 consecutive weeks.[24] From day 0 to 21 day after leukemia induction, MENS was administered orally. Burden of leukemia was evaluated by assessing hematological parameters, serum parameters, B.W, and organ weight in all experimental groups.

### Effect of MENS on B.W and Organ Weight in BIL Rats

B.W of all animals was assessed every week and weight of organs, namely, heart, lungs, pancreas, liver, spleen, and left and right kidney was taken on day of scarification.

### Effect of MENS on Hematological and Serum Parameters in BIL Rats

On the 21st day, blood was collected by retro-orbital puncture from each rat and centrifuged at 2000 rpm for 10 min to obtain serum which was used for estimation of uric acid, potassium, phosphate, and glutathione. On same 21st day, animals of different groups are sacrificed by the following ethical guidelines and blood was collected by cardiac puncture in EDTA tubes and used for the estimation of red blood cells (RBC), hemoglobin (HB), packed cell volume (PCV), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), white blood cells (WBCs), and in differential WBCs such as neutrophils, basophils, eosinophils, lymphocytes, and monocytes.

### Statistical Analysis

Data obtained were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison test. All values are expressed as Mean ± SEM.

## Results and Discussion

### Percentage Yield and Phytochemical Screening of Various Extracts

% Yield, color, consistency, and weight of extracts are presented in Table 1. All these extracts were found to contain alkaloids, carbohydrates, cardiac glycosides, tannins, phytosterols, and flavonoids [Table 2].

### Effect of MENS on B.W and Organ Weight in BIL Rats

On day 7, there was a significant increase ($P < 0.05$) in B.W of only cyclophosphamide (10 mg/kg) and MENS (1000 mg/kg) treated animals and on day 14 and day 21 B.W of all treatment groups increased highly significantly ($P < 0.0001$) as compared to negative control group [Table 3]. No significant change in weight of organs of all treatment groups throughout study as compared to negative control group (data not shown). Decrease in B.W of negative control group animals may be due to poisoning caused by repeated exposure of benzene whereas efficacy of MENS was ascribed by increase in B.W of treated rats during study. Same results were shown in female rats treated with camel's milk after inducing leukemia with benzene.[25]

### Effect of MENS on Hematological and Serum Parameters in BIL rats

In all treated groups, there was no significant change in RBC, HB, MCH, and PCV as compared to negative control group (data not shown). MCV decreased in MENS and cyclophosphamide-treated groups as compared to negative control group. However, there was significant increase in MCHC levels in both treatment groups viz., MENS and cyclophosphamide. [Figure 1]. Effect of WBC and differential WBCs is presented in Table 4. Potassium, phosphorous,

**Table 1:** Color, consistency, weight, and % yields of various extract

| *Extract* | *Color of extract* | *Consistency of extract* | *Weight of sample (gm)* | *Weight of extract (gm)* | *% yield* |
|---|---|---|---|---|---|
| Petroleum ether | Dark brown | Semi-solid | 120 | 8.9 | 7.41 |
| Chloroform | Grayish | Powder | 120 | 1.8 | 1.5 |
| Ethyl acetate | Black | Semi-solid | 120 | 2.0 | 1.6 |
| Methanol | Brownish-black | Semi-solid | 120 | 7.8 | 6.5 |
| Water | Greenish-brown | Semi-solid | 120 | 3.7 | 3.08 |

gm: Grams

**Table 2:** Phytochemical constituents screening results

| *Phytochemical constituents* | *PENS* | *CENS* | *EAENS* | *MENS* | *WENS* |
|---|---|---|---|---|---|
| Alkaloids | | | | | |
| Mayer's test | +ve | +ve | +ve | +ve | +ve |
| Wagner's test | +ve | +ve | +ve | +ve | +ve |
| Dragendorff's test | +ve | +ve | +ve | +ve | +ve |
| Hager's test | +ve | +ve | +ve | +ve | +ve |
| Carbohydrates | | | | | |
| Molisch's test | +ve | +ve | +ve | +ve | +ve |
| Benedict's test | −ve | −ve | −ve | +ve | −ve |
| Fehling's test | −ve | −ve | +ve | +ve | −ve |
| Saponins | | | | | |
| Foam test | −ve | −ve | +ve | −ve | −ve |
| Tannins | | | | | |
| Iodine test | +ve | +ve | +ve | +ve | +ve |
| Gelatin test | −ve | −ve | −ve | −ve | −ve |
| Lead acetate test | +ve | +ve | +ve | +ve | +ve |
| Acetic acid test | −ve | −ve | −ve | −ve | +ve |
| Dil. KMNO4 test | +ve | +ve | +ve | +ve | +ve |
| Cardiac glycoside | | | | | |
| Legal's test | −ve | +ve | +ve | +ve | +ve |
| Killer–Killani test | −ve | +ve | +ve | +ve | +ve |
| Phytosterol | | | | | |
| Salkowski's test | +ve | +ve | +ve | +ve | +ve |
| Liebermann–Burchard Test | +ve | −ve | −ve | +ve | +ve |
| Phenol | | | | | |
| Ferric chloride test | −ve | −ve | −ve | −ve | −ve |
| Proteins and amino acids | | | | | |
| Ninhydrin test | −ve | −ve | −ve | −ve | −ve |
| Millon's test | −ve | +ve | −ve | −ve | −ve |
| Flavonoids | | | | | |
| Alkaline reagent test | + | − | + | + | − |
| Lead acetate test | − | − | + | + | + |
| Sulfuric acid test | + | + | + | + | + |

MENS: Methanolic extract of *Nigella sativa*

**Table 3:** Effect of MENS on B.W. in BIL rats

| *Experimental groups* | *Day 0* | *Day 7* | *Day 14* | *Day 21* |
|---|---|---|---|---|
| Normal control | 140±2.19$^{ns}$ | 142.7±4.83$^{ns}$ | 150.1±4.04**** | 152.3±3.73**** |
| Negative control | 139.3±6.799 | 127±4.36 | 100±4.416 | 97.98±4.42 |
| Cyclophosphamide 10 mg/kg | 122.8±2.689$^{ns}$ | 146.0±2.944* | 161.0±1.225**** | 160.5±1.3**** |
| MENS 500 mg/kg | 123.3±1.436$^{ns}$ | 140.8±3.44$^{ns}$ | 152.1±3.71**** | 155.5±2.09**** |
| MENS 1000 mg/kg | 139.5±7.006$^{ns}$ | 144.0±4.564* | 163.7±2.75**** | 160.4±2.19**** |

All values are expressed as mean±SEM, $n$=4, analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test, *$P$<0.05 and **** $P$<0.0001, as compared to negative control group, ns: Non-significant, MENS: Methanolic extract of *Nigella sativa,* B.W: Body weight, BIL: Benzene-induced leukemia

and uric acid levels were increased in negative control group; these are significantly decreased in treatment groups as compared to negative control group. Significant decrease in GSH levels as compared to normal control group represents successful induction of leukemia. Levels of GSH were restored to normal in stand group. Whereas, in MENS treated groups these levels are significantly are increased as compared to negative control group [Table 5]. Overproduction of reactive oxygen species (ROS) without satisfactory response from innate antioxidant system which maintain body's homeostasis results in oxidative stress environment (OSE), leading to the interruption of cellular proliferation and host defense mechanism. Increased production of ROS was due to either exogenous (chemical carcinogen, e.g., benzene)[26] or endogenous factors (mitochondria catalyzed electron transport reaction).[27] By extrapolating this above discussion to our study, we can foretell that leukemia was induced due to benzene exposure which caused increased production of ROS justified by decreased production of GSH in negative control group as compared to normal group, similar effect in decrease of GSH level was also evidenced previously in acute myelogenous leukemia patients as compared to healthy individuals.[28] One more study report depletion of GSH in B lymphocytes affected with chronic lymphocytic leukemia.[29] In

**Table 4 :** Effect of MENS on WBC and differential leukocytes in BIL rats

| *Experimental groups* | *WBC (millions/cm3)* | *Neutrophils (%)* | *Basophils (%)* | *Eosinophils (%)* | *Lymphocytes (%)* | *Monocytes (%)* |
|---|---|---|---|---|---|---|
| Normal control | 9.45±0.22**** | 25.00±0.57**** | 1.25±0.25** | 3.25±0.47**** | 67.50±1.25$^{ns}$ | 3.00±0.40$^{ns}$ |
| Negative control | 15.28±0.30 | 38.50±1.44 | 0.0±0.0 | 0.50±0.28 | 63.00±0.57 | 3.25±0.47 |
| Cyclophosphamide 10 mg/kg | 14.03±0.34** | 39.25±1.31$^{ns}$ | 0.0±0.0$^{ns}$ | 1.00±0.0$^{ns}$ | 54.75±2.05$^{ns}$ | 2.50±0.28$^{ns}$ |
| MENS 500 mg/kg | 10.45±0.02**** | 30.75±0.75*** | 1.25±0.25** | 2.00±0.40* | 53.75±5.10$^{ns}$ | 3.25±0.47$^{ns}$ |
| MENS 1000 mg/kg | 13.25±0.08**** | 29.50±0.86**** | 1.50±0.28*** | 2.00±0.0* | 62.25±0.75$^{ns}$ | 5.50±0.28** |

All values are expressed as Mean±SD, *n*=4, analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test, *$P$<0.05, **$P$<0.01, ***$P$<0.001, and ****$P$<0.0001, as compared to the control group, ns: Non-significant, WBC: White blood cell, MENS: Methanolic extract of *Nigella sativa,* BIL: Benzene-induced leukemia

**Table 5:** Effect of MENS on potassium, phosphate uric acid, and GSH levels in BIL rats

| *Experimental groups* | *Potassium (mEq/L)* | *Phosphate (mg/dl)* | *Uric acid (mg/dl)* | *GSH (mg/dl)* |
|---|---|---|---|---|
| Normal control | 4.150±0.0645**** | 4.010±0.5138**** | 0.5350±0.0086*** | 59.25±0.02887**** |
| Negative control | 7.350±0.4792 | 9.593±0.7250 | 3.125±0.5500 | 9.750±2.839 |
| Cyclophosphamide 10 mg/kg | 5.810±0.1039** | 7.375±0.3753** | 1.675±0.7217* | 53.75±6.921**** |
| MENS 500 mg/kg | 5.700±0.1730*** | 7.575±0.5196** | 1.600±0.4330* | 29.50±6.946* |
| MENS 1000 mg/kg | 5.620±0.1328*** | 7.150±0.2021*** | 1.775±0.3000* | 29.00±3.000* |

All values are expressed as Mean±SD, *n*=4, analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test, *$P$<0.05, **$P$<0.01, ***$P$<0.001, and ****$P$<0.0001, as compared to the control group, ns: Non-significant, MENS: Methanolic extract of *Nigella sativa*, GSH: Glutathione, BIL: Benzene-induced leukemia

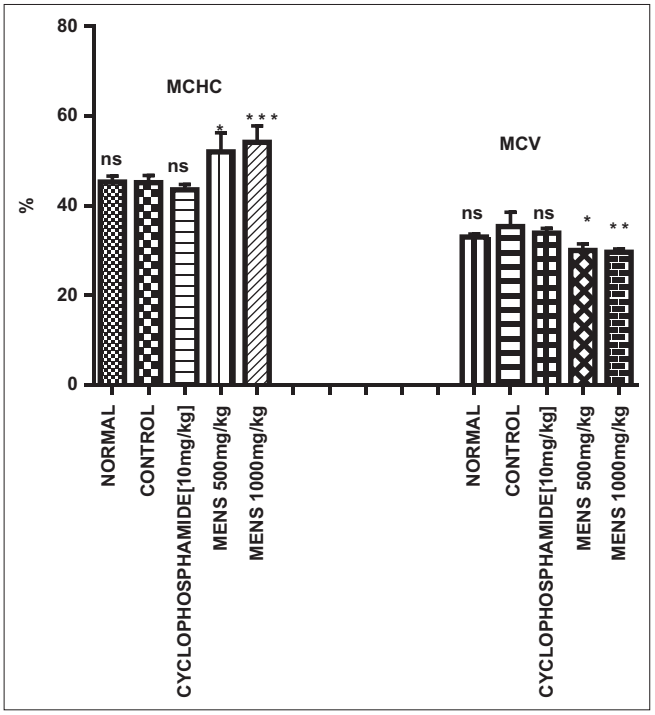


**Figure 1:** Effect of MENS on MCHC and MCV in BIL in rats. All values are expressed as Mean±SD, *n*=4, analyzed by analysis of variance followed by Dunnett's multiple comparison test, *$P$<0.05, **$P$<0.01, and ***$P$<0.001, as compared to the control group, ns: Non-significant. MENS: Methanolic extract of *Nigella sativa*, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean cell volume, BIL: Benzene-induced leukemia

treated groups, increased levels of serum GSH indicate diminished production of ROS after challenging animals with benzene and circumventing damaging effects produced by it.

Leukemia is capable of altering normal physiologic regulation of many systems, including serum levels of most electrolytes, due to rapid proliferation of leukemic cells along with OSE causes activation of innate immune system of body, leading to lysis of cells which results in release of nucleic acids, proteins, and electrolytes in blood stream.[30,31] Purine nucleotides which are released are catabolized by xanthine oxidase to hypoxanthine, xanthine, and finally to uric acid.[32,33] Thus, increased uric acid level is considered as a marker of progressiveness of leukemia. In treated groups, decrease in uric acid levels may be due to the prevention of episodes of OSE. Moreover, leukemia presented with uric acid abundancy is rarely manifested with a complication of hyperkalemia. Hyperkalemia in leukemia patients occurs due to two reasons, either due to rapid cell lysis induced by chemotherapeutic agents or as a result of hyperuricemia leading to acute renal failure (ARF). In case of serum-phosphate level, initiation of chemotherapy, lysis of the cells due to OSE, and hyperuricemia-induced ARF resulted in hyperphosphatemia.[34] Thus, we can conclude that hyperkalemia and hyperphosphatemia in negative control group may be due to OSE and relieving of these conditions in MENS treated animals signifies their efficacy in treating leukemia.

## Conclusion

In recent years, ethnobotanical and traditional uses of natural compounds, especially of plant origin, received much attention as they are well tested for their efficacy and generally believed to be safe for human use. Anticancer activity of *N. sativa* was discovered several years ago, but scientific research on hematological malignancies has been recently done. With the view of results obtained from this study, it can be stated that MENS demonstrated antileukemic potential that might be due to the presence of important phytoconstituents such as alkaloids, flavonoids, carbohydrates, and tannins. Furthermore, isolation and structural characterization of active constituents of seeds is necessary to be evaluated in scientific manner using specific experimental models and clinical trials to elucidate the molecular mechanism of action to explore therapeutic benefits.

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