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GENOTOXIC EFFECTS OF NEEM SEED OIL ON HUMAN LYMPHOCYTES

A. Bošković[1], A. Leskovac[2], O. Medić[1], T. Ganić[1], B. Nikolić[1] and S. Petrović[2]

[1]*University of Belgrade, Faculty of Biology, Studentski trg 12-16 11000 Belgrade, Serbia*

[2]*Vinča Institute of Nuclear Sciences – National Institute of the Republic of Serbia, University of Belgrade, Mike Petrovića Alasa 12-14, 11351 Belgrade, Serbia (sandra@vin.bg.ac.rs)*

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ABSTRACT

Neem (*Azadirachta indica*) seed oil is widely used as a biopesticide and in cosmetic formulations, yet data on its genotoxicity remain limited. This study assessed its effects in human peripheral blood lymphocytes using the cytokinesis-block micronucleus assay. A significant, concentration-dependent increase in micronuclei incidence was observed, accompanied by reduced cell proliferation across all tested concentrations. At the highest concentration, pronounced cytotoxicity was observed. These findings highlight the need for further in-depth safety evaluation of neem seed oil and clarification of the roles of its individual constituents in the observed biological effects.

INTRODUCTION

Neem (*Azadirachta indica*) seed oil is widely used in agriculture and cosmetic formulations and has also been incorporated into traditional medicinal and pharmaceutical preparations [1]. As a result of this broad, cross-sectoral application, human exposure to neem-derived products may occur through multiple routes, including dermal contact, inhalation, and oral intake as an auxiliary medical agent. Notably, certain *Azadirachta indica*-derived substances (e.g., neem seed extract) have been identified as carcinogenic, mutagenic, or toxic to reproduction (CMR) and have been prohibited in cosmetic products in the EU since 2023 [2]. Neem seed oil represents a highly complex mixture comprising more than 300 constituents, including fatty acids, triterpenes, flavonoids, saponins, catechins, and limonoids [3]. Given its complex chemical composition and increased use, concerns have arisen regarding human exposure and potential adverse effects, particularly at higher concentrations or with prolonged exposure. Genotoxicity is a critical endpoint in toxicological assessment, yet, despite its wide use, data on the genotoxic potential of neem seed oil remain limited and inconsistent, likely due to differences in composition, formulation, and experimental design. In this study, the genotoxic and cytotoxic

effects of neem seed oil in human peripheral blood lymphocytes were evaluated using the standardized cytokinesis-block micronucleus (CBMN) assay [4]. The findings provide insight into its potential genotoxic risk and contribute to its safety assessment under increasing environmental and human exposure.

EXPERIMENTAL

Cell culture and genotoxicity assessment

Commercial cold-pressed neem (*Azadirachta indica*) seed oil (Eterra, TemaCo d.o.o., Novi Sad, Serbia; origin: India) was used. Stock emulsions were prepared in DMEM (Thermo Fisher Scientific, USA) with Tween 20 (Sigma-Aldrich, France) as the surfactant. Working concentrations were obtained by serial dilution. Peripheral blood samples were collected from a healthy male donor after informed consent, in accordance with protocols approved by the Institutional Ethics Committee (approval no. 116-5-5/2023-000) and relevant ethical guidelines [5]. Heparinized whole blood was cultured in Panserin 701 (Pan-Biotech, Germany) medium. Neem seed oil was tested at concentrations ranging from 0.025 to 1 mg/mL. Untreated cultures served as controls. Tween 20 was used at final concentrations below the cytotoxicity threshold (<0.01%) [6]. Experiments were performed in triplicate. Genotoxicity was assessed using the CBMN assay of Fenech [7], following the OECD 487 Guideline [4]. At least 2000 binucleated cells per sample were analyzed using a light microscope (Optech, Germany) at 400× or 1000× magnification. Cytostatic/cytotoxic effects of neem seed oil were assessed by estimating the cytokinesis-block proliferation index (CBPI) [8], using the formula: CBPI = [MI + 2MII + 3(MIII +MIV)]/N, where MI-MIV represent cells with one to four nuclei, and N is the total number of cells scored. At least 500 cells per sample were analyzed [4]. Cytotoxicity (%) was calculated as: $100 – 100[(CBPI_T – 1)/(CBPI_C – 1)]$, where $CBPI_T$ is the CBPI of the test compound, and $CBPI_C$ is the CBPI of the untreated control cells [4]. Statistical analyses were performed using Student's t-test and Pearson and partial correlation analyses in Statistica 8.0 and OriginPro 8.5. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The genotoxic potential of neem seed oil was evaluated in human lymphocytes across a concentration range of 0.025–1 mg/mL. The results (Fig. 1.) show a significant, concentration-dependent increase in micronuclei (MN) incidence. The effect was evident even at the lowest tested concentration and increased progressively; at 1 mg/mL, near-complete inhibition of cell proliferation precluded reliable MN scoring, indicating cytotoxicity beyond the evaluable range (data not shown). CBPI analysis showed a parallel, concentration-dependent reduction in proliferative capacity (Fig. 1.). CBPI decreased progressively up to 0.5 mg/mL, while 1 mg/mL caused ~ 100% inhibition of proliferation (data not shown). Cytotoxicity increased accordingly, ranging from

mild effects at lower concentrations to pronounced cytotoxicity at 0.5 mg/mL (54.41 ± 1.47%) and near-complete cell cycle arrest at 1 mg/mL (Table 1).

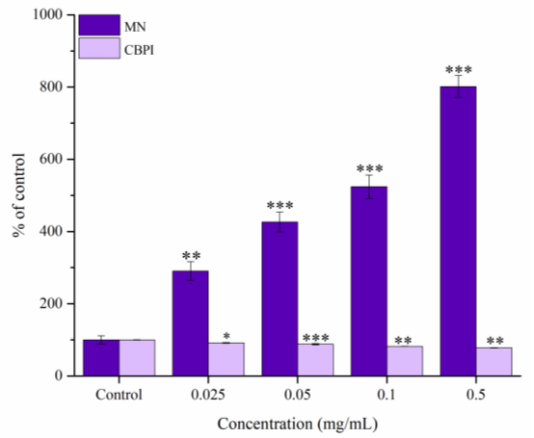


Figure 1. The MN incidence (mean ± SD) and CBPI (mean ± SD) in cultured human lymphocytes treated with neem oil, expressed as percentages relative to the control, set at 100%. *p<0.05, **p<0.01, ***p<0.001 compared with the control.

A significant inverse correlation between MN incidence and CBPI ($r = -0.96$; $p = 0.0001$) suggests activation of cell cycle checkpoints, particularly at G_2/M, in response to DNA damage. Damaged cells likely undergo arrest, senescence, or apoptosis, thereby reducing proliferation and increasing genomic instability. The observed genotoxic responses align with reports on neem nanoformulations, in which DNA damage has been linked to oxidative stress and ROS generation [9]. Notably, these effects occurred at higher concentrations (1.2–2 mg/mL) than in the present study, suggesting that formulation, bioavailability, and cellular uptake influence toxicity. Evidence for constituents, such as azadirachtin, indicates that genotoxicity may arise from mitotic spindle disruption and chromosome missegregation rather than from direct DNA damage, leading to micronuclei formation [10].

Table 1. Neem seed oil–induced cytotoxicity (%) in human lymphocytes.

	Concentration of neem seed oil (mg/mL)			
	0.025	**0.05**	**0.1**	**0.5**
Cytotoxicity (%) (mean±SD)	22.06	26.47	45.59	55.88
	23.53	27.94	44.11	52.94
	17.65	35.29	44.11	54.41
	21.08±3.06	29.90±4.72	44.60±0.85	54.41±1.47

Collectively, these findings suggest that neem oil–associated genotoxicity may involve both clastogenic and aneugenic mechanisms, likely driven by multiple bioactive constituents rather than a single compound.

CONCLUSION

The present findings indicate that neem seed oil compromises genomic stability and cell cycle progression in a concentration-dependent manner, raising potential toxicological concerns associated with its increasing use and possible human exposure.

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