



## The In-vitro Micronucleus Assay for Antigenotoxic Effect Evaluation of the *Nigella sativa* L. seeds extract on Cultured Human peripheral blood Lymphocytes.

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### Article info

### Abstract

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This study aims to assess the antigenotoxic effects of the *Nigella sativa* L. seeds extract on human peripheral blood lymphocytes using Methotrexate as the positive mutagen. Human lymphocytes cells cultures were treated by different concentrations of the methotrexate ranging from  $5,5 \cdot 10^{-8}$  to  $5,5 \cdot 10^{-6}$   $\mu\text{mol/l}$ , either alone or together with the concentration 80mg/ml of the *N. sativa* seed extract. The DNA damages (cytogenetic aberrations) and the cells viability were assessed by the micronucleus (MN) assay and the cell proliferation kinetics (CPK) respectively. Results showed that the *Nigella sativa* L. seed extract decreases the genotoxic effect of the MTX and possesses significant genoprotective effect of DNA at decreasing of the (MN) frequencies and increasing of the (CPK).

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### INTRODUCTION

*Nigella sativa* Linn (*N. sativa* L.) is a high-profile plant across the Eastern Mediterranean and especially the Arab world as a panacea with interesting medical properties. This is a dicotyledon belonging to the family of Ranunculaceae, used for thousands of years as a spice

for food preservation and especially as protective and curative element as a liver tonic and against several disorders such as heart and skin disease, inflammation and arthritis [1, 2, 3]. *N. sativa L.* is one of the plants that generated the most interest in terms of study, including the effects of extracts of the seeds of this species as well as the main constituents (especially the thymoquinone) on various systems *in vivo* and *in vitro*. This plant has several biological properties, namely an antioxidant effect [4], antimutagenic [5, 6], anti-inflammatory [7] and anti-tumor [8].

Therefore, knowledge of the genotoxic potential of natural and antigenotoxic substances would open new horizons in the search for new anticancer molecules or, to find a natural substituting equally effective as chemotherapy but with fewer side effects. To evaluate the genotoxic and/or antigenotoxic of the aqueous extract of the seeds of *N. sativa L.* positive control using methotrexate (MTX), we characterized the genotoxic effect of this chemical before and after addition of 80mg/ml of *N. sativa L.* aqueous extract on *in vitro* human peripheral blood lymphocytes. This dose was chosen as the median dose of the range of concentrations tested in our previous study [9].

## MATERIALS AND METHODS

### Plant material

Seeds of *Nigella sativa L.*, (family Ranunculaceae), were cultured in region Arfoud City in Morocco.

### Genotoxic agent

The mutagenic agent used as a positive control in this study, is the Methotrexate (Sigma Chemical).

### Preparation of *Nigella sativa* seeds extracts

*N. sativa* seeds were ground to obtain a fine powder. The 80 mg/ml was prepared by dissolving the seed powder in 10ml of PBS (phosphate buffer saline) at pH 7.4 [10]. The extracts were filtered and centrifuged at 4500 rpm for 10 min to remove any insoluble debris and the supernatant was sterilized by filtration through a 0.22 µm Millipore system, before use to avoid contamination.

### Preparation of the dilutions of Methotrexate

The method consists to prepare a dilution series at a rate of 10 for each compound from their mother solutions. Dilutions are made by adding DMSO (99, 5%). The chosen concentrations are part of a wide range with 10 concentrations (Table I). The stock solution concentration is  $5,5 \cdot 10^2 \mu\text{mol/l}$ .

**Table 1: The concentrations of the Méthotrexate used in treatment of lymphocytes.**

Tube N°	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	M <sub>4</sub>	M <sub>5</sub>	M <sub>6</sub>	M <sub>7</sub>	M <sub>8</sub>	M <sub>9</sub>	M <sub>10</sub>
[ ] µmol/l	5,5.1	5,5	5,5.10	5,5.10	5,5.10	5,5.10	5,5.10	5,5.10	5,5.10	5,5.10
	0		-1	-2	-3	-4	-5	-6	-7	-8

### **Lymphocytes donor**

Blood samples were obtained from a consenting nonsmoking healthy 22 years old female.

### **Cell culture**

Lymphocytes cell culture has been made in RPMI 1640 medium supplemented with 15% foetal calf serum, 1 % of Phytohemagglutinin (PHA) (Mitogen agent) and 1% of antibiotics (penicillin/streptomycin) to inhibit any bacterial growth. The RPMI medium with the entire additive reagents was sterilized by filtration through a 0.22µm millipore system. Under aseptic conditions, 10 tubes of 5 ml of this prepared medium and 0.5 ml of the total peripheral blood were prepared. After homogenization, these tubes were incubated at 37°C for 72 h.

### **Lymphocytes treatment**

The micronucleus (MN) test was done according to the method of [11]. After 24 h of culture, cell culture tubes were treated by adding 100 µl of each concentration tested of the positive control (mutagenic agent) and the other series of the cell culture tubes are treated with 100 µl of the mixture of the dose 80mg / ml of the extract with each concentration of the range of the positive control. The negative control corresponds to two controls (treatment of cells with a mixture of DMSO with PBS, untreated cells). The cell culture re-incubated at 37°C. After 44 h of culture 0.1 ml of Cytochalasin-B was added to each cell culture tube and the cultures were incubated at 72h. Giemsa-stained slides were coded, analyzed for MN and scored in continuous fields. Scoring for MN was restricted to binucleate cells with distinct preserved cytoplasm and included all smooth edged MN smaller than 1/3 the diameter of the main nuclei.

### **Cell proliferation kinetics and micronuclei computation**

To evaluate the DNA damages rate, MN frequency and cell proliferation kinetics (CPK) were assessed. These two parameters were calculated respectively according to 1000 observed binucleate cells and 1000 analyzed lymphocytes for each concentration.

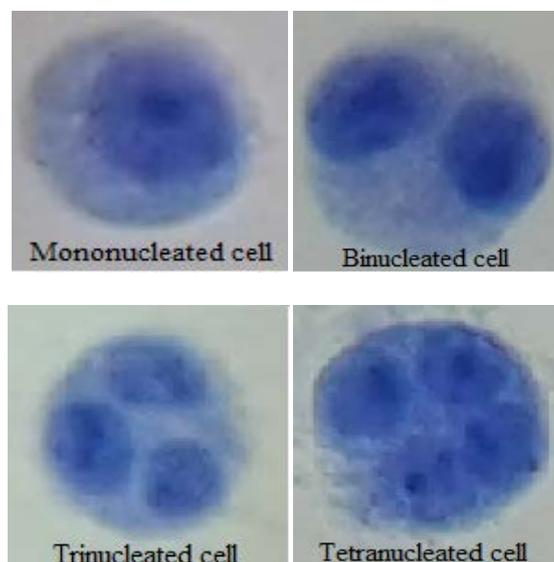
### **Statistical analysis**

The Statistica-10 software was used to analyze obtained data. The linear regression test, the Pearson correlation, the anova variation test at probability 5% and the student test was performed.

## **RESULTS AND DISCUSSION**

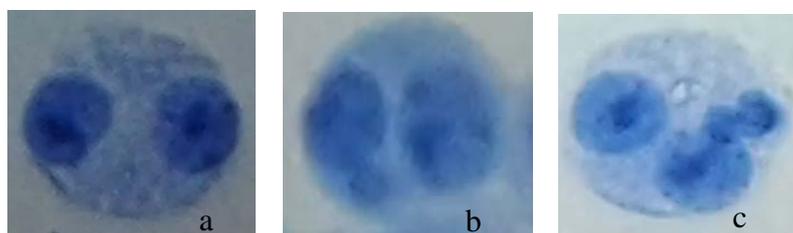
The results of the antigenotoxic effect of the *N. sativa L.* seeds extract are presented on a dose-response relationship. The micronucleus test is effective as an indicator of induced DNA damage following exposure to genotoxic agents. Thus, it can assess the antigenotoxic activity of the *N. sativa L.* seeds extract with the mutagenic agent used on cells of human

peripheral blood lymphocytes *in vitro*. This test was used to calculate the rate of micronucleus (MN) and Cell Proliferation Kinetics (CPK) as biomarkers for estimating the cell cycle time and assess the cellular behavior of exposed cells compared to controls. Any reduction in the frequency of biomarkers studied through these assay gives an indication of the antigenotoxicity of a particular compound (12-15). In fact, the cell proliferation kinetics (CPK) is commonly used



**Fig. 1:** Lymphocytes analyzed for evaluation of the cell proliferation kinetics of the *N. sativa* L. seed extract (Gx40).

as a parameter for measuring the cytotoxicity [6]. This decrease is a probable lethal cytotoxicity. The gradual decrease in the frequency of MN confirms this cytotoxicity. The CPK is estimated based on the number of cell in the first, second, third and fourth division (Figure 1). The frequency of micronucleus (MN) was calculated for each concentration in 1000 analyzed cells binucleate, between normal cells and binucleated cells with micronucleus 1, 2 or 3 micronuclei (Figure 2).



**Fig. 2:** Binucleated cells observed under the microscope: (a) normal, (b) with 1 micronucleus and (c) with 2 micronucleus (Gx40).

### Effect of Méthotrexate on the cell proliferation kinetics

Figure 3 shows that of the obtained values of the CPK for the different concentrations of MTX, is lower than that of the control. A progressive increase was noted as a function of

dose concentration range of  $5,5 \cdot 10^{-8}$  to  $5,5 \cdot 10^{-6}$ . However, the recorded values of CPK seem to be almost stable between the two doses  $5,5 \cdot 10^{-6} \mu\text{mol}$  and  $5,5 \cdot 10^{-1} \mu\text{mol}$ . This could be due to the weakness of the interval between doses. However a decrease in the proliferation index has been registered in the concentration  $5,5 \cdot 10^{-1} \mu\text{mol}$  to  $5,5 \cdot 10 \mu\text{mol}$ . This decrease could be due to cell death.

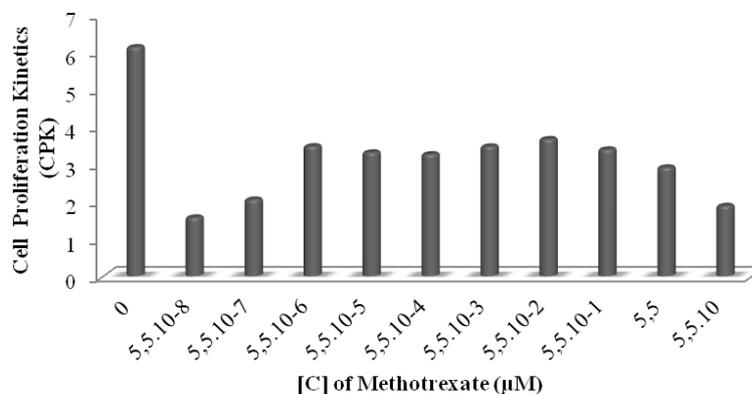


Fig. 3: Variation of the cell Proliferation kinetics versus concentrations of *Methotrexate*.

#### Effect of Methotrexate on the frequency of micronuclei

Figure 4 shows that the values of the frequency of MN in cells treated with MTX, increase with the increasing in concentrations of MTX. However, the difference between the MN values recorded at doses ranging from  $5,5 \cdot 10^{-4}$  to  $5,5 \mu\text{mol}$  is very low. This could be due to insufficient interval between doses does not segregate the effect of MTX. However, the increase with the increasing in concentrations of MTX, could be the cause of a genotoxic effect on normal lymphocytes and there may also induce damage in the genetic material. Other studies show that methotrexate causes a mutagenic effect becomes particularly severe when the cells are continuously exposed to high concentrations of MTX (17).

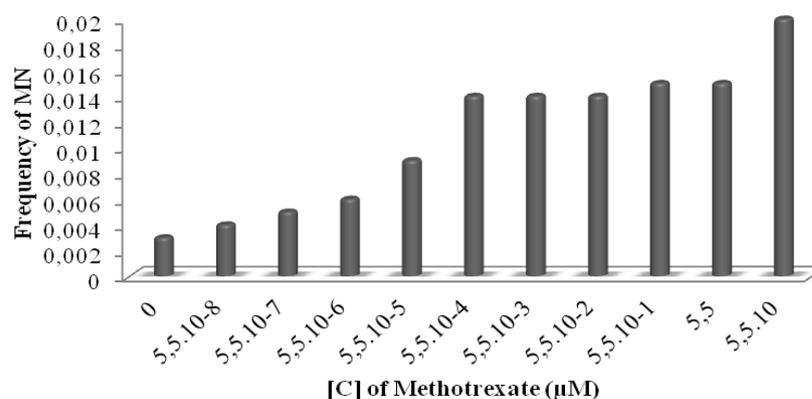


Fig. 4: Variation of the frequency of micronuclei versus concentrations of *Methotrexate*.

### Evaluation of antigenotoxic effect of the *N. sativa L.* seeds extract

To quantify and qualify the effect of the addition of the tested dose of PBS from *N. sativa L.* extract, on the MTX effect, we compared the compartment of each parameter studied with and without *N. sativa L.* extract. This comparison realized according to two logics. The first logical regards the comparison of evaluation in each parameter as a function of the selected doses. Thus, a correlation has been tested for the various parameters studied before and after addition of the *N. sativa L.* extract (Table 2). In the second logic, we considered that different doses selected are repeats in order to realize a comparison of average of each parameter before and after addition of the *N. sativa L.* extract (Table 3).

The correlation test revealed a significant difference between the change in the frequency of the MN according to the MTX doses chosen with and without the *N. sativa L.* aqueous extract. This difference becomes larger with the increase of the dose with larger values of without the *N. sativa L.*, seeds extract. Similarly, the comparison of means showed that the CPK values were maintained at the starting level with an average of the CPK after addition of the aqueous extract of *N. sativa L.* very significantly low. This may reflect a slowing or delaying effect of *N. sativa L.*, it's not a cytotoxic effect.

**Table 2: Correlations of the results of each parameter between the methotrexate effect and the combined effect of the *N. sativa L.* extract with methotrexate.**

		<i>N. sativa L.</i> extract and methotrexate	
Methotrexate	Variables	CPK	MN
	CPK	0,187573	
	MN		0,037732

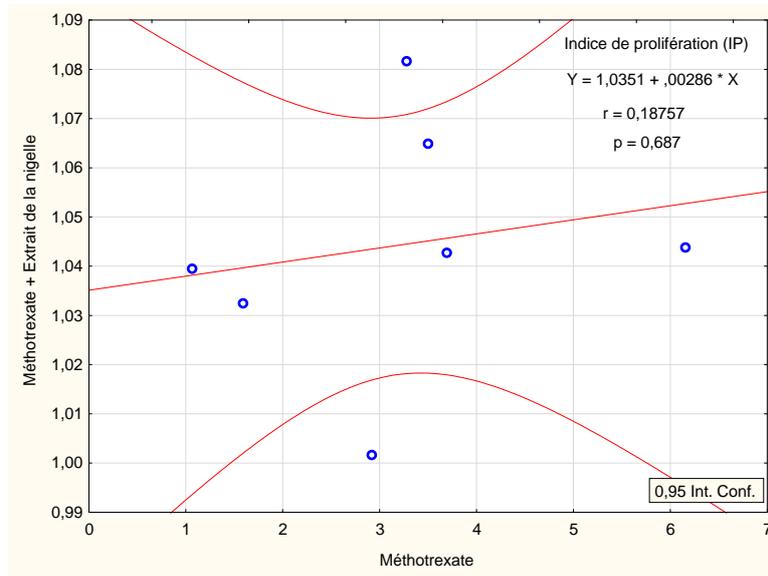
**Table 3: Results of the Student test of comparison.**

Tests	Average - G1	Average - G2	Value t	DI	P	E-Type - G1	E-Type - G2	Ratio F Variances	P Variances
IP	3,151714	1,044143	3,384	12	0,005423	1,647360	0,025123	4299,8	<0,001
MN	0,009143	0,004857	1,817	12	0,094293	0,005113	0,003579	2,0	0,406562

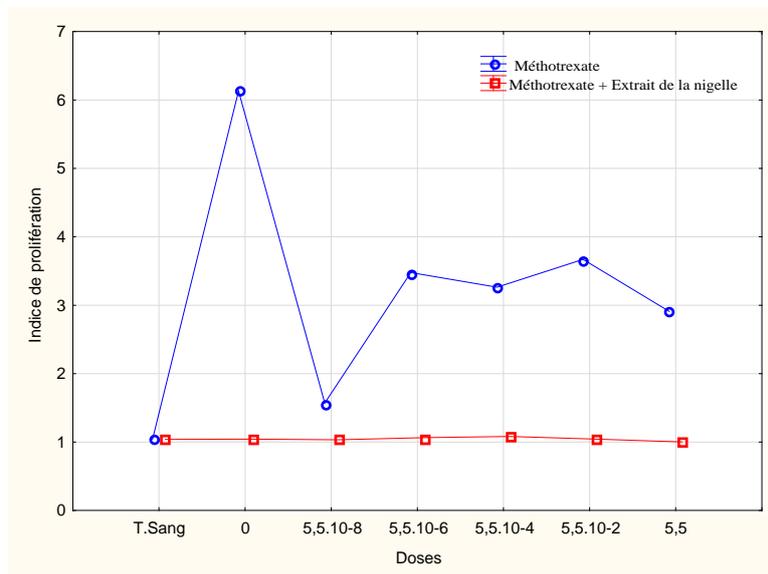
### Evaluation of the combined effect of the *N. sativa L.* extract with methotrexate on CPK.

No significant correlation was revealed for the CPK (Figure 7). The values of the highest CPK are recorded for the test without aqueous extract of *N. sativa L.* (Figure 8). In fact, CPK values were maintained at their starting level with an average of CPK after addition of the *N.*

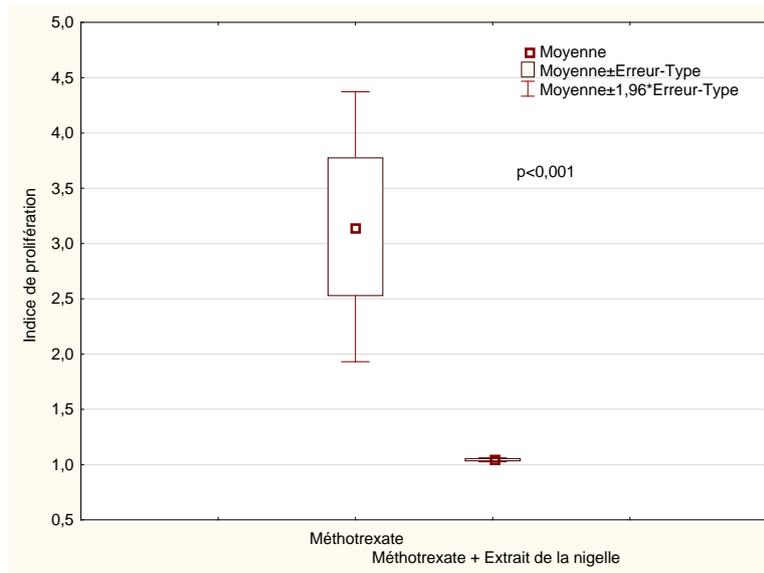
*sativa L.* aqueous extract, very significantly lower (Figure 9). This may reflect a slowing or delaying effect of *N. sativa L.*, it's a cytotoxic effect.



**Fig.5:** Correlation of the CPK values between experience of the methotrexate and experience of the combination of methotrexate with *N. sativa L.* extract.



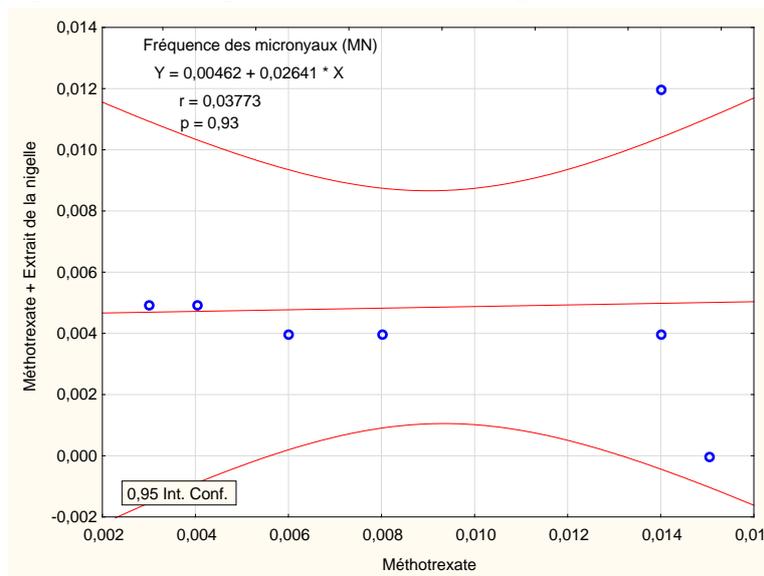
**Fig. 6:** Curves CPK values according doses for the experiment of the methotrexate and experience of the methotrexate combined effect with *N. sativa L.* extract.



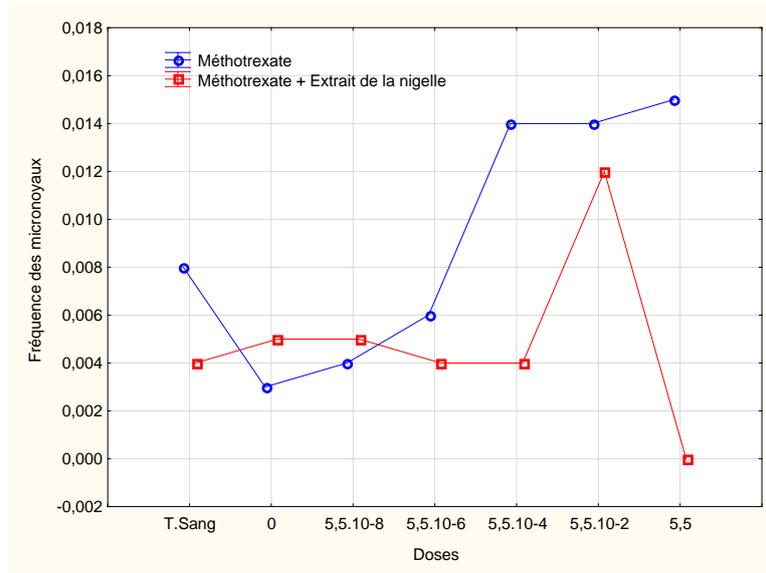
**Fig. 7:** Comparison of CPK average values between the experience of methotrexate and the experience of combined effect for the methotrexate with *N. sativa L.* extract.

**Evaluation of the combined effect of the *N. sativa L.* extract with methotrexate on the frequency of MN**

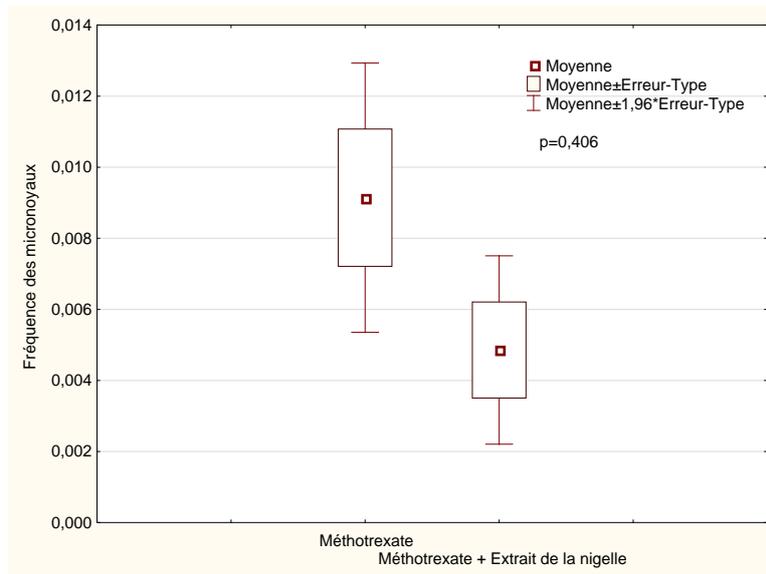
The correlation test revealed a significant difference between the evolution of the frequency of micronuclei MN according to the selected doses with and without *N. sativa L.* extract (Figure 10). This difference becomes larger with the increase of the dose with important values of MN for the test without *N. sativa L.* aqueous extract (Figure 11). In fact, the comparison of average showed that the MN means is higher in the test without Nigella extract, there being provided a significant difference (Figure 10).



**Fig. 8:** Correlation of the MN values between the experience of the methotrexate and the experience of the combined effect of methotrexate with *N. sativa L.* extract.



**Fig. 9:** curves of the MN values according the doses for the experience the methotrexate and the experience of combined effect of methotrexate with *N. sativa L.* extract.



**Fig. 10:** Comparison of MN average values between the experience of methotrexate and the experience of the combination of methotrexate with *N. sativa L.* extract

In general and taking into consideration the complexity and the delicacy of cytogenetic biomarkers used in terms of interpretation for the multitude of factors affecting our results underline and confirm the antigenotoxic effect of *N. sativa L.* aqueous seeds extract. This result could in a preventive action for cancer and/or reducing the cytotoxicity of conventional antineoplastic drugs. This is consistent with the study has shown that hepatocytes cell cultures, which has a treatment with the *N. sativa L.* extracts combined with a mutagen (MNNG), show a significant reduction in the rate of AC and MN induced by the mutagen [6].

In fact, pharmacologically the active constituents of the *N. sativa L.* seeds extract are: the thymoquinone TQ, the dithymoquinone (MTQ), the thymohydroquinone (THO) and thymol (THY) [18]. Thus, the aqueous extract of *N. sativa L.* used in this study is very rich in phenolic compounds, in fact, the average content of the total phenols is (163.33 ± 10.26 mg/mg), whereas for the methanol extract is (163.33 ± 3.05 mg/mg) [19].

*In vitro* studies have shown that thymoquinone and dithymoquinone are highly cytotoxic against various human tumor cell lines, cited: Pancreatic adenocarcinoma, sarcoma uterine, parental and multidrug resistant cancer and leukemia [20, 21, 22]. Thymoquinone carries the *in vitro* and *in vivo* inhibitory effect on the carcinogenesis of the stomach and fibrosarcoma induced by methylcholanthrene-20 in mice [21, 22].

## CONCLUSION

The use of MTX as a positive control for the evaluation of antigenotoxic activity of *N. sativa L.* seeds extract shows that it slightly reduces the genotoxic activity of MTX. The *N. sativa L.* seeds extract to have an antigenotoxic protective effect the DNA by reducing the rate of MN. The *N. sativa L.*, by its antimutagenic and antioxidant proprieties, could play an important role in the prevention of carcinogenesis, and may also provide protection against oxidative stress, and protection of genetic material from damage caused by genotoxic agents.

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