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In vitro studies on the modulatory effects of nutraceuticals and gene polymorphisms on organophosphate pesticide-induced genotoxicity as analyzed by the comet assay

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Abstract

Malathion and parathion are organophosphate pesticides known for their high toxicity to insects and low to moderate toxicity to humans and other mammals. We studied the antigenotoxic potential of curcumin and carvacrol to combat malathion and parathion-induced DNA damage. Tail moment (TM) as measured by the comet assay was used as a biomarker of genotoxicity. To determine the antigenotoxic potential of curcumin and carvacrol, heparinized fresh blood from healthy individuals was treated with malathion (30 µg/ml) and parathion (2.5 µg/ml) in the presence of curcumin and carvacrol. Curcumin at concentrations of 25 and 50 µg/ml significantly reduced (p<0.05) TM compared to samples exposed to malathion only. Curcumin at concentrations of 10 and 15 µg/ml also exerted an antigenotoxic effect on parathion-exposed samples. Similarly, carvacrol at concentrations of 2.5 and 5.0 µg/ml showed a significant (p<0.05) protective effect against both pesticides. We observed a significant (p<0.05) reduction in TM when samples were treated with malathion and parathion in the presence of curcumin and carvacrol. We also studied the effect of polymorphisms of the genes *GSTM1* and *GSTT1* on the genotoxicity induced by both pesticides. However, we did not observe any significant association (p>0.05) of *GSTM1* and *GSTT1* polymorphisms with malathion and parathion genotoxicity.

Introduction

Most people are exposed to pesticides through environmental contamination or occupational use. Chemical pesticides are used extensively to control agricultural pests and should be assessed for their potential hazardous effects. However, epidemiological data on cancer risk in farmers are conflicting. Meta-analyses showed that farmers were at risk for specific tumours including leukaemia and multiple myeloma [1, 2]. Malathion and parathion are man-made organophosphate insecticides which are commonly used to control a variety of insects that attack vegetables, fruits, shrubs and garden plants. Malathion is reported to be genotoxic under both *in vivo* and *in vitro* conditions, but reports are conflicting [3]. Undeger and Ba-

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saran [4] found methyl parathion to be genotoxic to freshly isolated peripheral blood lymphocytes (PBL).

The comet assay is a rapid, sensitive and inexpensive technique for qualitative and quantitative assessment of DNA damage in single cells. It has been used in many *in vivo* and *in vitro* studies to describe the genotoxic effects of various pesticides [5, 6].

The term nutraceuticals encompasses many products, ranging from isolated nutrients, diet supplements and specific diets to genetically engineered designer foods and herbal products. Curcumin or diferuloylmethane ([1,7-bis(4-hydroxy-3-methoxy phenyl)-1-6-hepatadine-3-5-dione]) is a polyphenolic molecule extracted from the rhizome of the plant *Curcuma longa*. It is reported to inhibit lipid peroxidation and oxidative DNA damage.

Carvacrol is a monoterpene phenolic constituent of essential oils extracted from *Origanum vulgare*, thyme, pepperwort, and wild bergamot. It has been reported to exhibit anti-proliferation activity in the human metastatic breast cancer cell line MDA-MB by inducing apoptosis [7]. Carvacrol reduces DNA damage by scavenging free radicals generated by xenobiotic compounds [8].

Individuals demonstrate varying responses to environmental chemicals due to their different genotypes. Hereditary differences in the detoxification of carcinogens play a crucial role in host vulnerability. As glutathione-S-transferases (GSTs) participate widely in the metabolic detoxification of xenobiotics, their genetic polymorphisms could play an important role in determining individual sensitivity to various reactive chemicals. *GSTM1*(mu) and *GSTT1*(theta) are candidate cancer-predisposing genes and have received much attention because of the high prevalence of homozygous deletions resulting in null genotypes with a decreased ability to detoxify carcinogenic compounds, placing null individuals at increased cancer risk [9].

Our study focused on the antigenotoxic effects of curcumin and carvacrol against DNA damage caused by malathion and parathion in PBL by using the comet assay to measure a biomarker of genotoxicity. We also investigated the effect of *GSTM1* and *GSTT1* polymorphisms on the genotoxicity of malathion and parathion as measured by TM values.

Materials and methods

Sample collection

Two 5 ml samples of venous blood were each taken from 60 healthy individuals and placed in two separate vacutainer tubes containing sodium heparin and dipotassium ethylenediamine tetraacetic acid (EDTA) for lymphocyte culture set-up and DNA extraction, respectively. Subjects exposed to diagnostic x-rays, drugs or vaccination during the 6 months before blood sampling were excluded from the study. All participants signed an informed consent form. All individuals participating in the study were Aryan males aged 18–30 years and resident in Haryana state, North India. The protocol was approved by the research ethics committee of Kurukshetra University.

Human lymphocyte culture

Short-term PBL cultures were grown using the technique of Moorhead *et al.* [10] with minor modifications. Cultures were established in duplicate by adding (0.4 ml) whole heparinized blood into 5 ml of RPMI 1640 culture medium (Himedia) containing L-glutamine (1%), fetal calf serum (20%) (Himedia), penicillin (100 IU/ml) and streptomycin (100 µg/ml) solution (Himedia), and phytohaemagglutinin (2%) (Bangalore Genei). Malathion (Sigma) and parathion (Sigma) were added at the beginning of culture at various concentrations. The maximum genotoxic doses of malathion and parathion (30 and 2.5 µg/ml, respectively) were chosen to determine the ameliorative effects of curcumin (Sigma) and carvacrol (Sigma). To examine the antigenotoxic potential of curcumin and carvacrol against malathion and parathion, separate cultures were established with various combinations of both the pesticides and curcumin/carvacrol. Heparinized fresh blood was treated with 30 µg/ml malathion together with either 25 or 50 µg/ml curcumin, and in a different set-up with 30 µg/ml malathion together with either 2.5 or 5.0 µg/ml carvacrol. Similarly, PBL were also treated with 2.5 µg/ml parathion together with 10 or 15 µg/ml curcumin or with 2.5 or

5.0 µg/ml carvacrol to examine the protective effect of curcumin and carvacrol against parathion. The combined effects of both curcumin and carvacrol were also studied against both pesticides. Blood was also treated with curcumin or carvacrol alone to check their genotoxic effects, if any. Blood not treated with pesticides, curcumin or carvacrol acted as control, while blood treated with dimethyl sulfoxide (DMSO) was used as negative control. Cultures were incubated for 24 h at 37°C in 5% CO_2. Cells were harvested by centrifugation and washed in phosphate buffered saline (PBS). Lymphocytes were then resuspended in 1 ml PBS.

Comet assay

To observe the effects of the chemicals on lymphocytes, PBL were cultured in the presence of malathion and parathion as described above and the alkaline comet assay was performed according to the method of Singh *et al.* [11] and Tice *et al.* [12] with minor modifications. Briefly, lymphocytes (10–20 µl) were mixed with 80 µl of warm 0.5% low melting temperature agarose (Sigma) and this mixture was layered as a second additional layer over a first layer of 1% normal melting temperature agarose (Sigma) and gelled at 4°C for 15 min. A third additional layer of 150 µl of 0.5% low melting temperature agarose was added on top and gelled again at 4°C for 15 min. The slides were treated for 2 h at 4°C in freshly prepared, chilled lysis buffer solution (25 mM sodium chloride, 100 mM sodium EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO added before use and pH adjusted to 10) followed by incubation in an alkaline electrophoresis buffer (10 N sodium chloride, 200 mM EDTA, pH adjusted to 13) for 20 min and electrophoresis (25 V and 300 mA) for 30 min in the same buffer. The slides were then neutralized with Tris buffer (0.4 M Tris, pH adjusted to 7.5), rinsed with distilled water, and stained with ethidium bromide (20 µg/ml) for 5 min under dark conditions. A total of 50 individual cells from each of the duplicate slides per subject were examined randomly under an *Olympus fluorescence* microscope. The extent of DNA damage was measured quantitatively as tail moment (TM) using Lucia comet assay software (version 7.12).
TM is defined as the percentage of DNA in the tail multiplied by the length between the centre of the head and the tail [13].

GSTM1 and *GSTT1* genotyping

Multiplex PCR was used to determine the presence or absence of the genes *GSTM1* and *GSTT1*. A part of exon 7 of the constitutional gene CYP1A1 was co-amplified as an internal control [14]. The PCR products were analyzed in 2% agarose gel. The occurrence of the genes *GSTM1* and *GSTT1* was detected by the presence or absence of a band at 215 and 480 bp, respectively.

Statistical analysis

All treatments were performed in duplicate and the results were expressed as means±SD. The Student's t test was used for calculating statistical significance using SPSS 16.0.

Results

DNA damage was examined in terms of TM using the comet assay in order to assess the genotoxicity of malathion and parathion. Damaged DNA resembling comets and undamaged intact DNA in samples treated and not treated with pesticides are shown in Fig. 1.
Samples only treated with various concentrations of malathion or parathion were observed for mutagenicity. PBL treated with malathion showed more DNA damage as evidenced by TM. As the concentration of malathion increased from 10 to 20 µg/ml, the mean value of TM also increased from 2.67±0.43 µM to 4.78±0.21 µM, reaching its maximum value (7.08±0.16 µM) at 30 µg/ml, which is significantly (p<0.05) higher than the mean TM value (0.52±0.12 µM) of the untreated sample. Similarly, parathion also induced DNA damage in a dose-dependent manner: the mean value of TM increased from 1.91±0.16 µM to 3.84±0.21 µM as the concentration of parathion rose from 0.5 to 1.0 µg/ml. At 2.0 µg/ml of parathion, the mean value of TM further increased to 7.17±0.12 µM and reached its maximum value of 8.78±0.19 µM at 2.5 µg/ml, which is significantly (p<0.05) higher than the mean TM value (0.40±0.09 µM) of the untreated sample. As 30 µg/ml malathion and 2.5 µg/ml parathion demon-

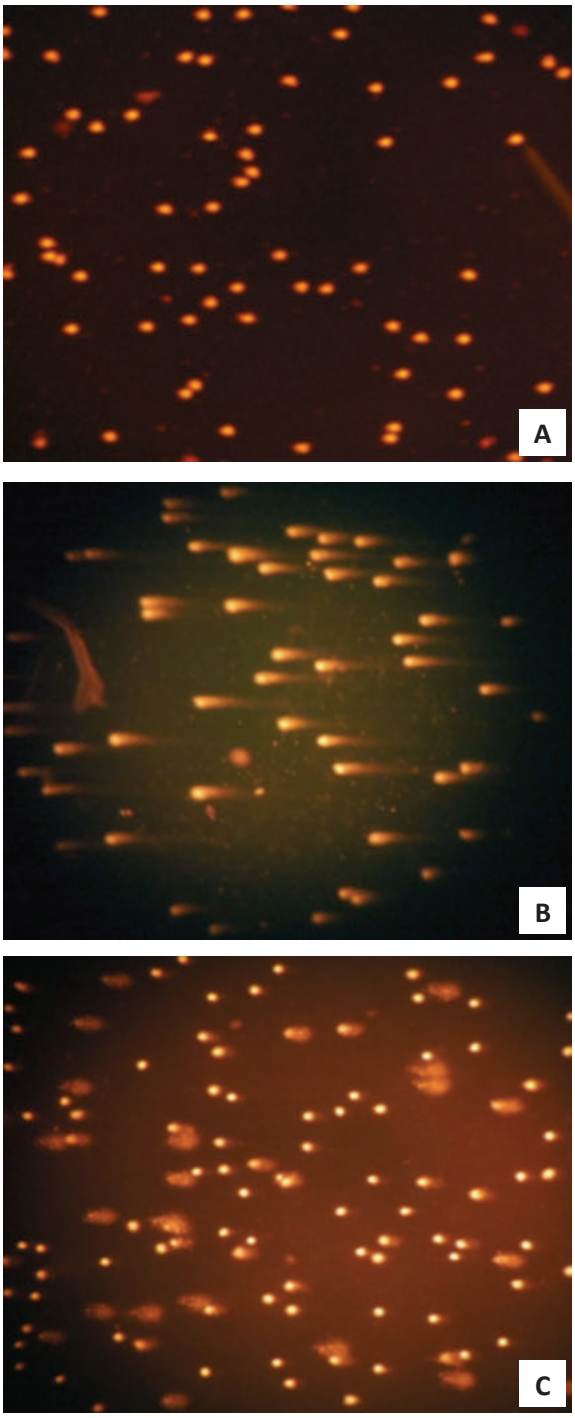

Figure 1 - Comets indicating DNA damage in cultured human peripheral blood lymphocytes. **A** Untreated sample: cells with intact DNA (without comets). **B** Pesticide- treated sample: cells with damaged DNA (with comets). **C** Sample treated with pesticide and curcumin: less DNA damage compared to pesticide-treated sample

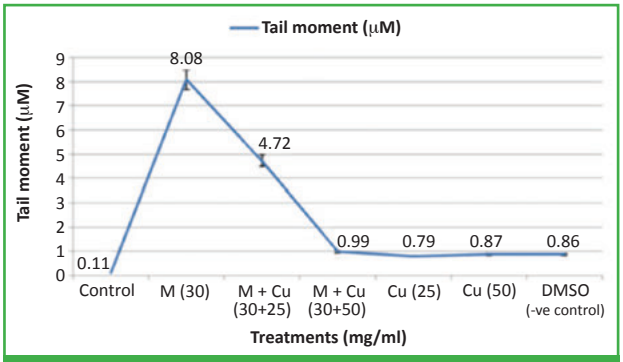

Figure 2 - Antigenotoxic effect of curcumin against malathion-treated cultured human lymphocytes. *Control* untreated, *M* malathion, *Cu* curcumin, *DMSO* dimethyl sulfoxide

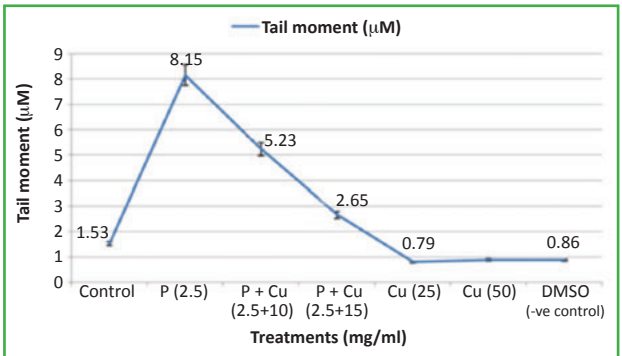

Figure 3 - Reduction in tail moment with curcumin treatment against parathion-treated cultured peripheral blood lymphocytes. *Control* untreated, *P* parathion, *Cu* curcumin, *DMSO* dimethyl sulfoxide

strated the maximum DNA damage to PBL, these concentrations were chosen for analyzing the antigenotoxic potential of curcumin and carvacrol. The reduction in TM in the presence of each pesticide was used to determine the antigenotoxic effects of curcumin and carvacrol. Curcumin at the concentrations of 25 and 50 μg/ml significantly reduced TM compared to malathion only treated samples (Fig. 2), while it exhibited a protective effect against parathion at the concentrations of 10 and 15 μg/ml (Fig. 3). Carvacrol exerted an antigenotoxic effect against malathion and parathion at the concentrations of 2.5 and 5.0 μg/ml (Figs. 4 and 5). Both curcumin and carvacrol were also analyzed for genotoxic effect in absence of both pesticides: neither were observed to be genotoxic. The combinatorial antigenotoxic effect of both curcumin and carvacrol was also studied against both pesticides (Tables 1 and 2). We did not observe any reduction in TM with curcumin and carvacrol combined compared to separate treatment against malathion and parathion. A small increase in TM was statistically non-significant ($p>0.05$).

Effect of *GSTM1* and *GSTT1* polymorphisms on malathion and parathion-induced genotoxicity

We found no significant effect of *GSTM1* and

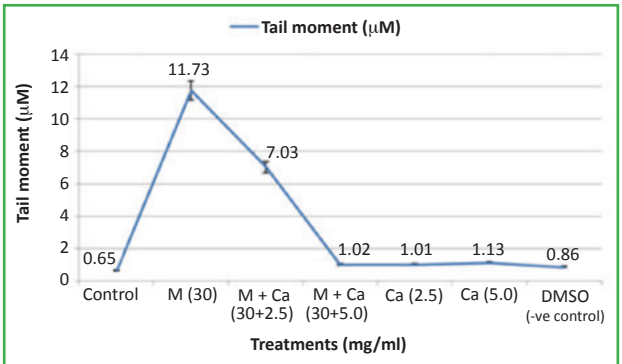

Figure 4 - Protective effect of carvacrol against malathion. *Control* untreated, *M* malathion, *Ca* carvacrol, *DMSO* dimethyl sulfoxide

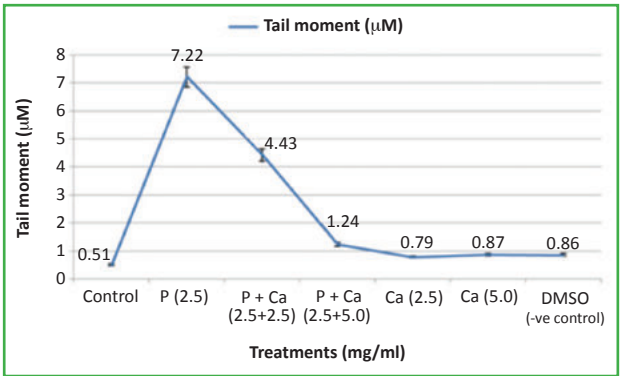

Figure 5 - Protective effect of carvacrol against parathion. *Control* untreated, *P* parathion, *Ca* carvacrol, *DMSO* dimethyl sulfoxide

GSTT1 polymorphisms on genotoxicity induced by both pesticides under *in vitro* conditions (Table 3). The extent of DNA damage induced by malathion and parathion in non-null *GSTT1* and *GSTM1* genotypes was higher but was non-significant ($p>0.05$).

Discussion

In the present study, we found that curcumin and carvacrol had ameliorative effects against malathion and parathion-induced genotoxicity, while their combined treatment did not show any significant reduction in TM compared to treatment by each separately. Also, no significant effect of *GSTT1* and *GSTM1* polymorphisms on the genotoxicity of malathion and parathion was observed. An increase in genotoxic damage is associated with an increased overall risk of cancer [15]. We observed that PBL exposed to 10–30 μg/ml malathion and 0.5–2.5 μg/ml parathion showed a dose-dependent increase in TM.

Treatment	Concentrations used (μg/ml+μg/ml)	Tail moment (μM) (mean±SD)
Control	Untreated	1.10±0.55
Malathion	30	8.98±1.73[a]
Malathion + curcumin	30+25	4.72±1.34[b]
Malathion + curcumin	30+50	0.99±0.22[b]
Malathion + carvacrol	30+2.5	6.38±1.55[b]
Malathion + carvacrol	30+5.0	1.02±0.64[b]
Malathion + curcumin + carvacrol	30+25+2.5	5.86±1.73[c]
Malathion + curcumin + carvacrol	30+50+5.0	1.93±0.67[c]

[a]$p<0.05$ (significant compared to untreated)
[b]$p<0.05$ (significant compared to malathion treatment)
[c]$p>0.05$ (non-significant compared to treatment with curcumin or carvacrol alone)

Table 1 - Combinatorial antigenotoxic effect of curcumin and carvacrol against DNA damage induced by malathion

Treatment	Concentrations used (μg/ml+μg/ml)	Tail moment (μM) (mean±SD)
Control	Untreated	1.20±0.82
Parathion	2.5	7.91±0.63[a]
Parathion + curcumin	2.5+10	5.23±2.29[b]
Parathion + curcumin	2.5+15	2.65±1.70[b]
Parathion + carvacrol	2.5+2.5	4.43±1.30[b]
Parathion + carvacrol	2.5+5.0	1.24±1.01[b]
Parathion + curcumin + carvacrol	2.5+10+2.5	5.67±1.29[c]
Parathion + curcumin + carvacrol	2.5+15+5.0	2.21±0.43[c]

[a]$p<0.05$ (significant compared to untreated)
[b]$p<0.05$ (significant compared to parathion treatment)
[c]$p>0.05$ (non-significant compared to treatment with curcumin or carvacrol alone)

Table 2 - Combinatorial protective effect of curcumin and carvacrol against parathion-induced DNA damage

Genotype	Malathion (30 μg/ml)	Parathion (2.5 μg/ml)
Relationship with *GSTT1*		
GSTT1 (non-null)	8.06±1.25	7.72±1.61
GSTT1 (null)	8.63±1.53[a]	7.81±2.34[a]
Relationship with *GSTM1*		
GSTM1 (non-null)	6.78±1.16	8.63±2.44
GSTM1 (null)	6.98±0.51[b]	7.94±1.38[b]

[a]$p>0.05$ (non-significant compared to *GSTT1* non-null genotypes)
[b]$p>0.05$ (non-significant compared to *GSTM1* non-null genotypes)

Table 3 - Effect of *GSTT1* and *GSTM1* polymorphisms on the genotoxicity of malathion and parathion

Similarly to our findings, several reports in the literature support the genotoxicity of both these pesticides. Błasiak *et al*. [3] found that malathion and its isomer isomalathion were genotoxic to cultured human PBL. Malathion was also found to be genotoxic to the freshwater teleost fish *Channa punctatus* (Bloch) in a study using the micronucleus test and the comet assay as a biomarker of genotoxicity [16].

Several cell culture studies using the comet assay to measure increased TM have also described the genotoxicity of malathion. Moore *et al*. [17] reported that malathion is genotoxic to human liver carcinoma (HepG2) cells. They observed a significant increase in DNA damage at 24 mM malathion exposure using the comet assay. The role of oxidative stress in malathion-induced cytotoxicity and genotoxicity was also examined by Moore *et al*. [17] who performed MTT, lipid peroxidation, and single cell gel electrophoresis (comet) assays to evaluate cell viability, malondialdehyde (MDA) production, and DNA damage, respectively, in HepG2 cells. HepG2 cells were treated with malathion (6, 12, 18, and 24 mM) and the comet assay was performed. Study results indicated that malathion is mitogenic at lower levels of exposure, and cytotoxic at higher levels of exposure. After 48 h of exposure to 24 mM malathion, the comet assay showed a significant increase in the percentage of DNA damage and comet tail length. The percentages of DNA cleavage were 7.93±4.51%, 9.71±5.48%, 13.16±7.87%, 14.65±5.29%, and 27.3±11.16% for 0, 6, 12, 18, and 24 mM malathion, respectively. These findings suggest that oxidative stress plays an important role in malathion-induced cytotoxic and genotoxic damage in HepG2 cells.

The genotoxicity of malathion was also demonstrated by Dalia and El-Monem [18] in male albino rats. The alkaline comet assay showed significantly increased TM in the liver cells of animals treated with malathion alone compared to a control group. Hence the results indicated that malathion treatment induces cytotoxic and genotoxic effects in the bone marrow cells and liver cells of albino rats. Similarly, Xian *et al*. [19] also evaluated the cytotoxic and genotoxic effects of five organophosphate pesticides or metabolites: acephate (ACE), methamidophos (MET), chloramidophos (CHL), malathion (MAT), and malaoxon (MAO), and clarified the role of oxidative stress using pheochromocytoma (PC12) cells of rat adrenal medulla. DNA damage studies were carried out using a comet assay. DNA damage as indicated by DNA single-strand breaks was demonstrated by an increase in TM. The results showed that 40 mg/l ACE, MET, MAT, and MAO significantly inhibited cell viability and increased DNA damage in PC12 cells. However, when the concentration was reduced to 20 mg/l, only MAO caused significant DNA damage. In contrast, when the concentration was increased to 40 mg/l, all other organophosphates, except for CHL, induced significant DNA damage.

Undeger and Basaran [4] evaluated the genotoxic potential of commonly used pesticides (i.e., dimethoate, methyl parathion, propoxur, pirimicarb, cypermethrin, and permethrin) by using single cell gel electrophoresis (the comet assay) in freshly isolated human PBL. They reported that the organophosphorus insecticide methyl parathion at 200 μg/ml significantly increased DNA damage (measured as tail length, tail intensity, and TM). Similarly, Mathew and Thoppil [20] evaluated the genotoxicity of methyl parathion in Swiss albino mice using the micronucleus test and comet assay. Single intraperitoneal doses of methyl parathion at ¼ LD_{50} and ½ LD_{50} concentrations elicited a statistically significant increase (p<0.001) in the frequency of micronucleated erythrocytes and DNA damage (measured as tail DNA %, tail length, TM, and olive TM). As methyl parathion proved to be more mutagenic and cytotoxic than the positive control ethyl methanesulfonate, the study confirmed the mutagenicity of methyl parathion.

The role of oxidative stress in methyl parathion and parathion-induced toxicity demonstrated using HepG2 cells as a test model was investigated by Edwards *et al*. [21] who performed the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay for cell viability, lipid peroxidation assay for MDA production, and comet assay for DNA damage. Results from the MTT assay indicated that methyl parathion and parathion gradually reduce the viability of HepG2 cells

in a dose-dependent manner, showing 48 h LD_{50} values of 26.20 and 23.58 mM, respectively. The lipid peroxidation assay revealed a significant increase ($p<0.05$) in MDA levels in methyl parathion and parathion-treated HepG2 cells compared to controls, suggesting that oxidative stress plays a key role in organophosphate insecticide-induced toxicity. For the comet assay, 1×10^6 HepG2 cells were cultured in six-well tissue culture plates and exposed to various concentrations (0–30 mM) of parathion and methyl parathion for 48 h and stored in a 37°C, 5% CO_2 incubator. Comet assay results indicated a significant increase in genotoxicity (as evidenced by an increase in the percentage of DNA damage and the length of comet tails) at higher concentrations of organophosphate insecticide exposure. Overall, the results indicated that methyl parathion is slightly less toxic than parathion to HepG2 cells.

The results of the present study support the antigenotoxic role of curcumin and carvacrol against malathion and parathion. Ameliorative effect of curcumin has been reported in many studies in agreement with our findings. Tiwari and Rao [22] evaluated the role of curcumin as a potential natural antioxidant to mitigate the genotoxic effects of arsenic (As) and fluoride (F) in human PBL using the comet assay and chromosomal aberrations (CA). Their results revealed that curcumin efficiently ameliorates the toxic effect of As and F by reducing the frequency of structural aberrations (by >60%), hypoploidy (by >50%), and primary DNA damage. Similarly, Siddique *et al.* [23] suggested the protective role of curcumin against the genotoxic damage caused by tinidazole in cultured human lymphocytes. Curcumin at doses of 5, 10 and 15 µM showed a dose-dependent decrease in sister chromatid exchanges/cell against 10 µg/ml tinidazole. Ahmed *et al.* [24] studied the antioxidant effect of curcumin and N-acetylcysteine (NAC) against malathion-induced oxidative stress in peripheral blood mononuclear cells (PBMC) under *in vitro* conditions. There was a significant decrease in MDA and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) levels in PBMC when co-treated with NAC and/or curcumin as compared to pesticide alone. These results indicate that malathion-induced oxidative stress is probably responsible for the DNA damage and that NAC or curcumin attenuate this effect by counteracting the oxidative stress.

In our study, carvacrol has also shown a protective effect against malathion and parathion, which is supported by Aristatile *et al.* [25] who observed the protective effect of carvacrol against oxidative stress and DNA damage caused by UV radiation in cultured PBL using the DPPH (2,2-diphenyl-1-picryl hydrazyl) and ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assays and the comet assay. Ozkan and Erdogan [26] also studied the antioxidant/prooxidant effects of various concentrations of carvacrol and thymol on the membrane and DNA of parental and drug-resistant H1299 cells. They reported that carvacrol and thymol protected the cells against H_2O_2-induced cytotoxicity and DNA damage when the cells were preincubated with these two compounds at lower concentrations ($<IC_{50}$) before H_2O_2 incubation.

Regarding the effect of *GSTM1* and *GSTT1* polymorphisms on pesticide-induced genotoxicity, there are few *in vivo* studies to support our findings. Falck *et al.* [27] did not find any genotypic effect exclusively in pesticide-exposed subjects: the *GSTM1* positive genotype was associated with an increased micronucleus frequency irrespective of exposure. Similarly in another study, no significant association of *GSTM1* and *GSTT1* genotypes with micronucleus frequency was found in a group of Spanish greenhouse workers exposed to pesticides [28].

To the best of our knowledge, there are no *in vitro* studies on the effect of *GSTM1* and *GSTT1* polymorphisms on malathion and parathion-induced genotoxicity. However, a few *in vitro* studies on other genotoxicants have reported both positive and negative results. As in our study, Park *et al.* [29] examined the association between *GSTM1* and *GSTT1* polymorphisms and benzopyrene-induced genotoxicity in cultured PBL under *in vitro* conditions but failed to find any correlation. Similarly, no significant relationship was found between *CYP1A1*, *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms and the genotoxicity of trichloroethylene under both *in vivo* and *in vitro* conditions [30].

Conclusion

In the present study, malathion and parathion (organophosphorus insecticides) were found to be genotoxic in human PBL. We found that curcumin and carvacrol have a protective effect against the genotoxicity induced by these organophosphorus insecticides, while there was no significant effect of *GSTT1* and *GSTM1* polymorphisms on the genotoxicity of malathion and parathion.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Human and Animal Rights

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975 and its later amendments.

Consent Form

Informed consent was obtained from all patients for inclusion in the study.

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