Original Article

Effects of *GST* Polymorphism on Ameliorative Effect of Curcumin and Carvacrol against DNA Damage Induced by Combined Treatment of Malathion and Parathion

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ABSTRACT

Background: Organophosphorus pesticides has been widely used in agriculture fields to control various crop insects and their extensive use pose human life at threat because of their adverse effects on human health. In this study, we checked the effects of GST polymorphism on ameliorative effect of curcumin and carvacrol against DNA damages.

Methods: Comet assay was used to assess the DNA damage and results were expressed as Tail moment. Heparinised fresh blood from healthy individuals was treated with combined concentration of malathion and parathion (i.e. 30 µg/ml of malathion and 2.5 µg/ml of parathion) in presence of combination of curcumin and carvacrol (25 µg/ml curcumin + 2.5 µg/ml carvacrol and 50 µg/ml curcumin + 5.0 µg/ml carvacrol) in order to observe the ameliorative role of curcumin and carvacrol. Multiplex PCR was performed for GSTM1 and GSTT1 genotyping.

Results: Curcumin in combination with carvacrol (i.e. 25 µg/ml curcumin + 2.5 µg/ml carvacrol and 50 µg/ml curcumin + 5.0 µg/ml carvacrol) significantly reduced the DNA damage caused by combined action of malathion and parathion which supports their antigenotoxic property. No significant relationship of *GSTT1* and *GSTM1* polymorphism with genotoxicity of both the pesticides and antigenotoxic potential of curcumin and carvacrol was observed.

Conclusion: Malathion and parathion were genotoxic in human PBL. Curcumin and carvacrol had an antigenotoxic effect against the malathion and parathion while there was not any significant effect of *GSTT1* and *GSTM1* polymorphism on genotoxicity of these pesticides and antigenotoxicity of curcumin and carvacrol.

Keywords: Carvacrol, Comet Assay, Curcumin, Malathion, Parathion, Tail Moment Genotoxicity.

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INTRODUCTION

Humans are normally exposed to organophosphate pesticides (OPs) which increased concern about their tendency to elicit toxicity in humans. The primary target for OPs is acetylcholinesterase (AchE), an enzyme involved in conduction of nerve impulse. Inhibition of AchE by OPs causes accumulation of acetylcholine at cholinergic synapses, over-stimulation of muscarinic and nicotinic receptors and followed by "cholinergic syndrome" [1, 2]. OPs exert their toxicities via other pathways also include cytotoxicity, genotoxicity and disruption of sex hormones and reproduction [3-5]. Malathion and parathion are organophosphate insecticides widely used to control pests in agriculture and utilized in fumigation, veterinary practices and commercial extermination [6]. Both of these pesticides are well reported in literature for their genotoxicity. Giri et al., had demonstrated the genotoxic effects of malathion using chromosome aberration, sister chromatid exchange (SCE) and sperm abnormality assays in mice [7].

Similarly, Pamela et al. examined the role of oxidative stress in malathion-induced cytotoxicity and genotoxicity in Hep G2 cell lines using comet assay [8]. Like malathion, parathion was genotoxic [9]. The authors evaluated the genotoxic potential of commonly used pesticides (i.e., dimethoate and methyl

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parathion from the organophosphate class, propoxur and pirimicarb from carbamates, and cypermethrin and permethrin from pyrethroids) by using the comet assay in freshly isolated human PBL. Methyl parathion at 100 and 200 µg/ml significantly increases the DNA damage (measured as Tail length, Tail intensity and Tail moment) in human PBL.

During the last decade, the single cell gel electrophoresis (SCGE) or comet assay was introduced as a rapid, sensitive and inexpensive technique for qualitative and quantitative assessment of DNA damage in single cells [10]. This method, within a short time, has found wide usage in epidemiological and biomonitoring studies in humans, to determine DNA damage, because of endogenous factors and lifestyle, occupational exposure and environmental exposures [11-13].

The term nutraceutical was coined from nutrition and pharmaceutical in 1989 by Stephen Defelice [14]. According to him "a nutraceutical is any substance that is a food or a part of food and provides medical or health benefits, including the prevention and treatment of disease". "Such products may range from food supplements, isolated nutrients, and specific diets to genetically engineered designer foods and herbal products" [15].

Curcumin or diferuloylmethane ([1, 7-bis (4-hydroxy-3-methoxy phenyl)-1-6- hepatadine-3-5-dione]), a polyphenolic molecule extracted from the rhizome of the plant *Curcuma longa*. This natural compound was used over centuries in Ayurvedic, Chinese and Hindu traditional medicine. Its immunomodulatory properties including anti-oxidant, anti-inflammatory and anti-tumor properties are well-documented [16]. It shows anticancer properties as demonstrated in a plethora of human cancer cell lines/animal carcinogenesis model [17]. It is reported to inhibit lipid peroxidation, oxidative DNA damage [18] and it also exhibit free radical scavenging and antioxidant properties [19].

Carvacrol (2-methyl-5-(1- methylethyl)-phenol) is a natural member of monoterpene phenol and is present in the volatile oils of Thymus vulgaris, Carum copticum, origanum and oregano [20]. It has been safely used in our daily life such as cosmetic ingredient, safe food additive in baked goods, sweets, beverages, and chewing gum. As well known, carvacrol exhibits anti-microbial, anti-mutagenic, anti-platelet, analgesic, anti-inflammatory, anti-angiogenic, anti-oxidant, anti-elastase, insecticidal, anti-parasitic, cell-protective, AChE inhibitor and anti-tumor activity [21-22]. It is revealed that carvacrol has anti-proliferative properties on non-small cell lung cancer cells A549, chronic myeloid leukemia cells K562, Hep-2 cells, murine B16 melanoma cells and human metastatic breast cancer cells, MDA-MB231 [23-24].

In this study, we checked the effects of GST polymorphism on ameliorative effect of curcumin and carvacrol against DNA damages.

MATERIALS AND METHODS

Sample Collection

This was an *in vitro* experimental study consisted of healthy (2014) individuals only. Five ml venous blood was taken from healthy individuals in two separate vacutainer tubes containing sodium heparin and dipotassium ethylenediamine tetraacetic acid (EDTA) for lymphocyte culture set up and DNA extraction respectively. Subjects having exposure to diagnostic X-rays, drug intake and vaccination in last six month prior to blood sampling have not been included in study.

All the participants were asked to sign an informed consent for their participation and a questionnaire was filled by the donors for their health status. All the individuals participated in the study were men of age group 18 to 30 years and were bonafide resident of Haryana state, North India (Aryan race). The protocol was duly approved by human Ethical Committee of Kurukshetra University.

Human Lymphocyte Culture

Short-term peripheral blood lymphocyte (PBL) cultures were set up using earlier studied technique with minor modifications [25]. Culture was set up 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 UI/ml) and streptomycin (100µg/ml) solution (Himedia), and phytohaemagglutinin (2%) (Bangalore genei). Malathion (Sigma) and Parathion (Sigma) were added at the beginning of culture at the concentrations of 30 µg/ml and 2.5 µg/ml respectively to check the protective effect of curcumin (Sigma) and carvacrol (Sigma). To check the combined effect of both curcumin and

carvacrol against both the pesticides, cultures were set up separately having various combinations of both the pesticides and curcumin/carvacrol. In one set up, heparinised fresh blood was treated with 30 and 2.5 µg/ml concentration of malathion and parathion along with 25 and 2.5 µg/ml concentrations of curcumin and carvacrol respectively.

Similarly, in another culture set up, 50 and 5.0 µg/ml concentrations of curcumin and carvacrol respectively were added along with above said concentrations of malathion and parathion. Blood was also treated with curcumin and carvacrol alone to check their genotoxic effects if any. Blood without any mutagen/ curcumin and carvacrol acted as control while blood having Dimethylsulphoxide (DMSO) was taken as negative control. The cultures were then incubated for 24 h at 37˚C and 5 % CO_2. The cells were harvested by centrifugation and washed in phosphate buffer saline (PBS). These lymphocytes were then resuspended in 1ml PBS.

Single Cell Gel Electrophoresis or Comet Assay

Alkaline comet assay was performed according to previous method with minor modifications [26-27]. Slides were prepared in duplicate per sample. Dust free, plain slides were covered with a layer of 150 µl of 1% normal melting agarose (Sigma) and allowed to dry for 10 min in hot air oven. This layer served as an anchor for additional layers to prevent slippage. The fixed lymphocytes (10-20 µl) were mixed with 80 µl of warm 0.5% low melting agarose (Sigma) and this mixture was layered as second additional layer and gelled at 4 ºC for 15 min. A third additional layer of 150 µl of 0.5% low melting agarose was added on top and gelled again at 4 ºC for 15 min. The slides were treated for 2 h at 4 ºC in freshly prepared, chilled lysis buffer solution (25 mM sodium chloride, 100 mM sodium EDTA, 10 mM tris, 1% triton X - 100, 10 % DMSO added before use and pH adjusted to 10). The slides were then removed from the lysis solution and were incubated in alkaline electrophoresis buffer (10N sodium chloride, 200 mM EDTA, pH adjusted to 13) for 20 min followed by 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 (20µg/ml) for 5 min under dark condition.

Comet Scoring

A total of 50 individual cells from each of the duplicate slides per subject were examined randomly under Olympus fluorescence microscope with 535 nm absorbance green emission filter. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. The extent of DNA damage was measured quantitatively as tail moment (TM) value using Lucia comet assay software (Version 7.12). The TM is defined as the percentage of DNA in the tail multiplied by the length between the center of the head and tail [28].

GSTM1 & GSTT1 Genotyping

Genomic DNA was extracted from 200 µl of whole blood by DNA extraction kit (Bangalore genei). Multiplex PCR was used to detect the presence or absence of GSTM1 & GSTT1 gene. For the purpose of internal control, a part of exon 7 of the constitutional gene CYP1A1 was also co-amplified [29]. The amplification reaction was carried out in a 25 µl volume containing 50-100 ng of genomic DNA as a template, 20 pmol/ µl of each primer (GenXbio), 200 µM of each dNTP (Bangalore genei), 1X PCR buffer with 15 mM/L MgCl2 (Bangalore genei) and 0.7 units of Taq polymerase (Bangalore genei). PCR was performed by using reaction mixture with primers GSTM1 (Fw5'-GAACTCCCTGAAAAGCTAAAGC-3’ and Re5'- GTTGGGCTCAAATATACGGTGG-3’); GSTT1 (Fw 5’-TTCCTTACTGGTCCTCACATCTC-3’, Re 5’-TCACGGGATCATGGCCAGCA-3’) and CYP1A1 (Fw5' -GAACTGCCACTTCAGCTGTCT-3’ and Re 5’-CAGCTGCATTTGGAAGTGCTC-3’) yielding a 312-bp product. A total of 35 temperature cycles were used: 10 min at 94 ºC, : 1 min at 94 ºC, 30 sec at 59 ºC, and 1 min at 72 ºC. The last elongation step was extended to 10 min at 72 ºC. The PCR products were analyzed in 2% agarose gel.

Statistical Analysis

All treatments were performed in duplicates and results were expressed as means ± S.D. The student *t* test was used for calculating the statistical significance using SPSS 16.0 (Chicago, IL, USA).

RESULTS

We studied the protective effects of combination of curcumin and carvacrol against the genotoxicity induced by combined action of malathion and parathion in cultured PBL. DNA damage was examined in terms of TM value using comet assay. The damaged DNA in form of comet and undamaged intact DNA in pesticides treated and an untreated sample respectively is shown in Figure 1.

Ameliorative effect of curcumin and carvacrol was analyzed by reduction in TM in presence of malathion and parathion. Curcumin and carvacrol in combination (25 µg/ml curcumin + 2.5 µg/ml carvacrol and 50 µg/ml curcumin+ 5.0 µg/ml carvacrol) had significantly (P<0.05) reduced the TM as compared to malathion and parathion treatment. Both curcumin and carvacrol were also analyzed for any genotoxic effect in absence of malathion and parathion (Data communicated). None of these was observed to be genotoxic (Table 1, Figure 2).

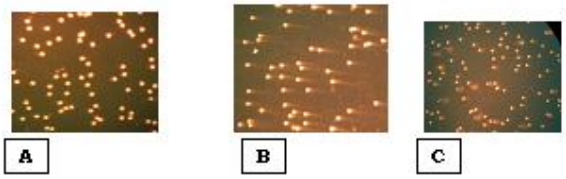

Figure 1. Comets representing DNA damage in cultured human PBL.
(A). Untreated sample [cells with intact DNA (without comet)]
(B). Malathion + Parathion treated sample [cells with damaged DNA (with comet)]
(C). (Malathion + Parathion) + (curcumin + carvacrol) treated sample (Less DNA damage as compared to parathion treated sample)

Table 1. Ameliorative effect of curcumin and carvacrol against combined treatment of malathion and parathion in cultured human PBL.

Treatment	Concentrations used (µg/ml)	Tail Moment (µM) (Mean±SD)
Control	Untreated	0.98±0.41
Malathion + Parathion	30 + 2.5	12.09±0.31[a]
(Malathion + Parathion) + (Curcumin + Carvacrol)	(30 + 2.5) + (25+ 2.5)	5.49±0.39[b]
(Malathion + Parathion) + (Curcumin + Carvacrol)	(30 + 2.5) + (50+ 5.0)	1.64±0.26[b]
DMSO (-ve Control)	20	0.86±0.21[c]

[a]P<0.05(Significance as compared to untreated)
[b]P<0.05(Significant as compared to Malathion and Parathion treatment
[c]P>0.05(Non-Significant as compared to untreated)

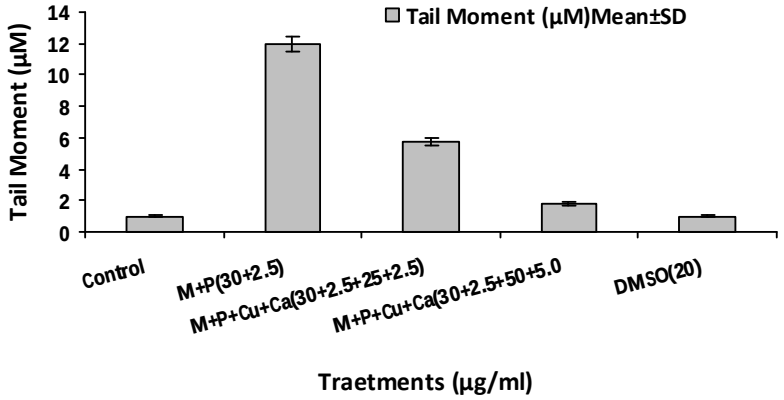

Figure 2. Antigenotoxic role of curcumin and carvacrol against combined treatment of malathion and parathion in cultured human PBL.

*M : Malathion, *P: Parathion, Cu: Curcumin, Ca: Carvacrol, DMSO: Dimethyl Sulphoxide

Effect of GSTM1 and GSTT1 Polymorphism on Malathion and Parathion Induced Genotoxicity and on Ameliorative Potential of Curcumin and Carvacrol

Individuals having different genotypes respond differently to environmental chemicals. Multiplex PCR was used to detect the presence or absence of *GSTM1* and *GSTT1* genotypes. The occurrence of *GSTM1* and *GSTT1* genes was detected by the presence or absence of a band at 215 & 480 bp respectively. We studied the ameliorative potential of curcumin and carvacrol against the genotoxicity induced by combined treatment of malathion and parathion and their relationship with *GSTT1* and *GSTM1* genetic polymorphism. We found no significant effect of *GSTM1* polymorphism on malathion and parathion induced genotoxicity and antigenotoxicity of curcumin and carvacrol under *in vitro* conditions (Figure 3 and 4). Similarly, *GSTT1* polymorphism did not influence the genotoxic potential of both the pesticides and antigenotoxic potential of curcumin and carvacrol (Figure 5 and 6). The extent of DNA damage induced by malathion and parathion in null *GSTT1* and *GSTM1* genotypes was more but it was non-significant (P>0.05). Reduction in DNA damage by curcumin and carvacrol against both the pesticides was more in *GSTT1* and *GSTM1* null genotypes as compared to *GSTT1* and *GSTM1* non null genotypes but that reduction was found non-significant.

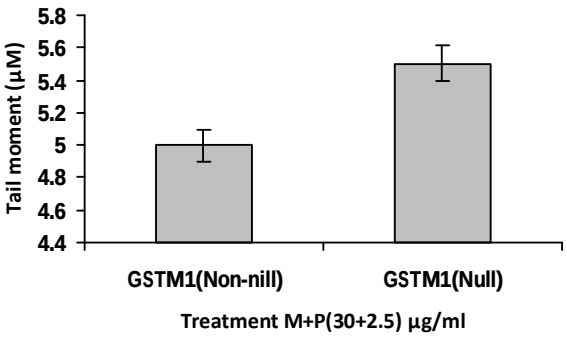

Figure 3. Effect of *GSTM1* polymorphism on DNA damage induced by combined action of malathion and parathion.

*M : Malathion, *P: Parathion

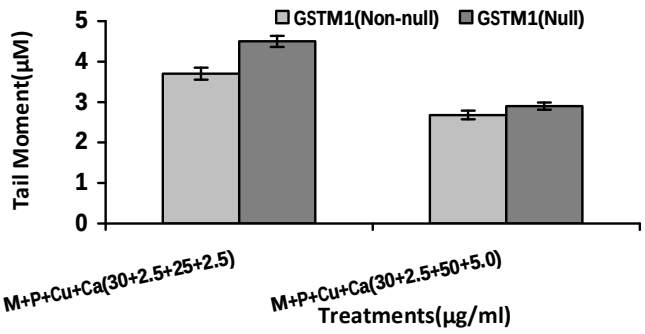

Figure 4. Effect of *GSTM1* polymorphism on combined ameliorative effect curcumin and carvacrol.

*M : Malathion, *P: Parathion, Cu: Curcumin, Ca: Carvacrol

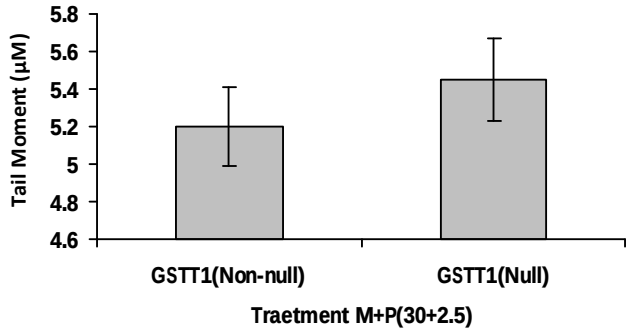

Figure 5. Effect of *GSTT1* polymorphism on DNA damage induced by combined action of malathion and parathion.

*M : Malathion, *P: Parathion

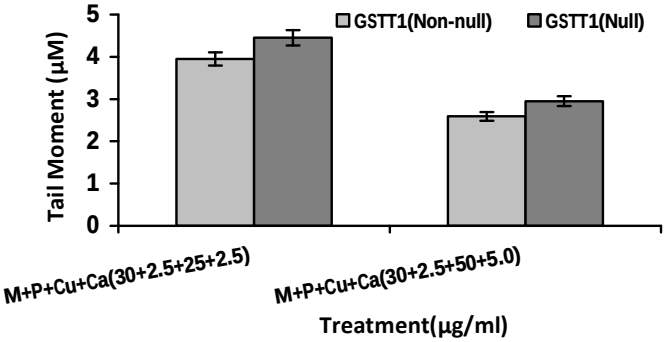

Figure 6. Effect of *GSTT1* polymorphism on combined ameliorative effect curcumin and carvacrol.

*M : Malathion, *P: Parathion, Cu: Curcumin, Ca: Carvacrol

DISCUSSION

In the present study, we investigated the protective effect of curcumin and carvacrol against the genotoxic damage caused by combined action of malathion and parathion using comet assay as a biomarker of genotoxicity. We observed that the cells exposed to 30 and 2.5 µg/ml concentrations of malathion and parathion respectively had shown increase in TM as compared to untreated sample. Several cell culture studies reported the genotoxicity of malathion using comet assay in terms of increased TM value which supports our findings. Malathion is genotoxic to human liver carcinoma (HepG2) cells [30]. The authors observed the significant increase in DNA damage at the 24 mM malathion exposure using comet assay. The role of oxidative stress in malathion-induced cytotoxicity and genotoxicity was also analyzed [10]. To accomplish this goal, MTT, lipid peroxidation, and single cell gel electrophoresis (Comet) assays were performed, respectively, to assess the levels of cell viability, malondialdehyde (MDA) production, and DNA damage in human liver carcinoma (HepG2) cells. HepG2 cells were exposed to malathion (6, 12, 18, and 24 mM) and comet assay was performed. Malathion was mitogenic at lower levels of exposure, and cytotoxic at higher levels of exposure. Upon 48 h of exposure with 24 mM malathion, the comet assay showed a significant elevation in percentage of DNA damage and comet tail length. The percentage of DNA cleavage was 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 of malathion, respectively. These findings imply that oxidative stress plays an important role in malathion-induced cytotoxic and genotoxic damage in HepG2 cells.

Xian et al. also evaluated the cytotoxic and genotoxic effects of five OPs 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 [31]. DNA damage studies were carried out using a comet assay. DNA damage represented by DNA single-strand breaks was reflected by an increase in tail moments. Their results demonstrated that MET, MAT and MAO caused significant inhibition of cell viability and increased DNA damage in PC12 cells at 40 mg/l. Considering tail length, at a concentration of 20 mg/l, only MAO induced significant effect on DNA damage, when the concentration was increased to 40 mg/l the other OPs, with the exception of CHL, all induced significant DNA damage.

Like malathion, parathion was also genotoxic in many studies. Undeger and Basaran investigated the genotoxic potential of usually used pesticides (i.e. dimethoate, methyl parathion, propoxur, pirimicarb, cypermethrin and permethrin) using the comet assay in freshly isolated human PBL [9]. They suggested that methyl parathion at 200 µg/ml had significantly increased the DNA damage (measured as Tail length, Tail intensity and Tail moment). Similarly, genotoxicity of the methyl parathion (MP) was also demonstrated in Swiss albino mice using micronucleus and comet assay [32]. Single intraperitoneal doses of MP at concentrations ¼ LD_{50} and ½ LD_{50} elicited statistically significant increase (P<0.001) in frequency of micronucleated erythrocytes and DNA damage (measured as Tail DNA %, Tail length, Tail moment and Olive tail moment). MP was proved more mutagenic and cytotoxic than the positive control ethyl methanesulphonate. Hence the study, confirmed the mutagenicity of MP.

Falicia et al. investigated the role of oxidative stress in methyl parathion and parathion induced toxicity using human liver carcinoma (HepG2) cells as a test model [33]. To attain this objective, they performed the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay for cell viability, lipid peroxidation assay for malondialdehyde (MDA) production, and comet assay for DNA damage, respectively. Outcome of 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 mM and 23.58 mM, respectively. Lipid peroxidation assay resulted in a significant increase (P<0.05) of MDA level in methyl parathion and parathion treated HepG2 cells compared to controls, suggesting that oxidative stress plays a key role in organophosphate insecticides (OPI) induced toxicity. For comet assay, 1×10^6 HepG2 cells

were cultured in 6-well tissue culture plates, 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 from increase in the percentage of DNA damage and the length of comet tail) at higher concentrations of organophosphate insecticides 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 investigations, which supports our findings. Tiwari and Rao assessed the function of curcumin as a potential natural antioxidant to diminish the genotoxic effects of arsenic (As) and fluoride (F) in human PBL by means of comet assay and chromosomal aberrations (CA) [34]. Accordingly, curcumin efficiently ameliorates the toxic effect of As and F by reducing the frequency of structural aberrations (>60%), hypoploidy (>50%) and primary DNA damage. Similarly, Siddique et al. evaluated the protective function of curcumin against the genotoxic damage caused by tinidazole in cultured human lymphocytes [35]. Curcumin at the doses of 5, 10 and 15 µM had shown the dose dependent reduction in SCEs/cell against 10 µg/ml concentration of tinidazole. Ahmed et al. 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 [36]. There was a considerable decrease in malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) levels in PBMC when co-treated with NAC or/and curcumin as compared to pesticide alone. These results indicate that malathion induced oxidative stress is probably responsible for the DNA damage and NAC or curcumin attenuate this effect by counteracting the oxidative stress.

In our study, carvacrol had also shown protective effect against malathion and parathion supported earlier [37]. They observed the protective effect of carvacrol against oxidative stress and DNA damage by U.V. radiations in cultured PBL using DPPH (2, 2-Diphenyl-1-picryl hydrazyl), ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay and comet assay. Ozkan and Erdogan also studied the antioxidant/prooxidant effects of carvacrol and thymol at various concentrations on membrane and DNA of parental and drug resistant H1299 cells [38]. They accounted that carvacrol and thymol defend the cells against H_2O_2-induced cytotoxicity and DNA damage when the cells were preincubated with these two compounds at lower concentration (< IC50) before H_2O_2 incubation**.**

Regarding the effect of *GSTM1* and *GSTT1* polymorphism on pesticides induced genotoxicity; few *in vivo* studies are available which supports our findings. Falck et al. did not observe any genotypic effect in the pesticide exposed subjects [39]. The *GSTM1* non-null genotype was associated with an increased MN frequency irrespective of exposure. Similarly, no significant association between *GSTM1* and *GSTT1* genotypes on the MN frequency was found in a group of Spanish greenhouse workers exposed to pesticides [40].

To the best of our knowledge, there is no *in vitro* studies available regarding effect of *GSTM1* and *GSTT1* polymorphism on malathion and parathion induced genotoxicity. However, few *in vitro* studies on other genotoxicants reported both positive and negative results. As in our study, Park et al. studied the effect of *GSTM1* and *GSTT1* polymorphism on benzopyrene induced genotoxicity in cultured PBL under *in vitro* conditions [41]. They did not find any association between *GSTM1* and *GSTT1* polymorphism and genotoxicity induced by the Benzopyrene (BaP). Similarly, no considerable relationship was found between CYP1A1, GSTM1, GSTT1, GSTP1 polymorphism and genotoxicity of trichloroethylene under both *in vivo* and *in vitro* conditions [42].

CONCLUSION

Organophosphorus pesticides (malathion and parathion), were genotoxic in human PBL when used in combination. We observed the combined antigenotoxic effect of curcumin and carvacrol against the malathion and parathion induced genotoxicity while there was not any significant effect of GSTT1 and GSTM1 polymorphism on genotoxicity of parathion and antigenotoxicity of curcumin and carvacrol.

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