PyrexJournals

Pyrex Journal of Biomedical Research
Vol 1 (6) pp. 068-073 December, 2015
http://www.pyrexjournals.org/pjbr

Original Research Article

DNA fragmentation and apoptosis caused by gasoline inhalation, and the protective role of green tea and curcumin

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Accepted 23rd November, 2015

This study aimed to investigate the toxic effects of gasoline on DNA in spleen and liver, on the other hand, studying the protective and the ameliorative role of some natural products on these toxic effects of gasoline. Green tea extract and powdered curcumin were chosen as antitoxicity natural products. CD1 mice were taken as an experimental model. Mice were exposed to gasoline vapor 2hours/day for 3 weeks in inhalation chamber. The concentration of gasoline is 9375 ppm and the concentration of benzene is 100 fold less than gasoline in equilibrium with pure benzene being 93.75 ppm. Green tea extract was provided to mice as their sole source of drinking water, and powdered curcumin was added to the diet, these were taken before starting inhalation with one week and along the time of experiment till sacrificing the animals. The study concluded that DNA fragmentation occurred as a result of gasoline toxicity in spleen and liver, these were protected by green tea and curcumin.

Key words: Gasoline, DNA, green tea, curcumin.

INTRODUCTION

Gasoline is a refined product of petroleum consisting of a complex mixture of hydrocarbons. A generic mixture contains about 54% of paraffins and isoparaffins (alkanes from C4 to C12), 36% of aromatic (principally benzene, toluene, ethylbenzene, and xylene), 6% of olefins (or alkenes), 5% of naphthenics hydrocarbons (or saturated cyclic hydrocarbons) and <1% of other compounds. Many of the toxicological effects associated with exposure to gasoline can be attributed to specific components of the mixture: e.g. benzene (ATSDR, 1995). Some populations as automobile mechanics, service station, filling station, workers, and taxi drivers are exposed to benzene through their contact with gasoline vapor and engine exhaust and by multiple routes. Automobile mechanics represent a population of workers exposed to modest levels of benzene through their contact with gasoline and engine exhausts. Although the concentration of benzene in gasoline is typically <1% (v/v) in the USA (Wallace, 1996), thermolytic dealkylation of alkylbenzenes raises the level of benzene in car exhausts to 5% of total hydrocarbon emissions (Wallace, 1996). Mechanics' benzene exposures have recently been reported to range from 0.01 to 13.6 mg/m3, with the vast majority of measurements well below the current OSHA standard of 1 p.p.m. (3.2 mg/m3) (Nordlinder and Ramnäs, 1987; Popp *et al.,* 1994; Mannino *et al.,* 1995; Hotz and Lauwerys, 1997; Javelaud *et al.,* 1998).

Benzene is one of the aromatic hydrocarbons, a colorless organic solvent derived from petroleum refining. Pure benzene is no longer widely employed in industries, but benzene-containing solvents, such as the extraction agent of vegetable oil and animal fat, and the solvents or thinners for rubber, resins, paints, and glues could be found as an industry production material. Occasionally, benzene-containing solvents are used as a degreaser for metal work-pieces (Phillips and Johnson, 2001; Wong, 2002; Kuang and Liang, 2005).

Benzene biotransformation produces numerous metabolites that can induce cytotoxicity and genotoxicity through diverse mechanisms (Smith, 1996; Valentine *et al.,* 1996; Ross, 2000; Snyder, 2000; 2002; 2004; Recio *et al.,* 2005 and Wan *et al.,* 2005). These reactive metabolites include quinones that can bind to cellular macromolecules, including DNA, tubulin, histones and topoisomerase II. Benzoquinones and other benzene metabolites can cause oxidative DNA damage, lipid peroxidation in vivo, formation of hydroxylated deoxyguanosine residues and strand breaks in the DNA of bone marrow cells, implicating a role for reactive oxygen species (ROS) and covalent binding in benzene-induced toxicity. Formation of

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DNA double strand breaks (DSB) by ROS and other mechanisms can lead to increased mitotic recombination, chromosomal translocations and aneuploidy (Zhang *et al.,* 2002; Roma-Torres *et al.,* 2006). Such genetic consequences may result in protooncogene activation, tumor suppressor gene inactivation, gene fusions, and other deleterious changes in stem cells that can ultimately result in leukemic responses (Wan *et al.,* 2005).

A major development over the past two decades has been the realization that free radical mediated peroxidation of membrane lipids and oxidative damage of DNA are associated with a variety of chronic health problems, such as cancer, atherosclerosis, neurodegenerative diseases and aging (Finkel and Holbrook, 2000; Perwez Hussain *et al.,* 2003; Barnham *et al.,* 2004). Therefore, inhibition of oxidative damage by supplementation of antioxidants becomes an attractive therapeutic strategy to reduce the risk of these diseases (Rice-Evans and Diplock, 1993; Brash and Harve, 2002). Curcumin is a powerful scavenger of many free radicals such as anion, hydroxyl radical and nitric oxide (Elizabeth and Rao, 1990; Sreejayan and Rao, 1997 and Barzegar *et al.,* 2011). Jayaprakasha *et al.,* (2006) demonstrated in vitro the antioxidant capacities and activities of curcumin, bisdemethoxycurcumin and demethoxycurcumin using the phosphomolybdenum method and linoleic acid peroxidation method. They reported that, by using phosphomolybdeum method curcumin, demethoxycurcumin and bisdemethoxycurcumin exhibited various degrees of antioxidant capacity. The antioxidant capacities of curcuminoids were found to decrease in the order: curcumin > demethoxycurcumin > bisdemethoxycurcumin. Also by using the linoleic acid peroxidation method, they found the same orders of antioxidant activities of the three curcuminoid compounds.

Recent studies provide scientific evidence regarding the potential pharmacological, prophylactic or therapeutic use of Cur, as anti-inflammatory, anti-carcinogenic, anti-tumoral, anti-viral, antifungal, anti-parasitic, anti-mutagen, anti-infectious, anti-hepatotoxic and anti-oxidant compound (Chen *et al.,* 2006; Aggarwal *et al.,* 2007; Ciftci *et al.,* 2010; 2011 and 2012; Shehzad *et al.,* 2011). Epidemiological and laboratory studies have reported that green tea presents diverse beneficial health effects including antioxidant (Sung *et al.,* 2000; Nakagawa and Yokozawa, 2002), hypocholesterolemic (Lin *et al.,* 1998; Riemersma *et al.,* 2001; Erba *et al.,* 2005 and Lee *et al.,* 2005), anti-hyperglycemic (Tsuneki *et al.,* 2004 and Li et al., 2006), hepatoprotective (Chung et al., 2003; Fujiki et al., 2005; Bun *et al.,* 2006, Kaviarasan *et al.,* 2007), anticarcinogenic (Wang *et al.,* 1992 ; Lou *et al.,* 1999; Hayakawa *et al.,* 2001 and Zaveri, 2006).

MATERIALS AND METHODS

Experimental animals

Sixty male mice (*Mus musculus*) weighting 20 – 25 g was purchased from the Egyptian Organization for Serological and Vaccine Production, Egypt, were used as experimental animals throughout the present work. The animals were housed individually in plastic cages and acclimated for 1 week before gasoline-fume exposure. Food and water were offered ad libitum. Animals were maintained at 22± 2 °C at normal light/dark cycle.

Preparation of green tea extract

Green tea (*Camellia sinensis*) was purchased from Shanghai tea import & export Corporation, China. The green tea extract was made according to Maity *et al.,* (1998), by soaking 15 gm of instant green tea powder in 1L of boiling water for 5 minutes. The solution was filtered to obtain 1.5% green tea extract; this solution was provided to mice as their sole source of drinking water

Inhalation of gasoline

A glass cubic box its length is 70cm, width is 70cm and high is 70cm, was manufactured to make as gasoline inhalation chamber, there are two orifices in both right and left sides of the box in the upper portion of the box to make aeration, each orifice 5cm in diameter covered with wire mesh to prevent mice escaping. At a 10cm distance from the bottom of the box, a wire mesh shelf 70x70 cm was fixed to put the mice on it. Under this shelf, 200 ml cans containing 150 ml of gasoline were placed in the exposure chamber and the animals were allowed to inhale the fumes evaporating from the cans. The gasoline, which evaporated during the time of inhalation, was about 80 ml/2hours.The time of exposure was 10.00 to 12.00 am and the cans were withdrawn and the inhalation stopped. The experimental fume gasoline inhalation was exceeded for successive three weeks as 2hours/day/three weeks.

The gasoline

The Egyptian commercial unleaded gasoline (octane 90) was purchased from a filling station. Gasoline is a petroleum-derived liquid mixture consisting mostly of more than 300 individual hydrocarbons primarily (in volume) of paraffins (30–90%), cycloparaffins (1–35%), olefins (0–20%), and aromatic (5–55%), distilling in the approximate range of 30°C–220°C. Composition of gasoline varies with the source of the crude oil, refinery processes, conditions, and the blending of refinery streams in the gasoline boiling range to meet performance criteria as well as regulatory requirements (Roberts et al., 2001). Volatile organic compound emissions from gasoline storage showed that total organic compounds per cubic meter gasoline loaded is 35 g/m3 saturated vapor at 25 °C.

Gasoline Dose

Based on analysis reported by Johnson et al. (1990) the concentration in equilibrium with gasoline is 9375 ppm. Benzene is 100-fold less than in equilibrium with pure benzene being 93.75 ppm. This dose of benzene is in equilibrium with gasoline in the inhalant mice cages in the current study. However, gasoline fraction differs from whole gasoline by containing far less aromatic, longer chain and longer aliphatic hydrocarbons. Analysis of workplace exposure to gasoline vapors revealed that C4–C5 length hydrocarbons constitute from 67 to 74% by weight of the typical vapor (Halder *et al.,* 1986).

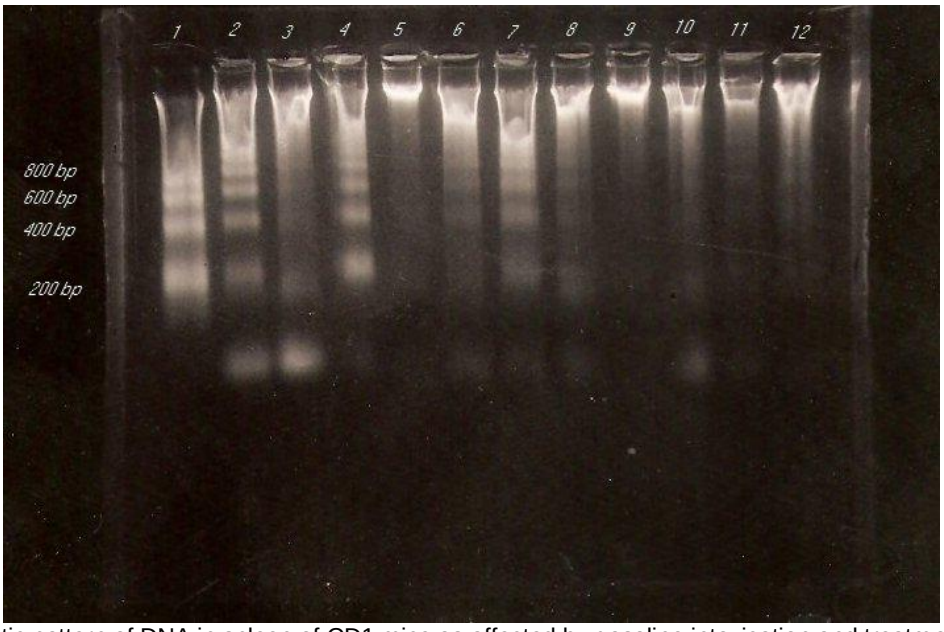

Plate 1: Electrophoretic pattern of DNA in spleen of CD1 mice as affected by gasoline intoxication and treatment with green tea and curcumin
lane1:ladder 2:gasoline 3:gasoline+green tea 4:gasoline 5:green tea 6:gasoline+curcumin 7:gasoline 8:gasoline+curcumin 9:curcumin 10:curcumin 11:control 12:control

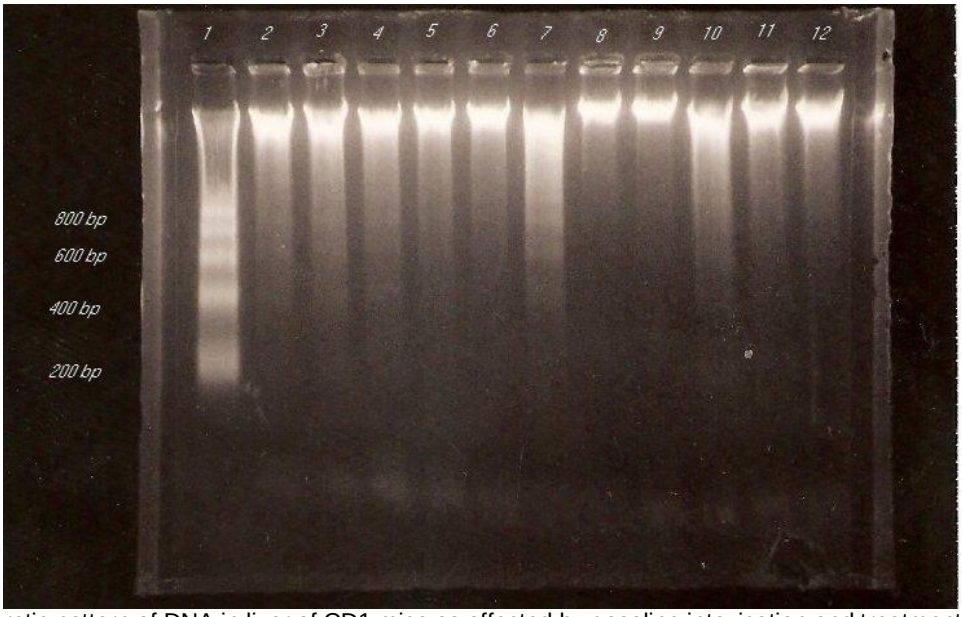

Plate 2: Electrophoretic pattern of DNA in liver of CD1 mice as affected by gasoline intoxication and treatment with green tea and curcumin
Lane 1:ladder 2:gasoline+green tea 3:gasoline+green tea 4:green tea 5:green tea 6:gasoline+curcumin 7:gasoline 8:control 9:control 10:gasoline+curcumin 11:curcumin 12:curcumin

Animal Groups

After an acclimation period for 1 week, animals were classified into six groups; each group consists of ten mice as follow:

1. **Control group:** received only the ordinary mice diet and drink water without any additions and kept two hours daily in the inhalation chamber without gasoline for three weeks.
2. **Green tea group:** received ordinary diet, drink green tea extract (1.5%) as a sole source of drinking water and kept two hours daily in the inhalation chamber without gasoline for three weeks.
3. **Curcumin group:** these animals received powdered dried ground rhizomes of Curcuma longa (turmeric) in the diet (3%) and kept two hours daily in the inhalation chamber without gasoline for three weeks.
4. **Gasoline inhalation group:** this is the intoxicated group with gasoline inhalation; these mice were kept 2 hours daily in an inhalation chamber with gasoline

for three weeks. This group drinks water and eat the ordinary diet.

5. **Gasoline and green tea group**: these animals exposed to gasoline 2 hours daily in an inhalation chamber for three weeks and received green tea extract (1.5%) eat the ordinary diet.
6. **Gasoline and curcumin group:** this group exposed to gasoline in the inhalation chamber, 2 hours daily for three weeks and received powdered dried ground rhizomes of Curcuma longa in their ordinary diet along the time of the experiment and drink water.

Molecular Studies on Liver and Spleen

Gel preparation

The gel was prepared with 1.8% electrophoretic grade agarose (BRL). The agarose was boiled with Tris borate EDTA buffer (1 x TBE buffer; 89mM boric acid, 2mM EDTA, pH 8.3). 0.5µg/ml ethidium bromide was added to the gel at 40 °C. The gel was poured and allowed to solidify at room temperature for 1 hour before samples were loaded.

Nucleic acid extraction and running

Nucleic acid extraction was based on salting out extraction method of Aljanabi and Martinez (1997) modified by Hassab El-Nabi (2004), whereas protein was precipitated by saturated solution on NaCl (5M). A piece of 5mg of liver or spleen tissue was squeezed by blue tips in an Eppendorf tube with 600µl lysing buffer (50mM NaCl, 1mM Na2EDTA, 10% SDS, pH 8.3) and was shacked gently. The mixture was kept overnight at room temperature. For protein precipitation, an amount of 200µl of saturated NaCl was added to the samples and was gently shacked and centrifuged at 12000 x g for 10 min. The supernatant was transferred to new Eppendorf tubes and DNA was precipitated by 600µl cold iso-propanol. The mixture was inverted several times till fine fibers of nucleic acids appeared and centrifuge for 5 min at 12000 x g, the supernatant was then removed.

For washing, an amount of 500µl 70% ethyl alcohol was added to the pellet and centrifuged at 12000 x g for 5 min, the alcohol was decanted or tipped and the tubes blotted on Whatman paper for 15 min, when the tubes were seen to dry, the pellets were re-suspended in 50µl of TE buffer (10mM Tris, 1 mM EDTA, pH 8). As usual in methodology, the extracted genomic DNA of mammals was dissolved in TE buffer or distilled water, this required overnight incubation of DNA pellets with TE at 37°C. The pellets of DNA were re-suspended by double gentle pipetting in TE buffer supplemented with 5% glycerol for 30 minutes. To get rid of RNA, an appropriate volume of Rnase was added and incubated at 37°C for 1 hour. The re-suspended DNA was loaded directly on the gel. An amount of 5µl from 6X loading buffer was added on the cell lysate. Electrophoresis was performed for 2 hours at 50 volts using 1X TBE buffer as a running buffer. The gel was photographed using a polaroid camera, while the DNA was visualized using a 312 nm UV light under transilluminator. The ladder bands were 200, 400, 600, and 800 bp.

RESULTS AND DISCUSSION

As shown in plates (1), inhalation of gasoline induced DNA fragmentation (apoptosis), whereas, the fragments of DNA appeared at 200, 400, 600 and 800 bp. The optical density of apoptotic bands was increased by gasoline compared to control. Curcumin and green tea had ameliorative effects on DNA, where the intensity of the bands was decreased. In plate (2), gasoline intoxication caused DNA fragmentation and increased the optical density of bands at 200, 400, 600 and 800 bp. On the other hand all the other groups had not visible fragmentation bands. Benzene metabolites bind covalently to proteins and DNA in biological systems such as cells or tissues, thereby inducing intracellular toxic effects, such as the inhibition of cell replication or carcinogenesis. Benzene is also believed to act as a mutagen via an indirect mechanism, resulting in oxidative DNA damage through the formation of hydroxyl radicals via hydrogen peroxide (Sul *et al.,* 2005). Proper repair of benzene-induced DNA lesions in the target cells or initiation of programmed cell death of severely damaged cells is essential for preventing possible malignant transformation. Several DNA repair pathways exist in mammals to restore genome integrity following genotoxic stress. Lesions that affect only one of the DNA strands, such as oxidized DNA and adduct formation can be repaired by base excision repair and/or nucleotide excision repair pathways. Double strand break is repaired by nonhomologous end joining or, after replication when a second identical DNA copy is present, homologous recombination. Cells with DNA damage that cannot be completely repaired by these pathways may then undergo apoptosis (Hoeijmakers, 2001).

The mechanism that leads to leukemia in some individuals following benzene exposure is unclear, but several aspects of benzene toxicity are certain. Benzene must undergo biotransformation to exert its toxic effect. While the exact metabolites responsible for the carcinogenic, hematotoxic, and genotoxic effects of benzene are uncertain, several reports discussing benzene toxicity have demonstrated interactions between combinations of phenol and HQ, phenol and catechol, and HQ and muconaldehyde. The quinones and free radicals generated from the metabolism of benzene can interact with cellular constituents, including DNA and macromolecules such as tubulin and histones (Faiola *et al.,* 2004). Ultimately, DNA strand breaks occur, which, if not repaired properly, can lead to chromosomal aberrations. The resulting chromosomal translocations and mitotic recombination events may lead to protooncogene activation or tumor suppressor gene inactivation and in the presence of other epigenetic changes can cause malignant transformation of a cell. Thus, key determinants of individual-to-individual variability and risk in response to the toxic effects of benzene may likely be the enzyme systems involved in the activation and detoxification reactions of benzene metabolism and the DNA repair enzymes required to restore genomic integrity following DNA damage (Smith, 1996).

In the present study DNA fragmentation and apoptosis were observed in both liver and spleen tissue of mice as a result of gasoline inhalation, which is in accordance with the epidemiological studies of Carere *et al.,* (2002), Sul *et al.,* (2005) and Roma-Torres et al. (2006) and also with the experimental study of Faiola *et al.,* (2004) on mice exposed to benzene inhalation. There are several possible inhibitory ways of green tea and curcumin on the in vivo binding of benzene to DNA. Modulation of carcinogen metabolism is often considered an important pathway for the inhibitory effects of many types of chemopreventive agents. These agents detoxify carcinogens through the phase I and/or phase II enzymatic systems: inhibition of the procarcinogen activation, which is catalyzed by

the phase I enzymes (cytochrome P-450s); induction of the detoxification pathway catalyzed by the phase II enzymes, such as GST, epoxide hydrolase, GPx and GR. They may also show their inhibition capacity through scavenging reactive intermediates, interfering with the interaction between the metabolites and DNA, altering the DNA repair rates and scavenging the reactive oxygen and other free radical species (Li *et al.,* 2003). The in vitro study of Anderson *et al.,* (2001) showed that catechins of green tea are highly active in reducing the amount of oxidative damage sustained by DNA through OH radical attack. Catechins, when compared with other classes of flavonoids, are found to be very active in reducing the amount of strand breakage and residual base damage by a mechanism other than direct scavenging of OH radicals before they react with DNA. The results of Anderson *et al.,* (2000; 2001) support the mechanism of electron transfer (or H-atom transfer) from catechins to radical sites on DNA. Both a high percentage and increased rate of electron transfer qualitatively correlate with increased efficiency in reducing DNA damage. Restitution of the DNA in this way results in the strand remaining intact and the range of free radical-induced base damage being reduced to forms which are no longer recognized by a range of endonucleases as damaged sites. While it is likely that the fast chemical reduction of DNA damage through the proposed mechanism results in a high degree of fidelity in repair.

Reaction of OH radicals with DNA gives rise to a wide range of radical intermediates on all of the DNA bases, as well as H-atom abstraction from different sites on the ribose moiety. In the case of thymidine, for example, 65% addition occurs at the C-5 position to yield the corresponding 6-yl radical, 20% 5-yl radical formation and 10% allylic radical through H-atom abstraction from the methyl group (O'Neill and Davies, 1986). Carbon-centered radicals may react at near diffusion-controlled rates with oxygen to produce peroxyl radicals, reaction. Peroxyl radicals formed at the 5 or 6 position on pyrimidines have been proposed to be precursors of DNA strand breaks and this proposal is directly supported by the demonstration of DNA strand breakage upon the in situ production of a 5-peroxyl radical on thymidine into DNA (Barvian and Greenburg, 1995). DNA strand breaks arising from the peroxyl radical formation on the ribose have been shown in the action of bleomycin (Stubbe and Kozarich, 1987). Catechins exert an antioxidant effect on peroxyl radicals, thus preventing DNA strand breaks or radical-induced base damage, through electron (or H-atom) transfer to form the hydroperoxide. Hydroperoxides, formed in free solution and on lipids, are known to induce DNA damage and mutations through a Fenton-type reaction with transition metals to produce peroxyl radicals. Such a reaction seems to be also possible with adventitious metal ions bound to DNA, with the peroxyl radicals produced from diffusing hydroperoxides being able to be scavenged by certain antioxidants (Yang and Schaich, 1996). Anderson et al. (2001) results support a similar antioxidant mechanism for catechins but in addition to them acting directly on peroxyl radicals formed in the DNA, these results indicated that EGCG does repair a similar proportion of the radical precursors for both strand breaks and base damage when present at high concentration. However, since formidopyrimidine and endonucleaseIII proteins both possess activity for AP sites, and that EGCG is relatively inactive at low concentrations on the radical precursors sites recognized by exonucleaseIII, it can be deduced that purine and pyrimidine damaged sites are more efficiently repaired by catechins.

In the present study green tea extract and curcumin in the diet resulted in decrease in the optical density of DNA apoptotic bands which means the protective effect of them on DNA in the spleen and liver tissue, and these results were in agreement with the studies of Li *et al.,* (2003) in their study of green tea, curcumin, grapestone, resveratrol and garlic protective potential effects on DNA against nitrobenzene-induced DNA adductions. Also the present study in agreement with the study of Wei *et al.,* (2006) on the synergistic effect of green tea polyphenols and trolox on free radical-induced oxidative DNA damage caused by 2,2'-azobis(2-amidinopropane hydrochloride), and also with the study of Glei and Pool-Zobel, (2006b) on the ameliorative role of EGCG on human leucocytes DNA damage induced by bleomycin.

CONCLUSION

The study concluded that DNA fragmentation occurred as a result of gasoline toxicity in spleen and liver, these were protected by green tea and curcumin.

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