Protective Effect of *Cinnamomum Camphora* Leaves Extract against Atrazine Induced Genotoxicity and Biochemical Effect on Mice

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Abstract: Atrazine (AT) is one of the most commonly used herbicides to control grasses and weeds. The widespread contamination and persistence of AT residues in the environment has resulted in human exposure. The present study was undertaken to investigate protective effect of *Cinnamomum Camphora* Leaves Extract (CLE) against AT-induced genotoxicity and biochemical changes in mice. Mice were given a 2% *Cinnamomum Camphora* hot water extract as their sole source of drinking water for 2, 4 and 6 weeks. After consumption of CLE animals were orally treated with AT at a total dose of 420 mg/kg body weight on 3 consecutive days. Our results showed that administration of CLE significantly reduced the percentage of DNA damage and chromosomal aberrations induced by AT. Also, it regulates glutathione and lipid peroxidase enzymes. These findings clearly demonstrated the protective effect of CLE in attenuating AT-induced genotoxicity and biochemical changes.
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1. Introduction

Triazine herbicides constitute one of the largest groups of herbicides used throughout the world. Among the triazines, atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine) is one of the most commonly used herbicides to control dicotyledonous weed plants in maize, cereals, lucerne and sugarcane **(Goldman, 1994) .** As a result of its widespread use, AT residues have contaminated not only plants, soil, water and cultivated ground but also agricultural products like fruits, milk, butter, and sugar beet **(Purcell et al., 2001).**

Various *in vitro* and *in vivo* studies have shown the ability of AT to cause serious health hazards like cancer, behavioral changes and reproductive abnormalities in human and animal cells **(Pino et al., 1988; Meisner et al., 1993; Ribas et al., 1995; Hayes et al., 2002).** Genotoxic effects of this herbicide on human and animals have been well documented in literature **(Kourakis et al., 1996; Antonucci et al., 2000; Singh et al., 2008).**

AT is believed to be an endocrine disruptor in human beings and wild life **(Cooper et al., 1999; Cooper et al., 2000; Sanderson et al., 2000) . Stoker et al., (2000) and Spanò et al., (2004)** mentioned that the highest AT exposure dose induced structural disruption in the testis that AT delays puberty in the male rat and its mode of action.

Knowledge has been built for decades on the use of herbal medicinal products and extracts in the treatment of human diseases **(Iwalewa et al., 2007).** *Cinnamomum camphora* Sieb (known as camphor) has long been prescribed in traditional medicines for the treatment of inflammation-related diseases such as rheumatism, sprains, bronchitis, asthma, indigestion and muscle pains. Indeed, 30–50 g of this plant is used three times a day as a form of extract for medicinal purpose in Korea **(Choi, 1997).**

According to previous phytochemical studies, it has been reported that *Cinnamomum camphora* contains alkaloids and essential oil, such as camphor **(Miyazawa et al., 2001)** and Type II ribosome-inactivating proteins (cinnamomin and camphorin) **(He and Liu, 2003)**. Sesquiterpenoid **(Lin et al., 2009)**, diterpenes **(Ngoc et al., 2009b)**, butanolides, lignans, flavanoids, benzenoids, steroids and aliphatic compounds **(Chen et al., 2007)** are widely distributed in plants of the genus *Cinnamomum***.** Constituents of the *Cinnamomum camphora* have shown biological activities such as antitubercular **(Chen et al., 2005)**, tyrosinase-inhibitory **(Ngoc et al., 2009a)**, anti-nociceptive **(Atta and Alkofahi, 1998),** antiarrhythmicaction **(Su et al., 1999)** and antioxidant **(Lee et al., 2006)** activities.

Reactive oxygen species are the causative factor involved in many human degenerative diseases, including cancer, and antioxidants have been found to have some degree of preventive effect on these disorders. However, no evidence has yet been reported to suggest the antigenotoxic properties of *Cinnamomum Camphora* leaves extract (CLE) on somatic and germ cells in any experimental protocol.

Therefore, the aim of this study was to evaluate the protective effect of CLE in AT-induced oxidative DNA damage and biochemical changes in mice.

2. Materials and Methods

2.1. Chemicals

Atrazine (Technical grade, 80.0%) was obtained from Syngenta company, Basel; Switzerland. The leaves of *Cinnamomum camphora* – identified by staff members of the Hebarium of the department of botany, faculty of science, Cairo university- were collected from Menofia region, Egypt in May 2010, air-dried, pulverized and stored for further use. All other chemicals used were of analytical grade.

2.2. Animals treatment:

8-10 week-old laboratory-bred strain Swiss albino male mice (25 ± 30 g) were obtained from the Animal House, National Research Center, Egypt. Animals were maintained under standard conditions of temperature, humidity, light and *ad libitum* on standard lab diet. After an acclimatization period of one week, the animals were divided into eight groups (5 mice /group) and housed in filter-top polycarbonate cages. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre, Dokki, Cairo, Egypt.

AT treated group were orally given AT dissolved in corn oil on 3 consecutive days for a total AT dose of 420-mg/kg b.wt. AT was given at this dose based on the doses reported in literature **(Singh et al., 2008)**. CLE treated animals were administered a 2% (w/v) *Cinnamomum Camphora* leaves hot water extract as their sole water supply. The aqueous extract was prepared every other day by adding 100ml of boiling water to 2 g of dried leaves, let to stand for 30 min. at room temperature, followed by filtration. After 2, 4 and 6 weeks of consumption of CLE or water alone, animals in the AT+CLE groups were orally treated with AT on the last 3 consecutive days of camphor groups treatment. Control mice received corn oil only.

2.3.Cytogenetic analysis

2.3.1.Chromosome aberrations assay in somatic and germ cells

For somatic and germ cells preparations, animals from the different groups were injected i.p. with colchicines, 2h before sacrifice. Chromosome preparations from bone marrow cells (somatic cells) carried out according to the method of **Yosida and Amano, (1965).** Chromosomal preparations from spermatocytes were made according to the technique developed by **Evans et al., (1964)**. 100 well spread metaphases were analyzed per mouse in five mice per group. Metaphases with gaps, chromosome or chromatid breakage, fragments, deletions and polyploidy were recorded in bone marrow cells and diakinesis metaphases-I with univalents were recorded in germ cells.

2.3.2.Mouse bone marrow micronucleus assay

Micronucleus assay was carried out on bone marrow according to the method described by **Valette et al., (2002).** The femurs were dissected out and the bone marrow was flushed out, vortexes and centrifuged. The pellet was resuspended in a few drops of fetal calf serum. Smears were made on pre-cleaned dry slides, air dried and fixed in absolute methanol and the slides were stained with Giemsa stain. Scoring the polychromatic erythrocytes and the percentage of micronucleated polychromatic erythrocytes (MNPCEs) was determined by analyzing the number of MN cells from 1000 PCEs per animal.

2.3.3. DNA fragmentation % (Diphenylamine Assay)

The colorimetric estimation of DNA content was detected according to **Perandones et al (1993)** with some modifications. Both supernatant and the pellet were used for Diphenylamine assay after acid extraction of DNA from liver tissue. The percentage of DNA fragmentation was expressed by the formula :

$$\% \text{ DNA fragmentation} = \frac{\text{O.D. of supernatant}}{\text{O.D. of supernatant + O.D. of pellet}} \times 100$$

2.4.Biochemical analysis

2.4.1. Blood samples

Blood samples were collected from the retro orbital venous plexus of all animals of the different groups. The collected blood samples were received into heparinized tubes. heparinized blood was centrifuged at 3000 r.p.m. for 10 minutes and the clear supernatant plasma was kept at – 20 ^{o}C till assayed for determination of lipid peroxide and glutathione reduced (GSH) levels.

2.4.2.Glutathione reduced (GSH)

Glutathione reduced (GSH) was determined according to **Beutler et al., (1963)** using commercial kits (Biodiagnostics, Egypt). The method based on the reduction of 5, 5 dithiobis (2- nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

2.4.3.Lipid peroxide

Lipid peroxide was determined using commercial kits (Biodiagnostics, Egypt) according to **Ohkawa et al., (1979).** In this test thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95 C for 30 min to form thiobarbituric acid reactive product the absorbance of the resultant pink product can be measured at 534 nm.

2.5.Statistical analysis

For DNA damage, the significance of treatment from the control data and treatment plus protective versus treatment alone was calculated using t-test. While, for biochemical analysis simple one way ANOVA and Duncan's Multiple Range test were used to differentiate between significant means using Statistical Package for Social Science (SPSS) version 16.0 (2007).

3. Results

3.1.Chromosomal aberrations in somatic and germ cells

The mean percentage of metaphases with chromosomal aberrations in bone marrow cells reached 18.2 % ($P < 0.01$) after treatment with AT. It was found to be highly significant also after excluding the number of metaphases with gaps. Treatment with AT induced structural and numerical chromosomal aberrations, where fragments were the most dominant chromosome aberration.

The mean percentage of diakinesis-metaphase I spermatocytes with abnormalities reached 16.8 ($P<0.01$) after treatment with AT. The main types of chromosome aberrations observed were XY and autosomal univalents. In the present study, the percentage of chromosomal aberration in somatic and germ cells induced by CLE, in all time intervals were not significant in comparing to control group. The mean percentage of chromosomal aberrations in somatic and germ cells were significantly decreased when animal groups were treated with CLE 2, 4 and 6 weeks prior to AT. The percentage of inhibitory index in somatic and germ cells increased from 22.3 and 52.3% within 2 weeks to 44.7 and 69.0% within 6 weeks of CLE treatment respectively Fig. (1).

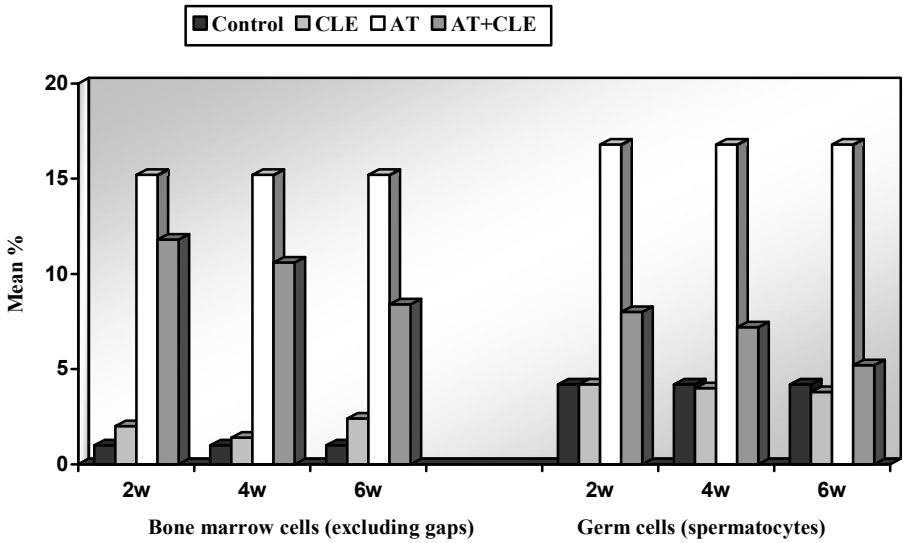

Fig (1): Mean percentage of chromosomal aberrations in mouse bone marrow cells and spermatocytes after treatment with CLE for 2, 4 and 6 weeks plus AT

3.2. Micronucleus and DNA fragmentation

A significant ($P<0.01$) increase in the percentage of micronuclei was observed after treatment with AT as compared to the control and CLE treated groups (Fig.2). Pretreatment with CLE 2 , 4 and 6 weeks significantly decreased the appearance of micronuclei in animals exposed to AT in a time dependent manner. A 40.9, 48.9 and 54.5% reduction in the percentage of micronuclei in PCE were observed after treatment with the three time intervals of CLE respectively.

Fig. (2) depicts the values of DNA fragmentation obtained after treatment with AT and/or CLE. It is clear that there was a significant ($P<0.01$) increase in DNA fragmentation in liver cells of mice treated with AT, while those treated with CLE were statistically not significant in comparing to the control group. Pretreatment with CLE for 2, 4 and 6 weeks significantly ($P<0.01$) decreased the percentage of DNA fragmentation induced by AT reaching 6.65, 5.24 and 4.8% respectively, compared to 9.98% for the group treated only with AT.

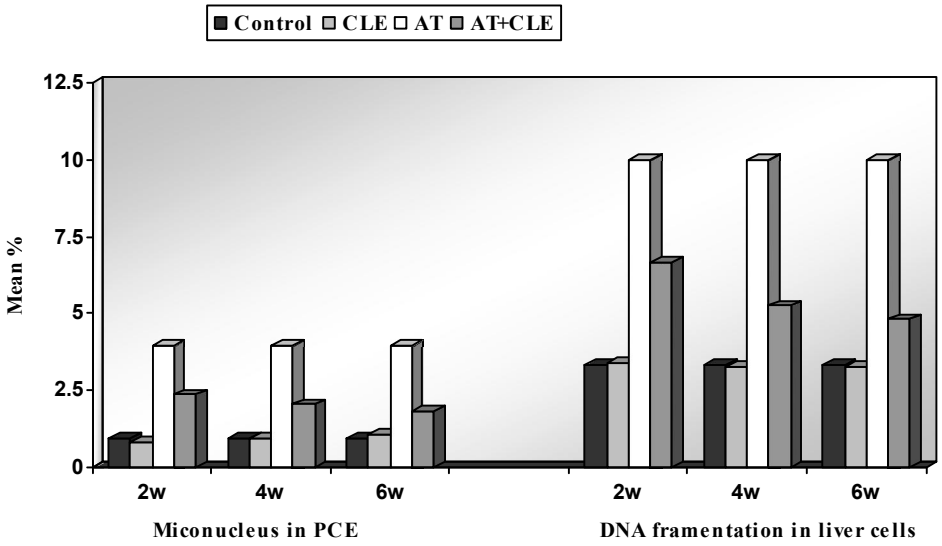

Fig (2): Mean percentage of micronucleus in PCE and DNA fragmentation in liver cells after treatment with CLE for 2, 4 and 6 weeks plus AT

3.3.Biochemical analysis

Table (1) showed the effect of atrazine and atrazine plus camphor on the levels of glutathione reduced (mg/dL) and lipid peroxide (nmol/ml, malonaldehyde) enzymes. There is a significant decrease in glutathione reduced is observed in mice treated with atrazine. Administrations of camphor regulate the decrease of glutathione reduced treated mice. Also, atrazine increased significantly lipid peroxide compared to control. Levels of lipid peroxide inhibit significantly in groups of atrazine treated with camphor.

4. Discussion

Atrazine is a selective triazine herbicide used to control broadleaf and grassy weeds in corn, sorghum, sugarcane, pineapple, and other crops, and in conifer reforestation plantings **(Kiely et al., 2004)**. Results of studies over the past two decades showed that atrazine is one of the most frequently detected pesticides in agricultural streams and rivers **(Comber, 1999; Vryzas et al., 2011).**

Our results showed that AT had the ability to induce significant ($P < 0.01$) elevation in the percentage of micronuclei, chromosomal aberrations in somatic and germ cells and DNA fragmentation ($P < 0.01$). These observations support the previous studies about the genotoxicity of AT **(Tennant et al. 2001; Liu et al. 2006; Singh et al., 2008)**.

For biochemical studies AT induced inhibition in the levels of testosterone hormone and reduced glutathione enzyme. Also; it elevates the level of lipid peroxide enzyme. AT cause endocrine disruption, **Orton et al., (2009).** It may act as an inducer of the enzyme aromatase, which converts androgens to estrogens. Also, it can alter hepatic metabolism, induce estrogenic effects and oxidative stress **(Salaberria et al., 2009).**

Table (1) Mean levels of glutathione reduced (mg/dL) and lipid peroxide (nmol/ml, malonaldehyde)

Treatments	Glutathione reduced	Lipid peroxide
control	12.04±0.73^{b}	22.79 ±2.01^{a}
atrazine	8.43±1.12^{a}	41.52±15.78^{b}
2 weeks camphor	15.57±3.78^{b}	23.58±1.03^{a}
2weeks camphor +atrazine	15.55 ±1.79^{b}	22.44±4.03^{a}
4 weeks camphor	19.39±1.79^{c}	25.89±2.25^{a}
4 weeks camphor +atrazine	14.18±5.88^{b}	26.01±3.9^{a}
6 weeks camphor	21.88±3.99^{c}	19.15±3.09^{a}
6 weeks camphor +atrazine	11.67±6.39^{ab}	16.69±0.69^{a}

Means with different superscripts are significantly different at P<0.05, * P=0.02

Exposure to low dose of AT (12.5 mg/kg/day) could delay the onset of puberty in juvenile male rats

and the higher doses significantly decreased serum and testicular testosterone **(Stoker et al., 2000; Trentacoste et al., 2001).** It was believed that AT and many other pesticides owe their mutagenic effect due to their conversion to nitrosamines **(Weisenburger et al., 1987)** or due to increase in oxidative stress and enhanced formation of free radicals **(Singh et al., 2008)**.

Cinnamomum camphora is an active, oriental herbal medicine used in various diseases **(Choi, 1997)**. In spite of its famous legacy, the pharmacological effects have not been fully explored from the antigenotoxical view point. Therefore, in this study we investigate the modulatory effect of CLE on AT-induced genotoxicity in mice. The results showed a significant and time-dependent decrease in the percentage of micronuclei, chromosomal aberrations and DNA damage in all tested tissues as compared to AT treated mice.

Cinnamomum camphora contains alkaloids and essential oil, such as camphor **(Miyazawa et al., 2001)**. **Goel et al., (1989)** reported that the radiation-induced SCE's frequency was significantly low after a single dose of camphor (0.5 μM/g b.w.) administered 30, 45 or 60 min before irradiation in mice, and that the effect was enhanced with increasing time intervals.

Eugenol is the main constituent (i.e, about 81-85%) of *Cinnamomum* leaf oil **(Mallavarapu et al., 1995).** Eugenol, a major phenolic component has been widely used in medical practice, due to its potent fungicidal, bactericidal, anesthetic, antioxidant and anti-inflammatory properties **(He et al., 2007)**. Eugenol ameliorates gamma radiation induced clastogenic effects **(Tiku et al., 2004)** and genotoxin-induced DNA damage **(Abraham, 2001)** *in vivo*. Eugenol pretreatment prevented DNA strand break and improves the antioxidant status in thioacetamide treated rats **(Yogalakshmi et al., 2010)**. It is also reported to induce the detoxifying enzymes namely glutathione-S-transferase in rat liver **(Yokota et al., 1988)**.

Recently, **Ho et al., (2008)** reported that *Cinnamomum* essential oil effectively protected certain biomolecules from peroxynitrite-mediated nitrating and oxidizing damage**.** The chemical constituents of the essential oils from *Cinnamomum* are monoterpenes, sesquiterpenes, and related oxygen derivatives of these two types of compounds. The major monoterpene are α-pinene, camphene, and limonene **(Miyazawa et al., 2001).**It is well documented that many monoterpenes from essential oils such as limonene and geraniol inhibit isoprenylation of proteins **(Crowell, 1999)**. Moreover, ras-oncogene induced carcinomas have been prevented by limonene, geraniol and 20-benzyloxycinnamaldehyde which are a constituent of *Cinnamomum* oil **(Gould et al., 1994; Carnesecchi et al., 2004; Moon et al., 2006)**. **Unlu et al., (2010)** concluded that *Cinnamomum* oil and its constituents may find application as an anticancer agent.

For biochemical analysis CLE showed the ability to regulate reduced glutathione and lipid peroxide enzymes affected by AT. **Banerjee et al., (1995) and Durgo et al., (2007)** reported that CLE may modulate the metabolism of mutagenic and/or carcinogenic chemicals via its modifying influence on endogenous-reduced glutathione concentrations and induction of liver carcinogen metabolising enzyme systems and this may be attributed to its inherent flavonoid and camphor content. Glutathione offers protection against oxygen-derived free radicals, and cellular lethality following exposure to an array of anti-cancer drugs or ionizing radiation **(Durgo et al., 2007)**. In addition, it can scavenge the electrophilic moiety involved in cancer initiation processes by conjugation reactions into water-soluble reduction products **(Coles and Ketterer, 1990; Orrneius and Moldeus, 1984)**. Also, camphor causes an increase in phase I as well as phase II enzyme system activities. The latter enzyme system promotes the rapid conversion of highly reactive metabolites into nontoxic conjugates.

Finally, we can conclude that CLE is a protective agent against AT induced genotoxicity and biochemical disturbances. Supplementation of CLE might be beneficial to AT exposed population and is effective modulator of chemical carcinogenesis. To our knowledge, this is the first report to describe the antigenotoxic function of CLE and further studies are needed to elucidate the detailed mechanism of action of CLE.

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