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Full Length Research Paper

Effect of *Artemisia alba* L. extract against ethinylestradiol induced genotoxic damage in cultured human lymphocytes

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In the present study, the antigenotoxic effect of *Artemisia herba-alba* was evaluated against the genotoxic effect induced by 10 µM of ethinylestradiol in the presence of metabolic activation using mitotic index (MI), chromosomal aberrations, sister chromatid exchanges and replication index (RI) as parameters. The treatment of 10 µM of ethinylestradiol along with 102, 212.5, 315 and 417µg/mL of *Artemisia alba* extract in culture medium resulted in a significant dose dependent decrease in the genotoxic effects induced by the treatment of 10 µM of ethinylestradiol. The results of the present study suggest that the plant extract *per se* does not have genotoxic potential, but can modulate the genotoxicity of ethinylestradiol in cultured human lymphocytes.

Key words: *Artemisia alba*, Methyltestosterone, ethinylestradiol, human lymphocytes, genotoxicity, natural products, chromosomal aberrations, sister chromatid exchanges.

INTRODUCTION

Artemisia herba alba (Composate) is commonly known by the Arabic name 'sheh' and is a popular folk remedy for the treatment of falling hair (crushed and applied to hair), chest, stomach, muscular pains (fumigations), cough, diarrhea, fever, poisoning (to drink and for irrigation), vomit, lungs, flatulence. For this purpose, the native use a hot water decoction made from the fresh leaves and branch lets. *A. herba alba* used by local population of some middle east countries as an antidiabetic activity (Iriadam, 2006), is also used as an antihelminitic (Khafagy et al., 1971). A literature survey revealed that certain species of Artemisia also showed antimalarial (Haynes, 2006), antibacterial (Kordali et al., 2005), insecticidal (Saadali et al., 2001) and antifertility effect (Motasem et al., 2007).

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Abbreviations: MI, Mitotic index; **SCE,** sister-chromatid exchanges; **RI,** replication index.

Phytochemical investigation of *A. herba alba* have shown that it contains santonin (Khafagy et al., 1971), sequiterpene lactones (Foglio et al., 2002) and flavonoids (stermitz et al., 2002). Components of the essential oil have also been investigated (Saleh et al., 2006).

Estrogens are used for treating many types of sexual disorders and as part of various oral contraceptive formulations (Schwend and Lippman, 1996). There is sufficient evidence for estrogen carcinogenicity and genotoxicity in various experimental models (IARC, 1979). Ethinylestradiol is commonly used in oral contraceptives and in other drug formulations (Siddique et al., 2010). The prolonged use of oral contraceptives has been reported to induce various types of cancers (IARC, 1979). There are also reports of the genotoxicity of ethinylestradiol in various experimental models (Siddique et al., 2005, 2008; Hundal et al., 2008; Drevon et al., 1981; Shyama and Rahiman, 1996).

In the present study, the effect of a leaf extract of *A. herba alba* on the ethinylestradiol induced genotoxicity in the presence of metabolic activation in cultured human peripheral blood lymphocytes using mitotic index (MI), chromosomal aberrations, sister chromatid exchanges

and replication index (RI) as parameters were evaluated.

MATERIALS AND METHODS

Chemicals

Ethinylestradiol (CAS: 57-63-6; Sigma); S9 mix from rat liver, Sprague-Dawley (Sigma); RPMI 1640, fetal calf serum, Phytohaemaglutinin-M (*In vitrogen*); dimethylsulphoxide, colchicine, 5-bromo-2-deoxyuridine and methanol.

Preparation of plant extract

Aerial parts of *A. herba alba* plants were collected from Mafraq area (East-north of Jordan) during summer of 2010. The aerial parts were dried and grinded into powder. Extraction was performed by soaking samples (30 gm of dry weight) in 300 ml of acetone for 8 to 10 h at 40 to 60°C in a Soxhlet apparatus. After filtration, the excess solvent was removed by rotatory evaporator. The extract concentrations of 107.5, 212.7, 315 and 417 µg/mL of culture medium were established (Siddique et al., 2008).

Human lymphocyte culture

Heparinized blood samples were collected from 10 healthy donors (five males and five females, non-smokers, with age range 20 to 25). Whole blood for each samples (0.5 ml) was added to 5 mL of culture medium (pH 6.8 to 7.0), supplemented with 10% fetal calf serum, 10% antibiotic-antimycotic mixture and 1% phyto-haemaglutinin of the final volume of cell culture (Carballo et al., 1993). The culture tubes were then placed in the incubator at 37°C for 24 h.

Chromosomal aberrations analysis

Following 24 h of incubation, about 10 µM of ethinylestradiol [dissolved in dimethyl sulfoxide (DMSO), 5 µl/mL] was given along with 107.5, 212.7, 315 and 417 µg/mL of *A. herba alba* extract, supplemented with the 0.5 mL of S9 mix. The cells were incubated with the S9 mix for 6 h. The cells were then collected by centrifugation and washed in the pre-warmed medium to remove the excess traces of S9 mix and added drugs and were further incubated for the remaining 42 h. Treatment of 0.2 mL of colchicine (0.2 µg/mL) was given to the culture tubes, 1 h prior to harvesting. Cells were then centrifuged at 1000 rpm for 10 min. The supernatant was removed and 8 mL of pre-warmed (37°C) 0.075 M KCl (hypotonic solution) was added and the cells were then re-suspended and incubated at 37°C for 15 min. The supernatant was removed after centrifugation at 1000 rpm for 10 min, and subsequently 5 ml of chilled fixative was added. The fixative was removed by centrifugation and the procedure was repeated twice. To prepare slides, three to five drops of the fixed cell suspension were dropped on a clean slide and air dried. The slides were then stained in a Giemsa solution in phosphate buffer (pH 6.8) for 15 min. The slides were coded before scoring and approximately 50 metaphases were scored for chromosomal aberrations according to the recommendations of EHC 51 for short term tests for mutagenic and carcinogenic chemicals (IPCS, 1985).

Sister chromatid exchange analysis

To study sister chromatid exchange analysis, bromodeoxyuridine (BrdU, 10 µg/mL) was added at the beginning of the culture. After 24 h of incubation, 10 µM of ethinylestradiol (dissolved in DMSO, 5 µl/mL) was given along with 107.5, 212.7, 315 and 417 µg/mL of *A. herba alba* extract, supplemented with the 0.5 ml of S9 mix for 6 h. The cells were then collected by centrifugation and washed in the pre warmed medium to remove the traces of S9 mix and drugs. Mitotic arrest was subsequently triggered by the addition of 0.2 mL of colchicine (0.2 µg/mL). Hypotonic treatment and fixation were performed in the same manner as previously described for chromosomal aberration analysis. The slides were coded before scoring and the sister chromatid exchange average was calculated from an analysis of metaphases during the second cycle of division (Perry and Wolff, 1974). A total of 25 well spread and complete (2n = 46) second division metaphases were scored for sister-chromatid exchange (SCE). The frequency of SCE/chromosome was recorded according to Carrano and Natarajan (1988).

Mitotic index and replication index

The MI was calculated as the number of metaphases in 1500 cells analysed per culture for each dose group and donor. A total of 100 metaphases per culture for each dose group and donor were scored to calculate the RI. Metaphase divisions were detected by the BrdU-Harlequin technique for differential staining of metaphase chromosomes (Crossen and Morgan, 1977; Tice et al., 1976). The RI, indirect measure of studying cell cycle progression was calculated by applying the following formula:

$RI = M_1 + 2M_2 + 3M_3/100$

Where, M1, M2 and M3 denote the number of metaphases in the first, second and third cycle, respectively.

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) and to study the dose response, the regression analysis was also performed by using the commercial software program Stat Soft Inc (2007).

RESULTS

The treatment of 10 µM of ethinylestradiol in the presence of S9 mix reduced the MI significantly as compared to the untreated. A significant dose dependent increase in the MI was observed when 10 µM of ethinylestradiol treatment was given along with the different doses of *A. herba alba* extract, that is, 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL. Ethinyl-estradiol induced a significant increase in chromosomal aberrations per cell as compared to untreated in the presence of S9 mix. A significant dose dependent decrease in the chromosomal aberration per cell was observed when 10 µM of ethinylestradiol treatment was given along with the different doses of *A. herba alba* extract, that is, 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL. A significant increase in SCE/ chromo-some was observed at 10 µM of ethinylestradiol as compared to untreated. A significant decrease in sister chromatid exchanges per chromosome was observed when 10 µM of ethinylestradiol treatment was given along with the different doses of extract of *A. herba alba*, that is, 1.075×10^{-4}, 2.125×10^{-4}, 3.15×10^{-4} and 4.17×10^{-4} g/mL

Table 1. Effects of *Artemisia alba* on sister chromatid exchanges and replication index by ethinylestradiol.

Treatment	SCE/Chromosomes ± SE	RI ± SE
EE (M)		
10	0.26 ±0.079[a]	160 ± 0.37 [a]
EE(M) + EAE(g/ml)		
10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}
10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}
10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}
10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}	10 +1.075 x 10^{-4}
EAE (g/ml)		
1.075 x 10^{-4}	0.114± 0.093 [b]	1.93 ± 0.17 [a]
1.075 x 10	0.104± 0.013 [b]	1.91 ± 0.36 [a]
1.075 x 10	0.124± 0.049 [b]	1.88 ± 0.49 [a]
1.075 x 10	0.135± 0.046 [b]	1.84 ± 0.64 [a]
UNTREATED	0.105± 0.013 [b]	1.96 ± 0.77 [a]
Negative control	0.118± 0.013 [b]	1.94 ± 0.33 [a]
Positive control	0.54± 0.013 [b]	1.32 ± 0.53 [a]

A total of 250 cells were scored for the sister chromatid exchange analysis and 100 cells were scored for replication index; [a]P<0.005 significantly different from the untreated; [b]P<0.005 significantly different from the ethinylestradiol; EE, ethinylestradiol; EAE, *Artemisia alba* L.extract.

Table 2. Summary of regression analysis for the dose effects of *Artemisia alba* L. on mitotic index, chromosomal aberrations, sister chromatid exchanges and replication index after the treatment along with 10 µM of ethinylestradiol.

S/N	Parameter	Regression equation	Beta	Standard error	F	P
1	Mitotic index	Y=3.664+0.09496X	0.930	0.78	11.39	<0.0005
2	chromosomal aberrations	Y=0.10216-0.0149X	-0.980	0.003	36.21	<0.0047
3	Sister chromatid exchanges	Y=0.21699-0.0162X	-0.990	0.008	94.57	<0.0005
4	Replication index	Y=1.6566+0.02930	0.995	0.009	70.60	<0.00001

(Table 1). A significant decrease in the RI was observed at the treatment of 10 µM of ethinylestradiol as compared to the untreated (Table 1). A significant dose dependent increase in the RI was observed at each of the concentrations of *A. herba alba* extract (Table 1). Regression analysis was also performed to determine the dose effects of *A. herba alba* extract on 10 µM of ethinylestradiol, for MI, chromosomal aberrations, sister chromatid exchanges and RI (Table 2). For MI, the treatments of various doses of *A. herba alba* extract were associated with an r value of 0.930 (Table 2). For chromosomal aberrations per cell the treatments of various doses of *A. herba alba* extract were associated with an r value of -0.98 (Table 1). For SCE per cell the treatments of various doses of *A. herba alba* extract were associated with an r value of -0.99 (Table 2). For RI, the treatments of various doses of *A. herba alba* extract were associated with an r value of 0.995 (Table 1). A decrease in the slope of linear regression lines for chromosomal aberration (F=35.84; *P<0.0047*) and sister chromatid exchange (F=94.57; *P<0.0005*) was observed as the dose of the *A. herba alba* extract increases. An increase in the slope of linear regression lines for MI (F=11.39; *P<0.0005*) and RI (F=69.89; *P<0.00001*) was observed as the dose of the *A. herba alba* extract increases.

DISCUSSION

The results of the present study reveal that the extract of *A. herba alba* is potent enough to reduce the genotoxic effects of ethinylestradiol at all the selected doses. The selected doses of *A. herba alba* extract were not genotoxic. Our previous study with ethinylestradiol has

shown that the metabolic activation and possible conversion of ethinylestradiol to a reactive species is responsible for the genotoxicity (Siddique et al., 2005). Medicinal plants and their products have been used for centuries to cure various ailments (Asolkar et al., 1992). Many plant products protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes (Morse and Stoner, 1993). The herb *A. herba alba* contains mainly coumestans, that is, wedelolactone (I) and demethylwedelolactone (II), polypeptides, poly-acetylenes, thiophene-derivatives, steroids, triterpenes and flavonoids (Bicoff, 1993). The wedelolactones are reported to possess a wide range of biological activities (Roncada et al., 2004). The verification of the possible mutagenic and/or antimutagenic effects of medicinal plants, infusions/extracts is an important factor in studies. Some plants may possess substances that can modulate the genotoxicity of the other compounds (Roncada et al., 2004).

The data obtained in the present study suggest that the compounds present in the extract of *A. herba alba* are not mutagenic. The protective effect of *A. herba alba* extract in the present study, that is, significant reduction in chromosomal aberrations and sister chromatid exchanges or increase in the MI and RI may be due to the direct action of the compounds present in the extract of *A. herba alba* on ethinylestradiol by inactivating it enzymatically or chemically. Our earlier studies with natural plant products and steroid toxicity are also encouraging (Siddique and Afzal, 2005). The compounds present in the extract may also scavenge electrophiles/ nucleophiles (Maurich et al., 2004). They may also enhance the DNA repair system or DNA synthesis or even may prevent the bio-activation of certain chemicals (Kuroda et al., 1992). The antigenotoxic potential of plant extracts have been attributed to their total phenolic content (Maurich et al., 2004). Medicinal herbs contain complex mixtures of thousands of compounds that can exert their antioxidant and free radical scavenging effect either separately or in synergistic ways (Romero-Jimenez et al., 2005). Identification and characterization of these active principles in the plant extract may lead to strategies to reduce the risk for developing cancer in humans (Dearfield et al., 2002). A study on oral contraceptives and liver cancer has revealed that the oral contraceptives may enhance the risk of liver carcinomas (Heinemann et al., 1997). The present study shows that the extract of *A. herba alba* reduced the genotoxic effects of ethinylestradiol, and hence suggests the possibility of having lower risk of carcinomas in the patients under-going ethinylestradiol therapy.

Flavonoids present in the extract may acts as a blocking agent, thus preventing the metabolic activation of promutagens. They can also form adducts or scavenge free radicals, thus preventing tumor formation (Noel et al., 2006). The compounds present in the extract may act synergistically, as compared to an isolated compound and this supports the indigenous system of medicine namely, Ayurvedic, Siddha and Unani that have been in existence for centuries (Chitravadivu et al., 2009). The identification and characterization of the compounds present in the *A. herba alba* extract to determine their particular function will be part of our future study.

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