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Original Article

Anti-mutagenic protection of *Quercus infectoria* galls against 2-Aminoanthracene induced genotoxicity in mice bone marrow

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ABSTRACT

The present study evaluated the mutagenic and antimutagenic protective effects of *Quercus infectoria* galls (QIG) against genotoxicity induced by 2-Aminoanthracene (2AA). Mice bone marrow mitotic index, chromosomal aberration, and micronucleus assay were employed to measure the genotoxicity, respectively. Mice were treated with aqueous extract of QIG (2, 5 and 7 gm/kg body weight) for 7 days. Without the dose of 2AA, no mutagenic effects were observed in bone marrow samples of the mice. QIG extract by itself did not show any harmful effects but it significantly improves mitotic activity in bone marrow cells at the dose 2 gm/kg body weight. Pre-, post- and simultaneous treatments with 2AA were carried out *in vivo*. It was observed that the dose 2gm/kg of QIG administrated 7 days before injection of 2AA (at the dose of 750 mg/kg) caused the most statistically significant decrease of 2AA induced chromosome aberration, micronuclei frequency and stimulate cell proliferation in bone marrow cells of mice as compared to 2AA group. Showing antimutagenic protective effects on 2AA induced mutagenicity in mice. Thus, it can be concluded from the study that aqueous extract of QIG exhibited no clastogenic effects but only possessing antimutagenic effects. This antimutagenic activity is an induction of its medicinal relevance.

Keywords: Antimutagens; Chromosomal aberration; Micronucleus assay; Mitotic index; *Quercus infectoria* galls; Mice bone marrow.

INTRODUCTION

Nowadays, a large number of anti-mutagens of plants origins are under investigation for the discovery of novel pharmacologically active compounds [1].Many natural bioactive chemicals with genoprotective activity are known to protect cellular components from genotoxic damage and prevent cancer and other diseases related to the incidence of mutations, such as atherosclerosis, emphysema, cardiomyopathies, neurodegenerative diseases, which are the leading causes of death in the human population [2]. The great interest primarily focused on the detection of anti-risk factors for humans in a variety of test systems [3]. These genoprotective substances commonly act as natural antimutagens or anticarcinogenes ,its mechanisms of antimutagencity have been classified into two major processes one is desmutagenesis: In which factors act directly on mutagens or inactivate them, by blocking or scavenging the final electrophile moieties in a nucleophilic reaction, generating innocuous products; in this manner, the exposure to these substances would protect against DNA damage ,the other is bio-anti-mutagenesis in which factors act on the processes of mutagenesis or repair DNA damages that result in a decrease in the mutation frequency [4] .

However, anti-mutagenesis is considered as one of the most feasible ways for inhibiting the negative effects of environmental genotoxicants including carcinogens. Furthermore, It was reported previously that several antitumor compounds act through the antimutagenic mechanism. Hence, searching for antimutagenic compounds represents a rapidly expanding field of cancer research [5]**.** Plants have been the source of several effective drugs for the treatment of cancer and over 60% of anticancer drugs originate from natural sources [6]. Therefore, extract of the *Quercus infectoria* galls, were investigated regarding their anti-mutagenic activities. *Quercus infectoria* is an oak tree of the family Fagaceae in the Mediterranean area, especially in Greece, Syria, Iraq and Asia Minor

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[7]. Its galls are round-shaped abnormal growth found arising on the young branches of the oak tree due to the attack by female gasp-wasp Adleria gallae-tinctoria and Cynips gallae tinctoria by deposition of the eggs [8].

Quercus infectoria, one of the popular medicinal plants used traditionally as an astringent, a moisture eliminator, an anti-inflammatory agent (i.e., to treat erysipelas), an antiseptic and an antidiarrheal agent, also in the treatment of intestinal dysmotility, Ulcerative Colitis (UC) as an enema, dysentery, functional enteritis, hemorrhagic sores, alopecia areata, dental caries, periodontitis, halitosis, pharyngolaryngitis and tympanitis [9] .The galls of *Q. infectoria* were documented to possess antibacterial [10,11], antiviral [12], antifungus [13], larvacidal [14,15] and antioxidant activity [16]. The main phytochemicals found in QI galls are tannin (50–70%), gallic acid (2–4%), ellagic acid, starch, and sugar [17].The pharmacological properties of water and ethanol extracts of QI gall extract are reportedly due to the presence of substantial amounts of phenolic compounds [18], Tannins (gallotannic acid), which is a phenolic compound, Gallotannic acid a tannic acid is known for its anti-mutagenic, anticancer and antioxidant properties [19]. We are unable to find any information on the antimutagenic properties of *Quercus infectoria* galls , Hence the main goal of the current study was directed to investigate the antimutagenic activity of QIG by moderating the genetic damage induced by 2-Aminoanthracene *in vivo*. For this goal, mice bone marrow cells were used, and Mitotic index, Chromosomal aberration, Micronucleus assays were applied.

MATERAILS AND METHODS

Chemicals: 2-Aminoanthracene (2AA) was purchased from Sigma Chemical Co. 2AA was first dissolved in 2% DMSO before use and diluted in corn oil in order to treat the mice with a final concentration of *750* mg/kg body weight (b.w.), injecting intraperitoneally (i.p.) .All other chemicals were of the highest analytical grades which were commercially available.

Plant material: *Quercus infectoria olivier* galls (Figure. 1) were collected locally from Qalasnj village on Safeen Mountain in Erbil governorate in September and December 2015 and authenticated by Professor Saleem Shahbaz, Taxonomist, University of Duhok . The galls were washed with distilled water, left dry at room temperature before they were crushed and ground prior to the extraction.

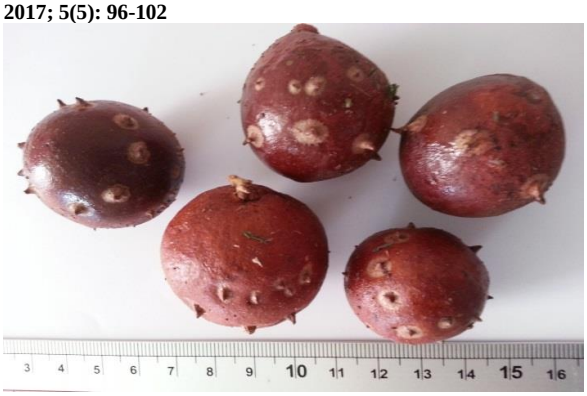

Fig (1): Gall of *Quercus infectoria* Olivier collected from Erbil governorate, Kurdistan region of Iraq.

Preparation of the QIG aqueous extract: The galls were crushed to small pieces using sterile pestle and mortar and powdered in an electric grinder. 100 grams of a pale yellow powder were soaked in 500 ml of sterile distilled water for 72 h. After this, the aqueous extract was brought to a boil point for30-minute period. The extract was filtered through a Buchner funnel using Whatman No.1 filter paper , The filtrate was concentrated to semi dryness under reduced pressure and controlled temperature (40 - 60 °C) using rotary evaporator, light brown colored residue was obtained, which was incubated in a clean Petri dish overnight at (37°C) to obtain about 25% yield of crude extract. The extract was stored at (-4°C) in air tight glass bottles and used during 1-5 days. Different QIG extract concentrations were freshly dissolved in physiological saline solution and given to Swiss albino mice by oral gavage.

Experimental animals: A total number of a hundred apparently healthy adult laboratory male Swiss albino mice (*Mus musculus*) Bulb/c strain of approximately 25 g bw were used in this study. These animals were obtained from the animal house of Science College, University of Duhok, Duhok, Iraq. The animals were housed in plastic cages, five per cage, and maintained on standard laboratory diet and water *ad libitum*.

Experimental design: Animals were divided into two experiments. The first experiment had twenty-five animals and was divided into five equal groups, each group 5 mice. Animals of the first group were orally treated by gavages with phosphate buffer saline (PBS) daily for seven days and used as negative control, animals of the second group, the positive control, were injected intraperitoneally with 2AA (750 mg/kg/day, halve of LD50), While Animals of the group three, four and fifth were treated with oral dose of QIG extract (2,5,7 gm/kg bw/day) for 7 consecutive days .

The second experiment had twenty-five animals, divided into five equal groups, each group five mice as follows: the animals of first and second groups were treated as those in the first experiment while the other groups distribution was as follow: group 3: (Pre-2AA treatment) animals administrated with QIG extract at concentration of 2gm/Kg bw for 7 days before injection with 2AA at concentration (750mg/kg) injected intraperitoneally at eighth day. Group 4: (simultaneous treatment) Animals treated with 2-AA 750 mg/kg/day combined with QIG extract (2gm/kg). Group 5: (Post-2AA treatment) animals administrated 2AA at concentration (750mg/kg) by injected intraperitoneally once (1 day) then, administrated animals with QIG extract at concentration 2gm/Kg for 7 days. However, all animals were sacrificed by cervical dislocation and samples of bone marrow were taken for cytogenetic analysis (MI and CA).The same experimental design was fallowed to calculate the number of polychromatous erythrocytes for the presence of micronuclei (MN).

Cytogenetic analysis:

A - Chromosomal aberration (CA) assay: Mice were injected intraperitoneal with 0.5 ml of 0.06% colchicine and three hours later were sacrificed by cervical dislocation. Both the femurs were fleshed out from the muscles .The femurs were then rinsed with 5 ml 0.075M KCl solution in a centrifuge tube. The tube was then incubated at 37°C for 20 minutes. After incubation, centrifugation at 1000 rpm for 10 minutes was carried out. Supernatant was discarded and fresh Carnoy's fixative was added (3:1 methanol: acetic acid). (The process of centrifugation was repeated three times. Chromosome slides were prepared by dropping the cell suspension onto cleaned slides, which were flame dried and all slides were coded and stained in dilute Giemsa solution. The microscopic observations, performed with a magnification of 100× oil immersion. Hundred well spread metaphase were scored per animal (500 metaphase per treatment group) at random [20].

B- Mitotic index (MI)assay: The slides were examined under light microscope with (40x) power, and 1000 of the divided and non-divided cells were counted and the percentage rate was calculated for only the divided ones according to this equation : M.I. %=No. of dividing cells in metaphase / {Total No.of dividing cells +No. of non- dividing cells (1000) cells} X 100 according to Becker [21].

C- Micronucleus test in bone marrow cells (MN) :

Micronucleus test were analyzed according to [22] All the animals were sacrificed and bone-marrow cells from both femur of each animal were flushed with bovine serum albumin. The obtained cell suspension was centrifuged (1000×g, 5 min), the supernatant was removed and the pellet re-suspended in bovine serum albumin. Then, a drop of the suspension was smeared on a clean slide, air-dried, fixed in methanol for 5 min then stained with Giemsa, microscopic examination with a magnification of 100X. About 1000 micronucleated polychromatic erythrocyte (MN-PCE) and polychromatic erythrocyte (PCE) were scored for the presence of micronuclei for each animal. The micronuclei frequency quantification was performed 24h after treatment.

Statistical Analysis: Statistical significance between the different groups was determined using one way analysis of variance (ANOVA) followed by Dunnett test. The results were considered statistically significant if p-values were less than 0.05.

RESULTS AND DISCUSSION

Mitotic index: The relationship between cell cycle progression and inhibition of cell proliferation was examined by determining the mitotic index. From the present study, it was found that a significant decrease in mitotic index of 2AA treated animals compared to the negative control group ($P > 0.05$), which can be due to the affected cell division in the bone marrow of mice, while the treatment with the extract at the dose (2 gm/kg b.w.) given a significant improvement in mitotic activity in bone marrow cells. From the result of Table (1), we concluded that QIG extract doesn't possess genotoxicity.

Chromosome aberration assay: The mammalian bone marrow chromosomal aberration assay can detect clastogenic or aneugenic effects of a test agent. Chromosomal aberrations occur because of lesions in the DNA that lead to discontinuities in the DNA double helix. The primary lesions, which include single and double strand breaks, base damage, DNA-DNA and DNA-protein cross links, alkylations at base or phosphate groups, intercalations, thymine dimers, apurinic and apyrimidinic sites, are recognized by DNA-repair processes. Therefore, the lesions may be corrected or transformed, to restitute the original base sequence or produce chromosomal aberrations and gene mutations [23] .The genotoxicity of three doses (2, 5, and 7 gm/kg b.w.) of the aqueous extracts of QI galls was analyzed and the results obtained were compared with that of solvent control group (Pbs). As shown in Table (1) all three extracts at the doses mentioned; had no significant effect on inducing chromosome aberrations in Swiss albino mice ($P > 0.05$). There was significant

increase in total no. of chromosome aberrations; rings, breaks, dicentric, deletions and fragmentation of 2AA treated animals (Figure .2) when compared with normal control animals. 2-Aminoanthracene gets metabolized to produce highly reactive O-substituted N-hydroxylamine intermediates, before it can act as a mutagenic agent to promote chromosome aberrations. Aberrations are due to DNA-adducts caused by 2-Aminoanthracene [24].

Table (1): Cytogenetic effects of different concentration of *Quercus infectoria* galls (QIG) extract in comparison with negative (PBS) and positive (2AA) controls on mouse bone marrow cells (*in vivo*).

Groups	Chromatid aberrations		Chromosome aberrations (ch.ab)						Total ch.ab	Mean MI%	Mean MN%
	gap	break	gap	break	del	ring	dc	frag			
Negative control	8.4 b	15.6 b	7.4 b	17.0 b	11.2 b	11.2 b	6.8 b	6.8 b	84.4 b	8.62 b	3.26 b
Positive control	39.8 a	55.6 a	50.4 a	40.8 a	70.8 a	81.8 a	78.2 a	19 a	455.2 a	3.82 c	26.0 a
QIG 2gm/kg	8.0 b	12.4 b	7.4 b	16.0 b	12.0 b	12.0 b	7.5 b	7.5 b	82.8 b	10.96 a	2.48 b
QIG 5gm/kg	7.4 b	14.0 b	7.7 b	18.0 b	9.8 b	12.0 b	7.0 b	8.0 b	83.9 b	9.46 b	2.50 b
QIG 7gm/kg	8.2 b	15.0 b	8.0 b	16.2 b	11.0 b	11.0 b	7.7 b	8.0 b	85.1 b	9.32 b	2.52 b

Different letters within each column differ significantly (P<0.05) according to Dunnett test. Abbreviation: del, deletion; dc, dicentric; frag, fragmentation.

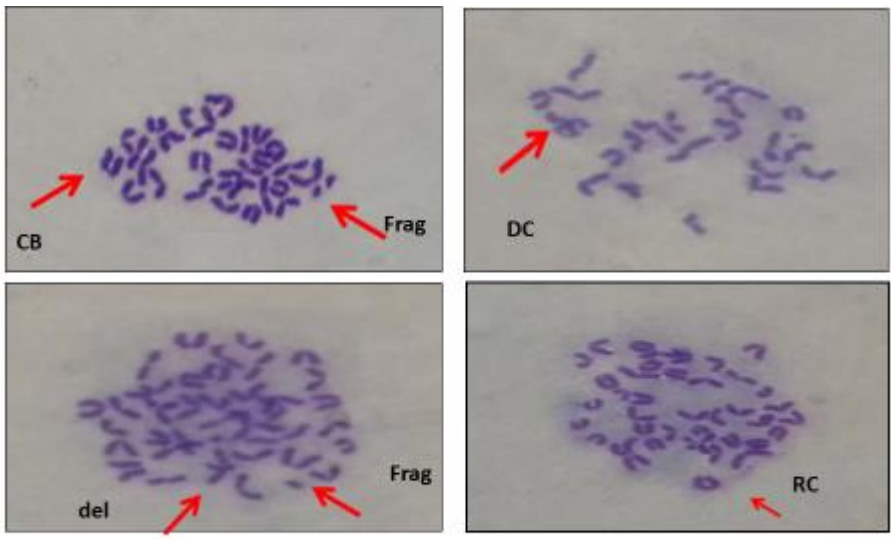

Figure (2) : Representative of different types of structural chromosome aberrations (chromatid break and gap -CB,Ring chromosome-RC,Dicentric chromosome-DC, Fragmentation –Frag, deletion –del)induced by 2AA in bone marrow cells of albino mice .

Micronucleus test in bone marrow cells: The micronucleus assay can be considered as a powerful tool to study both clastogenic and aneugenic effects of any toxicants .As micronuclei derive from chromosomal fragments and whole chromosomes lagging behind in anaphase and left outside the daughter nuclei in telophase [25;26]. After telophase the undamaged chromosomes, as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are included

in the daughter cells, too, but a considerable proportion is transformed into one or several secondary nuclei which are, as a rule, much smaller than the principal nucleus and are therefore called micronuclei [27;28] .Some micronuclei may be originated from fragments derived from broken anaphase bridges formed due to chromosome rearrangements such as dicentric chromatids, ring chromosomes or union of sister chromatids [29]. Treatment with three doses (2, 5, and 7 gm/kg b.w.) of the aqueous extracts of QIG had no significant effect on inducing micronuclei in Swiss albino mice ($P > 0.05$) as illustrated in Table (1). While the mutagenicity of 2-Aminoanthracene (2AA) was further confirmed from the results of micronucleus test (Figure .3).

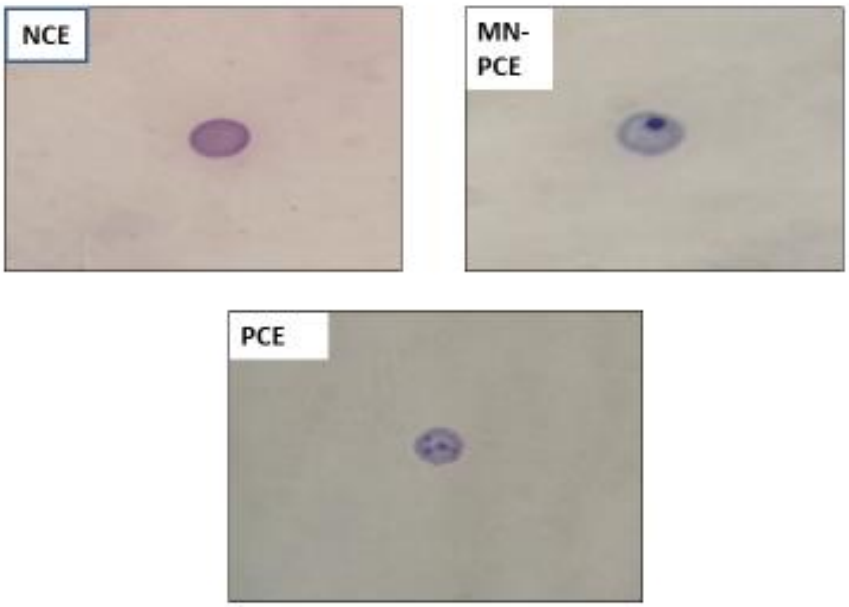

Figure (3) : Showing normal chromatic erythrocyte(NCE),micro nucleated polychromatic erythrocyte (MN-PCE)and polychromatic erythrocyte (PCE).

Antimutagenicity of QIG extracts: Our results demonstrate that QIG at the chosen dose (2 gm/kg b.w.) was able to significantly protect ($p < 0.05$) DNA from the mutagen studied when compared with positive control (2AA) as shown in Table (2).The Pretreatment with QIG exhibited the excellent inhibitory activity against the 2AA-induced chromosomal aberrations and give the most significant improvement in mitotic activity in bone marrow cells, the improvement in mitotic activity of bone marrow cells of animals treated with QIG may focus attention on the beneficial effect of QIG to overcome one of the most serious problems in cancer chemotherapy, which is the bone marrow suppression. Moreover the Pretreatment of mice with the same dose of QIG extracts showed the most significant reduction in the frequency of micronuclei compared with that of 2AA alone-treated group ($p < 0.05$) .The observed decrease in the incidence of Mn PCEs can be considered to indicate an inhibitory effect of QIG extract. Either 2AA Simultaneous or post-treatment had less significant effect on the frequency of 2AA induced cell proliferation, total chromosome anomalies and micronuclei (Table .2). In fact post treatment showed cell proliferation frequency similar to that found in positive control group.

Table (2): Interaction between *Quercus infectoria galls* (QIG) extract and 2AA (Pre-treatment, Simultaneous treatment and post-treatment).

Groups	Chromatid aberrations		Chromosome aberrations (ch.ab)						Total Ch.ab	Mean MI%	Mean MN%
	Gap	break	gap	break	del	ring	dc	Frag			
Negative control	8.4 d	15.6 d	7.4 e	17 c	11.2 e	11.2 c	6.8 e	6.8 c	84.4 e	8.62 a	3.26 d
Positive control	39.8 a	55.6 a	59.4 a	40.8 a	70.8 a	81.8 a	78.2 a	19 a	455.2 a	3.82 d	26 a
Pre-treatment	15 c	18 d	11.6 d	12.6 d	36.6 d	67.8 b	50.6 d	6.6 c	218.8 d	6.94 b	11 c
Simultaneous Treatment	24.2 b	29.8 c	22.6 c	34.6 b	46.6 c	75.2 a	59 c	11 b	303.0 c	5.52 c	17.6 b
post-treatment	37 a	46.8 b	54 b	38.6 a	61.8 b	68.2 b	65 b	10.4 b	381.8 b	4.36 d	20.2 b

Different letters within each column differ significantly (P<0.05) according to Dunnett test. Abbreviation: del, deletion; dc, dicentric; frag, fragmentation.

The antimutagenic potentiality of QIG is due to the bioactive components present in it. The previous phytochemical investigation of aqueous extract of QIG is found to contain Polyphenols, tannins, flavonoids, proteins. Hydrolysable tannins and polyphenols are one such class of compounds which are suspected of possessing protective properties [30;31]. Phenolic phytochemical compounds, possessing ability to remove free radicals, chelate metallic catalysts, activate antioxidant enzymes through a defense mechanism against reactive oxygen species (ROS) and inhibiting oxidases are given a considerable attention because of their diverse biological and beneficial health effects [32]. It is also known that the occurrence of cancer and degenerative diseases has been connected with generation of excess reactive oxygen species (ROS), inducing cell damage due to imbalance between antioxidants and oxidants [33; 34]. Hence earlier studies have been focused on natural antioxidants due to its interesting anticancer, anti-aging, and anti-inflammatory activities [35]. In a previous study *Q. infectoria* galls showed anti-oxidant and anti-inflammatory potential which confirm its protective effect against 2AA induced chromosome aberrations that optioned in this study[36]. Thus it is possible that common mechanisms of action of QIG extract, such as antioxidant activity, free radical scavenging and even gene regulation, could contribute to the direct and indirect antimutagenic profile of *Q. infectoria*.

CONCLUTIONS: We concluded the followings from the present work: first, QIG extract has no mutagenic effects on chromosomal aberrations and micronuclei presence under experimental conditions *in vivo*. Second, 2-Aminoanthracene has ability to produce chromosomal aberrations and micronuclei in bone marrow cells in male albino mice. Third, It had been suggested that inhibition in mutagenic effects of 2AA in animals receiving a pretreatment of QIG may be due to preventing the activation of 2AA before its binding with the DNA, which may be due to the antioxidant effect of tannic acid, gallic acid and phenols presence in galls of *Q.infectoria* .Thus the outcome of the present work ascertains the role of QIG as a desmutagen or potential genoprotectants against 2-Aminoanthracene. Further investigation to identify QIG antimutagenic compounds (especially at dose 2 gm/kg b.w.) should be performed.

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