

Anticlastogenic properties of *Quercus infectoria* galls extract against DMBA induced genotoxicity in bone marrow cells of mice *in vivo*

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(Received July 16, 2019; Accepted September 30, 2019; Available online May 21, 2020)

Abstract

This study aimed to evaluate the aqueous extract of *Quercus infectoria* galls extract (QIGE) as anticlastogenic. The effect of QIGE was tested in mice (5 groups for each test) treated with 7, 12-dimethylbenz (a) anthracene (DMBA), the strong site-specific carcinogenic agent. In this study, the QIGE show no signs of toxicity, a single dose of DMBA (50 mg/kg) was injected intraperitoneally to Swiss albino mice caused a great increase in number of chromosomal aberrations, micronucleated polychromatic erythrocytes (MnPCEs) and reduction in the percentage of mitotic index (MI) (cytogenetic markers). Oral pre-treatment and post-treatment of QIGE for 14 days at dose 2 gm/kg b.w. daily to DMBA-treated animals greatly reduced in number of micronucleus formation, chromosomal abnormalities such as chromosomal break, chromatid breaks, ring chromosome, dicentric chromosome and fragments. Besides, mitotic index frequency increased comparing with the positive control. The data suggest that QIGE has potent anti-clastogenic effect against DMBA-induced genotoxicity in bone marrow cells of albino mice and it may have a protective effect against the mutagenicity of the polynuclear aromatic hydrocarbons (PAH).

Keywords: *Quercus infectoria* galls, Anticlastogenic, Mutagenicity, Chromosome aberration, Micronucleus

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الخواص المضادة للطفرات الكروموسومية لعفص البلوط *Quercus infectoria* ضد السمية الوراثية التي يسببها DMBA في خلايا نخاع عظم الفئران داخل الجسم الحي

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الخلاصة

هدفت هذه الدراسة إلى تقييم قدرة المستخلص المائي لعفص البلوط *Quercus infectoria* كمثبط للكسور الكروموسومية. وقد تم في هذه الدراسة اختبار تأثير مستخلص العفص في الفئران (٥ مجموعات لكل اختبار) المعاملة مع ٧، ١٢- ثنائي ميثيلبنز (أ) أنتراسين هذا المسرطن القوي الذي يعمل على مواقع متخصصة. لم تظهر خلاصة العفص أي علامات للتسمم، وقد تم حقن جرعة واحدة من المسرطن وبتراكيز ٥٠ ملغم / كغم تحت البريتون للفئران البيضاء السويسرية مما تسبب في زيادة كبيرة في عدد من الانحرافات الكروموسومات وزيادة في عدد كريات الدم الحمراء ذات النويات الصغيرة وانخفاض في النسبة المئوية لمعامل الانقسام الخيطي (مؤشرات الوراثة الخلوية). كما أظهرت الدراسة بان المعاملة المسبقة بمستخلص العفص عن طريق الفم والمعاملة به بعد حقن ٧، ١٢- ثنائي ميثيلبنز (أ) أنتراسين ولمدة ١٤ يوماً وبتراكيز ٢ غم/كغم يومياً وجد بانها تقلل بشكل كبير من عدد النوى الصغيرة في كريات الدم الحمراء واختزلت التشوهات الكروموسومات مثل الكسر الكروموسومي والكسر الكروماتيدي والكروموسوم الحلقي والكروموسوم ثنائي السنتروميير وشظايا الكروموسومات فضلاً عن ازدياد تردد مؤشر الانقسام الخلوي مقارنةً بالسيطرة الموجبة. تقترح هذه البيانات بان مستخلص عفص البلوط

له تأثير قوي ضد السمية الوراثية التي يسببها ٧، ١٢- ثنائي ميثيلبنز (أ) أنتراسين في خلايا نخاع عظم ذكور الفئران البيضاء كما قد يكون له تأثير وقائي ضد الطفرات المستحدثة بواسطة الهيدروكربونات الحلقية متعددة النوى.

Introduction

Quercus infectoria (*Q. infectoria*) globally is referred as gall oak, traditionally was used as a common medical herbal in postpartum care and treatment of different diseases; the various *Quercus* species are originated in Iraq, Iran and Turkey, but now are widely spread and particularly common in Asia, Europe and North Africa. The galls growths on the new branch of the quercus tree in response to the attack by *Adleria gallaetinctoria*, the gall-wasp (1).

Pharmacologically, the galls of *Q. infectoria* reported as antidiabetic, and astringent agent (2), antimicrobial activities against viral, bacterial and parasites (3-5). Tannin 50-70%, gallic acid 2-4%, ellagic acid, starch, and sugar are the most common components showed within the galls of *Q. infectoria* (6). Tannins (gallotannic acid) is a phenolic compound characterized by its great roles in tissue protection from mutation, antitumor and protecting tissue from oxidation process (2).

Generally, any chemical that specifically or preferentially reduces the frequency of mutations is known as an antimutagen (7). Different polynuclear aromatic hydrocarbons (PAH) are found to be widely distributed in the atmosphere environment and have been known to cause cancer (8).

DMBA (strong site specific carcinogenic agent) found a secondary product within the environment as a result of incomplete burning of hydrocarbons (9). The product requires metabolic activation to become a carcinogen (6). Cytochrome P4501B1 converts DMBA to form diol epoxides and other toxic such as reactive oxygen species (ROS). Chromosomal damage has been reported as a result of binding of diol epoxides to adenine residues of DNA (6).

In addition, DMBA metabolism generates ROS which spread along the generation site or diffuse to outside of the intact cells (10). ROS cause lipid peroxidation directly or indirectly through production of free radicals leading to genotoxic and clastogenic effects on bone marrow (6,11-13). Recently, great attention has been focused to identify new antimutagenic and/or anticarcinogenic compounds through alternative pathways (14).

Many natural bioactive chemicals with genoprotective activity have been seen as a very effective way for halting human genetic diseases and cancer (7). These genoprotective substances commonly act as natural antimutagens and against processes of carcinogenesis. These substances are antimutagenic exert their effects through repairing mutation by inducing the mechanism of DNA repairing and replication (15).

Despite the fact, that QIGE has great health benefits with regards to its anti-microbial activities and the *Quercus* galls are widely distributed in northeastern of Iraq, to our knowledge no study yet has been done to understand the antimutagenic, anticlastogenic or anticarcinogenic activities of QIGE against DMBA. Accordingly, this study intended to evaluate the antimutagenic / anticlastogenic effects of QIGE against the potent carcinogen DMBA in *in vivo* study using bone marrow cells of mice by evaluation of the following cytogenetic biomarkers as mitotic index, chromosome aberration, and micronuclei.

Materials and methods

Used chemical materials

7,12-Dimethylbenz(a)anthracene and colchicine were supplied from ACROS organics company. DMBA was dissolved in corn oil then administered at 50 mg/kg dose intraperitoneally. All other chemicals were of the highest analytical grades which were commercially available.

Q. infectoria galls material

Q. infectoria galls are vegetable growths formed on twig of *Q. infectoria* tree, the galls are globular in shape and from 1.2 to 2.4 cm in diameter (Figure 1). The galls were collected locally from Gara mountain in Duhok province of Iraq in October 2016. The collected galls were washed with tap water and dried by air at home; then stored in a paper sack and kept at room temperature until use. The used galls were authenticated by Professor Saliem E. Shahbaz a taxonomist in Department of Forestry, College of Agricultural Engineering Sciences, University of Duhok, Iraq.



Figure 1: *Q. infectoria* Olivier (Fagaceae) galls.

Preparation of the QIG aqueous extract

Electric grinder was used for grinding the cleaned air-dried galls to make soft powder; the distilled water was used to homogenize the powder for 24 hours. The aqueous extract of the galls used in this study was prepared as follow: 200 ml of the distilled water was used to suspend 50 gm of the powdered galls in Erlenmeyer flask by using magnetic stirrer over night at 45°C. Following 24 hours, the obtained suspension was filtered by filter papers and gauzes firstly and then concentrated by rotary evaporator. The weight of the crude extract was determined by following equation: Yield of extract % = $\frac{\text{weight of Petri dish with extract} - \text{weight of empty Petri dish}}{50} \times 100$ (16). The crude extract then was kept at -20°C until the time of the use. In this study the extracts were dissolved in PBS as a solvent to obtain the experimental doses of 2 gm/kg body weight (b.w) that were given to Swiss albino mice by oral gavage.

Experimental animals

For the clastogenic and anticlastogenic assay, adult male Swiss albino mice Balb/c weighing 25-30 gm was used. The mice *Mus musculus* were provided by the animal house colony of College of Science, University of Duhok, Iraq. Five mice were housed per cage. The used mice were adapted to temperature and light in a well-controlled room, 12 hours dark /12 hours light, at temperature 25 ± 2 °C. Commercial pelleted was fed to mice and water *ad libitum*. This work was performed with the approval of Animal Ethical Committee of Duhok University, College of Veterinary Medicine, Iraq.

Cytogenetic Assay protocol

The main design of testing antimutagenic potential is based on standard test for evaluating the potential mutagenic properties of DMBA by induction of some cytogenetic markers in mice bone marrow *in vivo* (17). Twenty-five adult male mice were divided into five equal groups, each group of five mice. First group were orally treated by gavages with 0.5 ml of PBS daily for 14 days and used as negative control. Second group, the positive control was injected intraperitoneally once with 0.5 ml of DMBA (50 mg /kg b.w). Third group were treated with oral dose of QIGE (2 gm/kg b.w/day) for 14 consecutive days. Fourth group (Pre- DMBA treatment) animals administrated with QIGE at concentration of 2 gm/Kg b.w for 14 days before injection with DMBA at day 15th. Fifth group (Post-DMBA treatment) animals injected with single dose of DMBA once and then administrated with QIGE at concentration 2 gm/kg for 14 successive days. The used mice in each group were euthanized by cervical dislocation by the end of the experiment; samples of bone marrow were taken for cytogenetic analysis (MI and CA).

The same experimental design mentioned above was followed to calculate the number of polychromatic erythrocytes for the presence of micronuclei (MN).

Toxicity of *Q. infectoria* galls

To determine the toxicity of QIGE on mice used in this study, thirty-six Swiss Albino mice 25 - 30 gm weight were used and equally divided into six groups. Different QIGE doses 2, 4, 6, 8, 10 and 12 gm/kg b.w. were administered orally to the mice, respectively. A minimum tolerance dose (MTD) experiment was conducted. QIG aqueous extract was administered orally in individual doses of 2 gm/kg of body weight. All treated mice were kept under observation for a period of two weeks for any abnormality like behavioral changes, physical signs of toxicity or death.

Mitotic index (MI) assay

To study Mitotic Index (MI), light microscope with (40x) power was used to score 1000 cells/animal in percentages, 1000 cells were numerated (divided and non-divided were counted), the percentage rate was determined and calculated for only the divided cells according to this equation: $MI \% = \frac{\text{No. of dividing cells (P+M+A+T)}}{[\text{Total No. of dividing cells} + \text{No. of non-dividing cells (1000 cells)}]} \times 100$. where (P+M+A+T) is the collection of all cells in phase as prophase, metaphase, anaphase and telophase, respectively according to (18).

Chromosomal aberration assay

To detect the presence of CA; all treated mice were intraperitoneally injected with 1 ml of 4gm/kg b.w. colchicine, three hours later, the injected mice were killed by cervical dislocation. Femurs from the dead mice were fleshed out from the muscles. In a centrifuge tube, the femurs were soaked with 5 ml 0.075M KCl solution. The tube was then incubated for 20 minutes at 37°C, and then centrifuged for 10 minutes at 1000 rpm. Fresh Carnoy's fixative was added (3:1 methanol: acetic acid) following discarding the supernatant. The centrifugation process was done over three times. Later, the cells suspension was dropped onto clean slides that were dried by heating. The prepared slides were coded and stained by Giemsa stain then examined under oil immersion (100 x magnifications). For each animal, randomly 100 clearly distributed metaphase were scored/ animal (500 metaphase per treatment group) (19).

Micronucleus test in bone marrow cells (MN)

According to Schmid (20), MN test was investigated, bovine serum albumin was used to flush bone-marrow cells from femurs of sacrificed animals. Cells suspensions were centrifuged for 5 min at 1000 rpm, the obtained pellets were re-suspended in human albumin serum following discarding the supernatant. Giemsa stain then was used for air-dried,

methanol fixed smears for 5 minutes, then microscopically examined under high magnification 100x power. Nearly, 1000 micronucleated polychromatic erythrocyte (MnPCEs) and polychromatic erythrocyte (PCE) were numerated for detection of micronuclei for each animal.

Statistical Analysis

For normality and homogeneity, firstly, all data were checked using residual plots and Bartlett's test, respectively. Genstat 12th edition (VSN international Ltd, Hemel Hempstead, UK) was used for all statistical analyses. I. For multiple comparison between groups of mice tested in this study, one-way analysis of variance (ANOVA) was performed followed by Duncan's test. For all experiments $P \leq 0.05$ was considered as significant and all data are presented as Mean \pm S.E.M.

Results

Toxicity study

The toxic effect of QIG aqueous extract was firstly determined in this study; no mortality or great changes in

performance of treated animals was recorded in this study, despite of receiving high dose 12 gm/kg. Accordingly, LD₅₀ cannot be calculated. The recorded data suggested that the aqueous extract of QIG is safe, thus, the clinical experiment can be done safely.

Mitotic index

This study focused on bone marrow as a target tissue because it is extremely vascularized and it has high population of very active cells that can be easily isolated and prepared. To detect the rate of the cells with mitosis and calculate cellular proliferation, the mitotic index test was conducted. The recorded data showed that the Mitotic index value (QIGE= 8.23 ± 0.405) of animals treated with QIGE alone with the dose of 2 gm/kg was lower than that of negative control (PBS = 8.93 ± 0.52), but not significant. Moreover, it was found that a significant decrease in mitotic index of DMBA treated animals (pre = 5.23 ± 0.284) and (post = 5.7 ± 0.264) compared to the positive control group (DMBA alone = 2.86 ± 0.371) indicating anticytotoxicity effect of QIGE towards DMBA induced damage (Table 1).

Table 1: Chromosome aberration and Mitotic index in bone marrow of mice treated with *Q. infectoria* galls aqueous extract (2gm/kg), PBS (negative control), DMBA (50mg/kg) (positive control), pre-treatment (QIGE+ DMBA) and post-treatment (DMBA + QIGE). Different letters within each column differ significantly ($P < 0.05$) according to Dunnett test

	PBS (N.Co.)	QIGE	QIGE+DMBA	DMBA+QIGE	DMBA (P.Co.)
Chromatid break	3.36 ± 0.28^A	3.45 ± 0.24^A	6.35 ± 0.33^B	3.53 ± 0.41^A	5.78 ± 0.32^B
Chromosome break	3.38 ± 0.45^A	3.14 ± 0.57^A	6.25 ± 0.41^B	4.03 ± 0.94^{AB}	12.13 ± 0.99^C
Centromere break	2.05 ± 0.33^B	3.99 ± 0.09^C	0.57 ± 0.21^A	2.55 ± 0.31^B	4.78 ± 0.44^C
Ring chromosome	0.63 ± 0.29^A	1.04 ± 0.06^{AB}	0.48 ± 0.11^A	1.40 ± 0.35^B	0.54 ± 0.05^A
Dicentric chromosome	0.08 ± 0.08^A	0.32 ± 0.07^A	0.42 ± 0.07^A	1.13 ± 0.17^B	0.57 ± 0.29^A
Acentric fragment	0.52 ± 0.16^{AB}	0.93 ± 0.04^D	0.24 ± 0.02^{AB}	0.66 ± 0.17^{CD}	0.09 ± 0.09^A
Pulverization	0.00 ± 0.00^A	0.00 ± 0.00^A	0.00 ± 0.00^A	0.00 ± 0.00^A	4.33 ± 1.4^B
Total aberrant metaphase	10.02 ± 0.7^A	12.88 ± 0.4^{AB}	14.33 ± 0.85^B	13.30 ± 0.31^{AB}	31.23 ± 1.9^C
Mitotic index	8.93 ± 0.52^A	8.23 ± 0.41^{AB}	5.23 ± 0.28^B	5.7 ± 0.26^B	2.86 ± 0.37^C

Chromosomal aberrations

Concerning this study, the total aberrations with QIGE was 12.88 ± 0.49 , QIGE alone induced slightly significant chromosomal aberrations comparing with the negative control 10.02 ± 0.78 . While, the pre- and post-treatment of QIGE with the DMBA imparted protective effects which were found to be more significant in post-administration of the extract (post = 13.30 ± 0.31). The post-treatment caused a great reduction in the number of metaphases with chromosome aberrations relative to the DMBA such as chromatid break, chromosome break, centromere break, ring chromosome, dicentric chromosome, acentric fragment and pulverization defect comparing with DMBA treated animals (positive control). The decreases in aberrations after 14 days of QIGE could be due to the clearance of drug from the body, post-replication repair process.

The results of analysis of chromosome aberrations are presented in table1 and figure 2.

Micronucleated polychromatic erythrocytes (MnPCEs)

The data of micronucleus test are presented in table 2 and figure 3 which summarized the impact of DMBA alone, pre-treatment (QIGE+DMBA) and post-treatment (DMBA+QIGE) on the frequency of DMBA-induced micronucleated polychromatic erythrocytes (MnPCEs). A great elevation ($P < 0.05$) in the number of MN was detected in DMBA treated animals compared to untreated negative control; the pre- and post-treatment with QIGE data found a significant ($P < 0.05$) reduction in the incidence of the total MN cells compared to the positive control.

However, the pre-treatment (133.33±6.984) found to be more protective against DMBA-induced bone marrow micronuclei than that of post-treatment (181.67±4.91), thus,

the QIGE was found to play a potential protective role against the mutagenic agent, DMBA.

Table 2: Inhibition effects of *Q. infectoria* galls extract (QIGE) alone and in combination pre-treatment (QIGE+DMBA) and post-treatment (DMBA+QIGE) on DMBA induced bone marrow micronucleated polychromatic erythrocyte

	Single MN	Di MN	Tri MN	Tetra MN	Poly MN	Total abnormal
PBS (N.Co.)	43±8.51 ^A	15.67±7.056 ^A	11.67±1.20 ^A	3.33±2.02 ^A	17.00±5.29 ^A	90.67±19.2 ^A
QIGE 2gm/kg	39.33±4.10 ^A	15.33±5.89 ^A	11.33±1.86 ^A	2.33±1.33 ^A	16.33±2.63 ^A	84.67±10.2 ^A
QIGE+ DMBA	71.33±7.51 ^B	21.00±4.58 ^A	11.33±3.84 ^A	4.67±0.33 ^A	25.00±2.52 ^B	133.33±6.9 ^B
DMBA+QIGE	56.67±6.01 ^{AB}	13.33±4.26 ^A	11.00±7.02 ^A	3.67±0.88 ^A	97.00±4.16 ^A	181.67±4.9 ^C
DMBA (P.Co.)	102.00±7.02 ^C	46.00±3.056 ^B	16.00±5.29 ^A	9.33±1.33 ^B	80.00±13.01 ^B	253.33±13.8 ^D

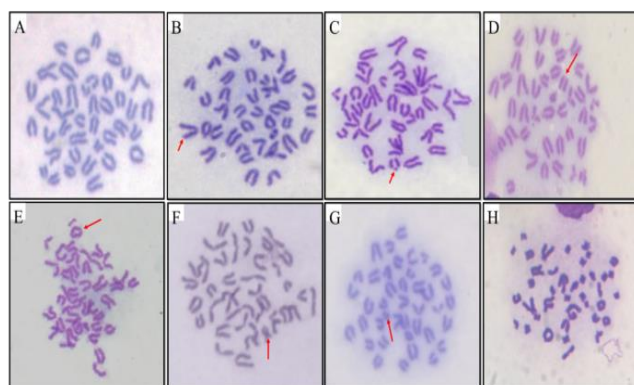


Figure 2: Metaphases from mice treated with DMBA showing different types of structural chromosomal aberrations. A: Normal chromosomes, B: Chromatid break, C: Chromosome breaks, D: Centromere break, E: Ring chromosome, F: Dicentric break, G: Acentric fragments, H: Pulverization. 1000x.

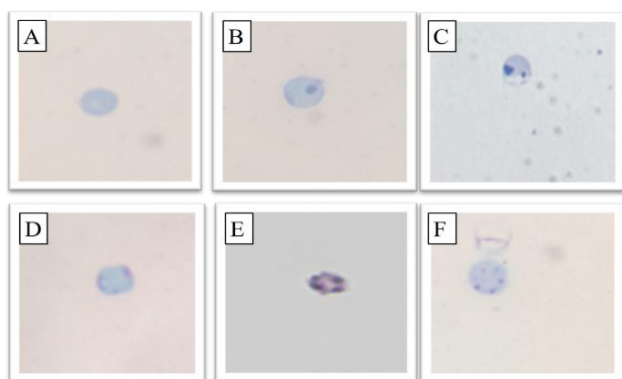


Figure 3: Types of micro-nucleated cell induced by 7,12-dimethylbenz(a)anthracene (DMBA) in male albino mice A: NCE normochromatic B: Mono micronuclei, C: Di micronuclei, D: Tri micronuclei, E: Tetra micronuclei, F: PCE polychromatic erythrocyte. 1000x.

Discussion

Generally, chromosome aberrations and point mutations are regarded as one of most common causes of tumors. (21). *In vivo* studies recommended to use mammals (mice) bone marrow cells to study the possible effects of mutagenic or antimutagenic agents on chromosomes (22).

The present study found that QIGE was safe to bone marrow cells of mice at the tested dose 2 gm/kg, also mice behavior was normal, the potential toxicity of QIG aqueous extract was also detected in mice at the dose 10 gm/kg through histopathological study, evaluation of hematological and biochemical parameters, which also confirm our result (23). Moreover, the present data showed that the administrating of QIGE strongly reduced the influence of DMBA-induced chromosome aberration; the pre-and post-treatment with QIGE combination significantly reduced number of bone marrow micronuclei induced by intraperitoneal injection of DMBA, suggesting the protective antigenotoxic properties of the QIGE. Polyphenols, tannins and flavonoids are the main components of the *Q. infectoria* galls that are characterized by antioxidant and antimutagenic activities and are regarded as antigenotoxic agents (24). Besides, great amount of polyphenols was found in galls extract with a strong antioxidant activities (25). Polyphenols and Hydrolysable tannins belong to a group of compound with protective characteristics (26,27). Phenolic phytochemical compounds have the ability to remove free radicals, activate antioxidant enzymes through a defense mechanism against reactive oxygen species (28). Cytochrome P450 was reported to be induced by polyphenols leading detoxification of the carcinogens inside cells (29). Furthermore, events have demonstrated that tannin molecules act as antimutagenic agent through reducing the mutagenic activity of some mutagens by its antioxidative properties (30). Thus, it is suggested that polyphenols, tannins and flavonoids component of QIGE act as potent antioxidant agents by scavenging of free radical, inducing

gene regulation, and by elimination of drug from the body and as a curative agent by activation the post-replication repair enzymes.

Conclusion

The present study found that the oral administration of QIG aqueous extract reported inhibitory and protective effects on the cytotoxic and clastogenic damage produced by DMBA which would be a promising approach toward inhibiting the genetic damage by chemical environmental pollutants. Surely, more works are required to study the same activity of *Q. infectoria* galls on different study models using various lab animals, to identify the active constituents of QIGE in details.

Acknowledgments

The authors are thankful to Duhok Research Center, College of Veterinary Medicine, Duhok University, for providing the needful lab facilities for this research. Also, the authors gratefully acknowledge the Department of Biology, College of Science, Duhok University for their support in providing their animal house laboratory and all its facilities to complete our practical work.

Conflict of interest

No conflict of interest.

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