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Determination of DNA damage induced after bitter orange *(Citrus aurantium)* essential oil administrated *in vivo*

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Abstract:

This research was carried out in order to investigate whether the purified bitter orange (*Citrus aurantium*) volatile oil purified from peels has a potentiality to induced DNA damage if administrated orally in vivo. White adult male rats (180-250g) were divided into five groups each group with five individuals. The first three groups administrated three different concentration of the purified citrus essential oil 100, 200 and 300µl/ kg Body weight (Bwt). The fourth group administrated olive oil and served as positive control group. The fifth was administrated water as negative control group. Dosing for all groups were two times weekly for one month period. To detected DNA damage, alkaline single cell gel electrophoresis (SCGE) for the blood samples was carried out for all groups. Parameters of nuclear olive moment, comet tail length, and DNA on comet tail were calculated. Results showed that there were no significant differences between treated and control untreated groups as well as olive oil administrated group. These results indicate that citrus volatile oil has no genotoxic effect and it was safe and tolerable in the highest dose used for the specified period of time conducted.

Key words: *citrus oil, rat, comet assay, olive oil, tail moment*

Introduction:

Many varieties of citrus fruits are used for their volatile oils or essential oils as used to be called in some literature. These compounds are used tremendously in perfumes, juices, and/or whenever fragrances needed either as a flavoring or as a solvent (1). Many researches have been conducted in order to detect the capability of different types of essential oils from dietary plants to contribute in different types of biological activity such as antioxidant, antimutagenic, enhancement of immune function and surveillance, enzyme induction and enhancing detoxification, and modulation of multidrug resistance synergistic (2). Besides the activity of essential oils as anti-fungous as well as ochratoxin inhibitor (3), antibacterial (4) either as natural compound or even modified to enhance antimicrobial activity, solubility and biocompatibility (5) was well documented know. Their anti-parasitic activity was recorded both in vitro and in vivo (7). Recently it has been found that essential oils can also induce apoptosis in leukemia cell line in a good selectivity index. But it was critical to the environmental and some production factors which significantly affect the secondary metabolite concentrations and the biological properties obtained (6). Their role as anti-diabetes was demonstrated (8). Beside essential oils from other plants, citrus essential oils are introduced recently as an alternative to some chemical preservatives in foods to reduce or even eliminate the dependent of food industry on these chemical compounds to elongate food and food products shelf live. Bitter orange Citrus aurantium essential oil has been recognized as the most frequently used sedative substances in some clinics of different south American countries, Spain and Italy Ambient odor of orange (C. sinensis) results in a lower level of state anxiety in dentistry patients. De Moraes et. al, (2006) demonstrated that essential oil from Citrus aurantium have anxiolytic activity in rodents without any motor impairment, even after 15 consecutive days of treatment. They recommended that studies of chronic treatment could be carried out in order to contribute information to guarantee the safer use of this essential oil (9) Aazza et. al, (2011) demonstrated a good antioxidant activity for Citrus aurantium essential oils (10). Now a days the health benefits resulting from the use of natural plant products rich in bioactive substances has promoted growing interest for pharmaceutical, food and cosmetic industries (11).

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The in vivo rodent alkaline single cell gel electrophoresis (comet assay) is used worldwide to detect DNA damage induced by genotoxic agents (12). The main advantages of the comet assay include: (a) the collection of data at the level of the individual cell, allowing more robust statistical analyses, (b) the need for a small number of cells per sample (<10,000), (c) sensitivity for detecting DNA damage and (d) use of any eukaryote single cell population both in vitro and in vivo, including cells obtained from exposed human populations and aquatic organisms for eco-genotoxicological studies and environmental monitoring (13, 14).

The purpose of this study was to evaluate the possible genotoxic activity of bitter orange (Iraqi cultivar) essential oils when feed in vivo and the capability of comet assay as a potential predictor of rodent carcinogenicity.

Material and Methods:

Plant oil extract

Bitter orange essential oil extracted using steam distillation method; the fruits were gathered from local market during the session of Nov. to Dec. 2014. Frutes were spliced and put in blender with cold water. The mixture then put on the Clevenger apparatus to extract the oil.

Different doses from this oil were prepared and randomly choses the concentrations 100, 200 and 300 mg/ kg b.w , by mixing the citrus oil with olive oil for experimental use. Olive oil was used as control group.

***In vivo* experiment and animals**

White rats (180-250g) was obtained from the animal house unite in Iraqi Center Cancer Medical Genetics Research (ICCMGR), animals were treated according to the ethical code implemented by the internal ethical board in ICCMGR. Animals were housed in cages for 30 days with free access to food and water with 12 hr light and 12 hr dark. The animals were divided into five groups, each group contain five individuals each group received the determined dose of essential oils orally using special gavage apparatus designed for this purpose, dosing for all groups were two times weekly for one month period (Table 1). All Animals were weighted every dosing time for 30 days.

Table 1: *In Vivo* Experiment dosing and Animals groups.

Group No.	Treatment dose	Number of individuals
I	Normal negative group	5
II	100 µl/kg Bwt essential oil	5
III	200 µl /kg Bwt essential oil	5
IV	300 µl /kg Bwt essential oil	5
V	Olive oil positive group	5

Single cell gel electrophoresis

The procedure was carried out in ICCMGR, molecular biology department, conducting the protocol of Rodrigo, et.al, (2007). (15). Peripheral blood collected from cardiac puncture and rapidly carried out the procedure. A freshly prepared suspension of cells in 0.75% low melting point agarose (USBiological, USA) dissolved in phosphate buffer saline (PBS). It was cast onto microscope slides and coated with 0.5% normal melting agarose (USBiological, USA). The cells were then lysed for 1hr at 4°C in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH 10). After the lysis, DNA was allowed to unwind for 40 min in electrophoretic solution (300 mM NaOH, 1mM EDTA, pH>13). Electrophoresis was conducted at 4°C for 30 min at electric field strength 0.73 V/cm (30mA). The slides were then neutralized with nutrilization buffer (0.4 M Tris, pH 7.5) and stained with 100 µl of ethidium bromides (2 ug/ml) (Sigma Chemicals, USA) and covered with cover slips. The slides were examined at 200 x magnification fluorescence microscope (Micros MCX 500, Austria) connected to a CCD camera (Infinity Capturer, Micros, Austria) which connected to a computer-based image analysis system, and images were analyzed using Comet Assay IV software (Perceptive, England). Fifty images were randomly selected from each sample; endogenous DNA damage was measured as the mean comet tail DNA of peripheral blood lymphocytes of the five groups. The following measurements were recorded. Olive tail Moment defined as the product of the tail length and the fraction of total DNA in the tail. Tail Moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail). Tail length defined as the distance of DNA migration from the center of the nuclear core and it is used to measure the value of DNA damage (15).

(23)

Statistical analysis

Results were analysed using one way ANOVA and the graphs were drawn by graph pad prism 6 (2012). Also Turkey multiple Comparisons have been carried out between the groups at ($p \leq 0.05$).

Results:

Bitter orange essential oil have been shown to be safe and did not show any comet tail or DNA damage on all blood samples for all concentrations that tested compared to olive oil which show DNA damage and comet tail appearance compared to control untreated group (Table 2). The results have no significant differences between bitter orange essential oil treated rats and untreated control group have as been estimated.

Table 2: parameters used in determine comet assay. G1 represent negative control rats untreated. G2 represent olive oil treated group. G3 represent 100 µl/kg Bwt citrus oil treated group. G4 represent 200 µl/kg Bwt represent the highest concentration of the bitter orange essential oil 300 ml/kg Bwt. All results did not show significant differences between groups. The parameters were represented as mean±SD.

Groups Parameters	G1 control	G2 100 ml/kg b.w	G3 200 ml/kg b.w	G4 300 ml/kg b.w	G5 olive oil treated	P
Olive moment Mean ±SD	0.356±0.529	1.014±0.837	0.8751.050±	0.809±1.325	0.05±0.170	0.05 N.S
Tail length Mean ±SD	1.00±1.633	3.3084.25±	1.50±2.153	2.067±3.195	4.251.179±	0.05 N.S
DNA tail Mean ±SD	30.8014.31±	4.213±3.583	5.249±5.90	25.57±10.61	6.603±0.957	0.05 N.S

Olive Tail Moment = (Tail mean – Head mean) ×Tail % DNA / 100. Extent Tail Moment= Tail Length × Tail % DNA / 100. Head % DNA = (Head Opt Inten/ (Head Opt Inten + Tail Opt Inten)×100 Tail % DNA = 100- Head % DNA.

The safeties of bitter orange essential oil in all examined concentrations (100, 200 and 300 µl/kg Bwt) have been observed when the comet assay parameters calculated in comparison to olive oil. Overall, from these results we can conclude the safety of bitter orange essential oil consumption without any genotoxicity effect or DNA damage (Figure 1).

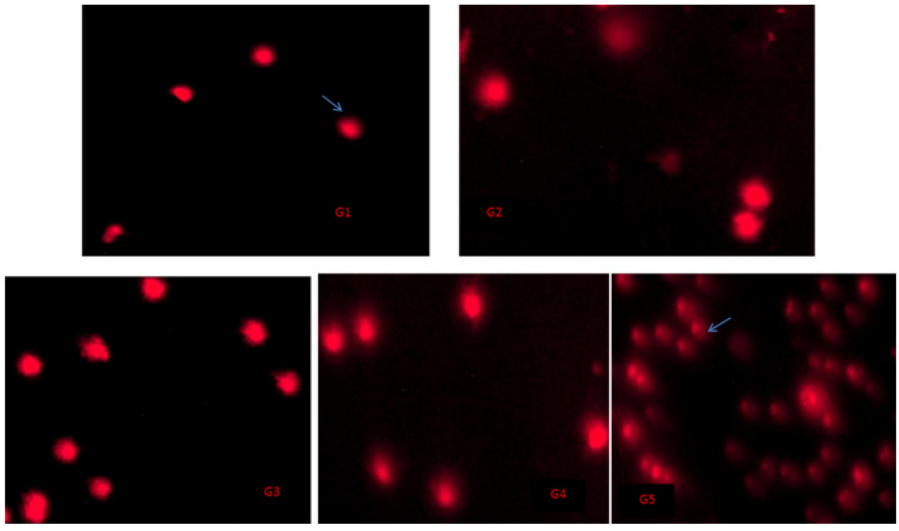

Figure 2: Shape movements of comet tails. G1 represent negative control untreated rats. G2 represent 100 µl/kg Bwt bitter orange essential oil treated group. G3 represent 200 µl/kg Bwt bitter orange essential oil treated group. G4 represent the highest concentration of the bitter orange essential oil 300 µl/kg Bwt. bitter orange essential oil. G5 represent olive oil treated group.

Discussion:

Throughout human history there has been increasing interest in natural alternative medicine, and nowadays, active principles of medical plants are the focus of scientific papers (13). In this regard, several studies have shown antioxidant properties derived from flavonoids, carotenoids and vitamin C, which are present at high concentrations in the Citrus genus (15, 16). In this study we focused on the bitter orange Citrus aurantium essential oil extraction benefits and wonder toward its safety using or not on rats treated with different prepared concentrations of this oil compared to olive oil genotoxicity.

There are several species of the Iraqian Citrus, which are found in most parts of the country. Although many studies have shown activity of limonene (15, 16) but there are few studies on cytogenetic and its cytotoxic activity of other components of Citrus essential oils (17, 18).

Citrus peels as mentioned in previous study (19), that determines the use of citrus peel as fed to livestock, scent perfumes and soap products and also described the contents of citrus species essential oil: terpens, aliphatic sesquiterpene, oxygenated derivatives and aromatic hydrocarbons.

We chose comet assay to determine the genotoxicity and its sensitivity to detect the presence of DNA strand breaks and alkali labile damages in the individual cells (20).

A study carried on Casearia sylvestris to determine its genotoxicity and examined how this plant will protect DNA from damage both in vitro and in vivo against DNA damage using the Ames test and the comet assay in HepG2 cells. Casearia sylvestris compound shown to be genotoxic to HepG2 cells at high concentrations while was protective of DNA at low concentrations (21).

As concluded from the study (22), were overcalled the mechanisms by which the compounds protect DNA from damage can act by, A) They can be considered desmutagens, which reduce the mutagenic/genotoxic effect by directly interacting with the mutagens, block their effects by inhibiting their metabolic activation or enhance their detoxification. The pre-treatment is commonly performed to assess the detoxificant properties of the compounds, since detoxificant enzymes can be induced during the pre-treatment period. Or B) these compounds can be classified as bio-antimutagens, which promote DNA repair after damage, increase DNA replication, inhibit error-prone replication or suppress the growth and replication of cells with damaged DNA .The post-treatment is useful to assess bio-antimutagenic effects, for instance DNA repair response can be activated after damage, and the post-treatment can help to improve this effect (22). Most carcinomas normally show a greater degree of DNA damage with extensive comet tails than that found in the tissue cells from controls (23).

Different studies have demonstrated the ability of single bioactive molecules found in Citrus fruit to inhibit cancerogenesis both in vitro and in vivo, as well as other investigations have suggested their capacity to hinder invasion and metastasis (24, 25).

Few studies have focused on the biological activity of Citrus aurantium. The concentrated orange juice decreased the chemically-induced mammary tumors burden in rats as concluded by So, et.al, (1996) (26). Some years later, a study indicated that orange juice inhibited azoxymethane (AOM)-induced colon cancer in rats (27).

Recently, Sesamol-reduced radiation-induced apoptosis and facilitated cell proliferation. In the comet assay, sesamol (20mg/kg) treatment reduced radiation-induced comets (% DNA in tail) compared with radiation only. The results strongly suggest the radio protective efficacy of sesamol to the haematopoietic system of mice (28).

The findings of the present study suggest that the proper use of herb products is safe and may provide some beneficial effects and more study must be continued on tumorized mice and also to determine the histopathological effects of organs in rats treated with bitter orange essential oil.

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تحديد مدى تضرر المادة الوراثية DNA داخل الجسم الحي بعد التجريع الفموي لزيت النارنج *(Citrus aurantium)* العطري

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الخلاصه:

جرى في هذا البحث التحري فيما لو كان للزيت العطري المنقى النارنج (*Citrus aurantium*) قدرة على إحداث ضرر في المادة الوراثية DNA عند تجريعه من خلال الفم داخل الجسم الحي . إستخدمت في التجارب الجرذان البيضاء بوزن تراوح بين 180 إلى 250 غرام والتي قسمت إلى خمس مجاميع كل مجموعة تحتوي على خمسة أفراد وجرى تجريع المجاميع الثلاثة الأولى تركيز مقدارها 100 و200 و300 مليغرام من الزيت العطري المنقى من قشور النارنج لكل كيلوغرام من وزن الجسم في حين جرعت المجموعة الرابعة زيت الزيتون التجاري المتوفر في الأسواق المحلية وأعتبرت مجموعة مقارنة موجبة وجرعت المجموعة الخامسة الماء النقي وعدت مجموعة مقارنة سالبة. جرعت كل المجاميع بجرعتين إسبوعا ولمدة أربعة أسابيع (شهر كامل) وبعد إنتهاء فترة التجريع جرى التحري عن مدى تضرر المادة الوراثية في الحيوانات المجرعة بإستخدم فحص هجرة الخلايا المنفردة في المجال الكهربائي القاعدي والمعروف بفحص المذنب أو الكومت comet assay لعينات دم الحيوانات تحت التجربة وقد أستخدم قياسات كل من مسافة حركة جسم النواة للخلايا و طول الذنب النووي في الخلايا وكمية المادة الوراثية DNA في الذنب الناتج لتحديد ذلك التضرر. أظهرت النتائج عدم وجود فروقات معنوية من حيث تضرر DNA بين الجاميع المجرعة بزيوت النارج العطرية ومجاميع المقارنة الموجبة والسالبة تحت الدراسة وبمختلف التراكيز مما يدل على عدم وجود سمية وراثية للزيوت العطرية المنقاة من النارنج وهناك قدرة تحمل للجرعة الاعلى المستخدمة وخلال الوقت المستخدم للتجريع .