Journal of Food Technology Research
2016 Vol. 3, No. 1, pp. 28-35
ISSN(e): 2312-3796
ISSN(p): 2312-6426
DOI: 10.18488/journal.58/2016.3.1/58.1.28.35

PROTECTIVE EFFECTS OF SWEET ORANGE PEEL (CITRUS SINENSIS L.) T INDUCTION OF MICRONUCLEI INDUCED BY CYCLOPHOSPHAMIDE IN HUM PERIPHERAL LYMPHOCYTES

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ABSTRACT

Recycling of fruit waste is one of the most important ways of utilizing it in a number of novel products , as well as in medicine, so came this research to verify the protective role of the extracts of sweet orange (Citrus sinensis L.) peels against genotoxicity induced by cyclophosphamide on human peripheral lymphocytes using the micronucleus and chromosomal aberration tests. Conducted overlap between Aqueous and alcohol extracts orange peel with the drug, through three types of transactions (before, after, with treatment). In order to test the effectiveness of the extracts, three concentrations of 5, 10 and 15 µg / ml where tested the prevention or minimization the effect of the drug (80 µg / ml) on human blood lymph . The extracts of the orange peel has antimutagenic potential induced by cyclophosphamide this may prevent the mutagenic effect of various genotoxic or carcinogenic agents, and thus utilization of fruit waste and products for therapeutic purpose.

Keywords: Cytogenetic, Sweet orange (*Citrus sinensis* L.) peels, Cyclophosphamide, Micronucleus.

Received: 15 February 2016/ **Revised:** 15 March 2016/ **Accepted:** 18 March 2016/ **Published:** 21 March 2016

Contribution/ Originality

This study is one of very few studies which have investigated of antimutagenic effect of fruit waste such as orange peel against cytotoxic effect of cyclophosphamide , The paper's primary contribution is its finding that these fruit waste can be used to prevent the mutagenic effect of various genotoxic or carcinogenic agents . These findings contribute to the understanding of the health benefits of fruit waste. This study originates new formula to recycling of fruit waste and benefit from it in the therapeutic purpose.

1. INTRODUCTION

Cyclophosphamide (CYP) is a cytotoxic alkylating agent that has been used extensively in veterinary medicine as an antineoplastic agent for the treatment of several tumors such as sarcomas and carcinomas of the lung and mammary gland (Withrow and MacEwen, 2001). However, this drug has serious side effects such as inducing genotoxic effects, renal (Kopecna, 2001) and hepatic damage (Gustafsson *et al.*, 1996) thereby limiting its therapeutic use. Its cytotoxic effects result from the reactive metabolites that alkylate DNA and form a variety of DNA adducts that sufficiently alter DNA structure or function (Hales, 1982) leading to formation of chromosomal aberrations and micronuclei formation (Madle *et al.*, 1986; Moore *et al.*, 1995). Besides that it is a well known carcinogen which bio-transformed principally in the liver to active alkylating metabolites by a mixed function microsomal oxidase system. These metabolites interfere with the growth of susceptible rapidly proliferating

malignant cells. The mechanism of action is thought to involve cross-linking of tumor cell DNA (Amador *et al*., 2012; Kocaman *et al*., 2012).

Orange constitutes about 60% of the total citrus world production . A large portion of this production is addressed to the industrial extraction of citrus juice which leads to huge amounts of residues, including peel and segment membranes. Peels represent between 50 to 65% of total weight of the fruits and remain as the primary by product. If not processed further, it becomes waste produce odor, soil pollutant, harborage for insects and can give rise to serious environmental pollution (Manthey and Grohmann, 1996; Mandalari *et al*., 2006). In Iraq and in many countries, major quantities of the peels are not further processed. Some attempts were made to insert these residues in some industrial product as drugs or as food supplements , because they are rich in nutrients and contain many phytochemicals (Hegazy and Ibrahium, 2012).

Citrus sinensis L is from rutaceae family and commonly known as sweet orange (Bakshi *et al*., 1999) it is the most commonly grown tree fruit in the world (Morton, 1987) and its fruit is strengthening, cardiotonic, Laxative, anthelmintic and removes fatigue (Kirtikar and Basu, 1984). It possesses anti inflammatory, antibacterial and antioxidant properties (Ramachandran *et al*., 2002). Oranges used to lower cholesterol and aid in the digestion of fatty foods (Cesar *et al*., 2010). The vitamin C in oranges is concentrated mainly in the peel and the white layer just under the peel. The peel contains citral, an aldehyde that antagonizes the action of vitamin A, therefore, anyone eating quantities of orange peel should make certain that their dietary intake of vitamin A is sufficient (Audrey, 1983). It consists of about 90% d-limonene (Okwu and Nnamdi, 2011). Peel of the orange Limonene now is known as a significant chemo preventive agent (Crowell, 1999) with potential value as a dietary anti-cancer agent in humans (Shamkuwar *et al*., 2012). There is evidence that oxidative damage is an important contributor to aging and various chronic diseases such as cancer and neuro degeneration (Scalbert *et al*., 2005; Kushwaha *et al*., 2011; Kumar *et al*., 2012). Both dietary antioxidant and those endogenous in the body are involved in controlling oxidative damage. In the context of nervous system, antioxidants have been shown to improve motor and cognitive functions in experimental animals and prevent ROS- medicated neuronal death (Socci *et al*., 1995; Joseph *et al*., 1999; Andres-Lacueva *et al*., 2005). Numerous studies were performed to assess the protective effects of plant extracts and/or their isolated bioactive components against geno toxicity induced by mutagenic agents using the Chromosomal Aberrations (CAs), Sister Chromatid Exchange (SCE) , and micronucleus (MN) tests in human peripheral blood lymphocyte cells (Sowjanya *et al*., 2009; Ananthi *et al*., 2010; Di Sotto *et al*., 2010; Kayraldız *et al*., 2010). However, a few studies are available in the scientific literature on the protective effects of peels on drug, Therefore, the aim of the present study was to evaluate the possible protective effects of peels sweet orange against the genetic damage induced by CP, known as alkylating mutagenic agents, in human peripheral lymphocytes using the MN tests.

2. MATERIALS AND METHODS

Cyclophosphamide (CP) was purchased from Sigma chemical Company (st. Louis, Mo, USA), one tablet of Cyclophosphamide (CP) was dissolved in 100 ml of sterilized distilled water to make the concentration of 80µg/ml, and then sterilized by filtration and kept at 4˚C until being used.

2.1. Preparation of Extracts

The plants used in this study were *Citrus sinensis* L. (sweet orange) , taken from local market, peeled and the peel were shade dried at (30- 35 C). A powder made from the peel and transferred into closed containers until use. 50 grams of the powder were extracted by means of Soxhlet using distilled water and alcohol as a solvent. The extract was evaporated using rotary evaporator (80°C), and the residue was dissolved in distilled water and alcohol to prepare the required doses (5, 10 and 15 µg / ml).

2.2. Peripheral Blood Culture Initiation

Heparinized blood samples were collected from four healthy females, non-smokers, (aged 25 - 28). Whole blood for each samples (0.5 ml) was added to 5 ml of culture medium RPMI 1640 (Sigma, pH 6.8 -7.0) , supplemented with 10% fetal calf - serum,10% antibiotic- antimycotic mixture and 1% phytohaemaglutinin of the final volume of cell culture (Carballo *et al*., 1993).

2.3. Experimental Design

Design of Experiments Divided tubes to implant aggregates with three replicates per treatment, these 8 groups were designed as the following manner

- Group "1" negative control (without treatment).
- Group "2' positive control, treatment with drug cyclophosphamide (CP) at a final concentration (80 µg /ml).
- Group "3" post - drug : treated with Cyclophosphamide (CP) after 24 hours adding ethanolic extract of peel sweet orange at three concentrations (5,10 and 15 µg /ml) respectively
- Group "4" post - drug : treated with Cyclophosphamide (CP) after 24 hours added aqueous extract of peel sweet orange at three concentrations (5,10 and 15 µg /ml) respectively
- Group "5" pre- drug: treated with ethanolic extract of peel sweet orange at three concentrations (5,10 and 15 µg /ml) respectively .Then after 24 hours added Cyclophosphamide (CP)
- Group "6" pre-drug : treated with aqueous extract of peel sweet orange at three concentrations (5,10 and 15 µg /ml) respectively , then after 24 hours adding Cyclophosphamide (CP)
- Group "7" simultaneous treatment: mixture of aqueous extract of peel sweet orange at three concentrations (5,10 and 15 µg /ml) respectively with CP at concentration (80 µg /ml)
- Group "8" simultaneous treatment: mixture of ethanolic extract of peel sweet orange at three concentrations (5,10 and 15 µg /ml) respectively with CP at concentration (80 µg /ml)

2.4. Micronuclei Assay

In order to detect the number of micronuclei in lymphocytes, cytochalasin B (4.5 µg/ml, Sigma®) were added to cultures at 44th hour of incubation. At the end of the 72 h incubation period, cells were harvested by centrifugation and treated with hypotonic solution (0.075 M KCl at 37.4°C). Then centrifuged and / fixated with methanol/acetic acid (3:1, v/v) hepeated three times.The resulting cells were resuspended and dropped onto a clean slides. 3–5 drops of the fixed cell suspension were dropped on a clean slide and air dried. The slides were stained with giemsa and scored. MN was scored in 1000 binucleated cells and the frequency of cells with MN was determined (Eroğlu, 2011).

3. RESULTS AND DISCUSSION

3.1. Drug Treatment (Pre- Post and Simultaneous)

The results of the present study reveal that the extract of sweet orange peel (aqueous and alcohol) is potent enough to reduce the genotoxic effects of cyclophosphamide at all the selected doses. The selected doses of peel extract were not genotoxic from our previous study, The data obtained in our earlier studies suggest that the compounds present in the extract of peel of *citrus aurantium* L (Bitter orange) and *citrus medica* L are not mutagenic in human lymphocytes (Ekhlas, 2014). The verification of the possible mutagenic and/or antimutagenic effects of medicinal plants / extracts is an important factor in studies (Roncada *et al*., 2004). Some plants may possess substances that can modulate the genotoxicity of the other compounds (Roncada *et al*., 2004). This experiment was designed to study the interaction of orange peel with the mutagenic effect of cyclophosphamide in human blood by used micronucleus test, which is one of the simplest short term assays for biomonitoring of the genotoxicity of chemical carcinogens and the effect of putative chemopreventive agents (Subapriya *et al*., 2004). The micronucleus

in young erythrocytes arises primarily from chromosomal fragments or lagging chromosomes that are not incorporated into the daughter nucleus at the time of cell division in the erythropoietic blast cells (Hayashi *et al*., 1994); (Aardema and Kirsch-Volders, 2001). The results of assay as shown in table (1) figure (1). Cyclophosphamide (positive control) significantly increased in micronucleus compared with untreated control (negative control), the reason of this increase was the drug causes harmful effects on DNA, these results were expected due to the cytotoxic and anti tumor effects of cyclophosphamide that's for this reason used as standard anti-cancer agent (Jayaseelan *et al*., 2012). This outcome is similar to the results obtained by others (Zhang *et al*., 2006; Alkan *et al*., 2012). While, it was clear from Table (2) The protective effect of peel extract in the present study to prevent the micronuclei induced by cyclophosphamide (CP) in peripheral blood lymphocyte , in three types of treatments (before, after, with treatment). may be due to the direct action of the compounds present in the extract of peel on cyclophosphamide (CP) by inactivating it enzymatically or chemically (Morse and Stoner, 1993). In post - treatment with peel sweet orange (aqueous and alcohol) extract respectively, there was a decrease in CP genotoxicity showed by a decrease of MN formation in three concentrations (5,10,15) of the peel . It is clear that the peel extract may activate the suppressing agent or activate the promoters of DNA repair mechanism, or may increase the error free repair fidelity in the cell (Bronzetti, 1994). On the other hand, the treatment with peel (aqueous and alcohol) extract at the same time with CP had significant ($p<0.05$) decreases of MN at each concentrations as compared with CP alone. The Pre- treatment with peel (aqueous and alcohol) extract respectively, had significant decreases ($p<0.05$) of the frequencies of MN as compared to group treated with CP alone. Statistical analysis showed that there were no significant differences for the frequencies of MN between the groups which treated with extract aqueous or alcohol in each interaction used (before, after, with treatment) only at high concentrations can consideration that the alcohol extract was the best.

Moreover, simultaneous treatment with peel extract and CP, can reduce the genotoxic effect of the CP. The ability of treat with peel extract to reduce the frequencies of MN was similar to the reduction ability of pre-treatment , figure (1), which means they have similar mechanism to reduce genotoxicity CP. The interaction (co and pre- treatment) was best than post - treatment , this mean that the peel may increase the DNA replication fidelity or induced repair system enzyme as result decreased in mutation frequency (Diplock, 1994). Peel sweet orange extract of phytochemicals that can protect cell component. The reason for this is that oranges are an important source of phytochemicals such as phenolics vitamin C, and carotenoids. These compounds also known as nutraceuticals provides health benefits due to a risk reduction of chronic illness such as cancer and cardiovascular disease (Gardner *et al*., 2000; Faulks and Southon, 2001). One of the mechanisms by which these phytochemicals exerts their beneficial effects in human health has been related to their antioxidant activity. Vitamin C contributes in 100% to the total antioxidant activity of orange juice (Francis, 2000). Vitamin C scavenges free radicals such as O2 oxygen, protecting the intracellular and extracellular structures (Francis, 2000; Jayaprakasha and Patil, 2007). Citrus fruits and juices are an important source of bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important to human nutrition (Karoui and Marzouk, 2013). Also, the results of pre- and post-treatments may qualify the present extract as a desmutagen and a bioantimutagen. As a desmutagen, the chemical constituents of the extract may be able to react with the mutagen or its metabolites, while as a bioantimutagen; the plant extract or its constituents may increase the error free repair fidelity in the cell or activate the promoters of DNA repair mechanisms (Bronzetti, 1994; Kuroda and Hara, 1999).

4. CONCLUSION

In conclusion, the present study demonstrated that dangerous effect of anticancer drug CP could be avoid by using the treatment of especially peel sweet orange which are considered as waste materials of the fruit at the same time with CP and pre - treatment , may be due to it contains natural antioxidant such as ascorbic acid, flavonoids, phenolic compounds and pectins. This strategy is necessary for diminishing the deleterious side effects of anticancer

drug with preservation of its chemotherapeutic efficacy , and also, could be of significance in human therapy, animal and plant diseases.

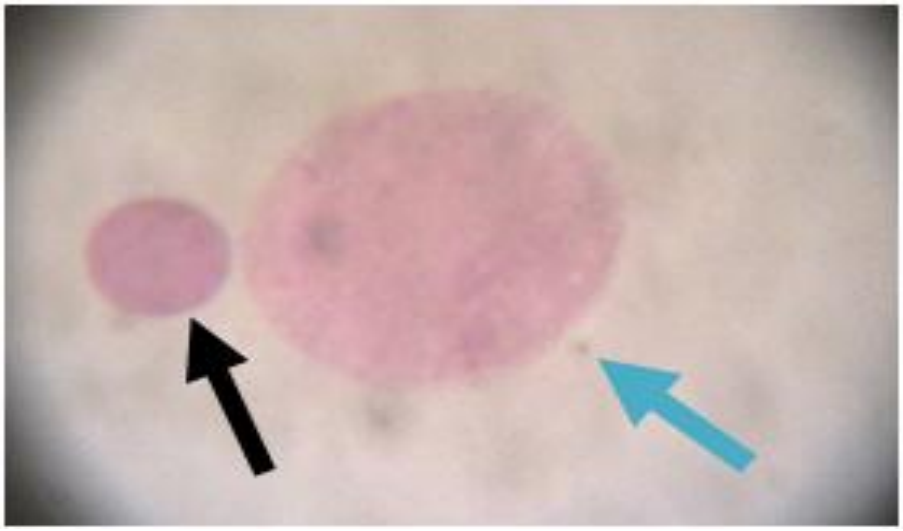

Fig-1. Giemsa-stained binucleated lymphocytes in human peripheral blood treated with Cyclophosphamide (CP) . Arrows indicate micronucleus (MN) and main nucleu
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Table-1. Effect aqueous extract of sweet orange peel on micronucleus formation in human peripheral lymphocytes cells

Test substance	Concentration µg /ml	Distribution of MN in BN				MN%
		0MN	1MN	2MN	3MN	
Control	0	995	5	0	0	0.5 a
Cyclophosphamide (CP)	80	928	18	12	10	7.2 b
Pre - drug treatment	5	986	10	2	0	1.4 a
	10	991	7	1	0	0.9 a
	15	991	8	1	0	1.0 a
Post - drug treatment	5	980	16	2	0	2.0 a
	10	986	12	1	0	1.4 a
	15	990	10	0	0	1.0 a
Simultaneous Treatment	5	991	9	0	0	0.9 a
	10	990	10	0	0	1.0 a
	15	993	8	0	0	0.8 a

Differences A, B, C, D, E are significant (P< 0.05) to compression row

Table-2. Effect alcohol extract of sweet orange on micronucleus formation in human peripheral lymphocytes cells

Test substance	Concentration µg /ml	Distribution of MN in BN				MN%
		0MN	1MN	2MN	3MN	
Control	0	995	5	0	0	0.5 a
Cyclophosphamide (CP)	80	928	18	12	10	7.2 b
Pre - drug treatment	5	992	8	0	0	0.8a
	10	990	8	1	0	1.0a
	15	993	7	0	0	0.7a
Post-drug treatment	5	991	7	1	0	0.9a
	10	989	9	1	0	1.1 a
	15	990	8	1	0	1.0 a
Simultaneous Treatment	5	993	7	0	0	0.7a
	10	994	6	0	0	0.6a
	15	995	5	0	0	0.5a

Differences A, B, C, D, E are significant (P< 0.05) to compression row

Funding: This study received no specific financial support.

Competing Interests: The authors declare that they have no conflict of interests.

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