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INVESTIGATION OF CYTOGENETIC EFFECT OF ORANGE COMMINUTED IN THE CULTURED HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

Orange comminuted, an orange derived paste which can be obtained by mixing several of its components, where juice is the major contributor at this product. It can be used as a natural basis to enhance sensory properties of soft drinks, despite the importance of this product. There are not available studies illustrate precisely the cytogenetic effects of this product. Therefore, in the present study, analyzed the comminuted spectrophotometrically, free and total phenolics, analyzed by Folin-Ciocalteu colorimetric method, compared with orange juice, then determined the cytogenetic effects of concentrations comminuted orange (5,10,15) µg / mL and the control (did not receive any additive) in cultured peripheral blood lymphocytes (in vitro), using cytogenetics parameters mitotic index(MI), replication index (RI), and micronucleus (MN). Results showed that the concentrations used did not induce an increase in the frequency of micronucleus (MN), replication index (RI) and mitotic index(MI). The results came, almost similar to that of control which indicates that the orange comminuted product, has no significant cytotoxicity and genotoxicity effects in vitro on human lymphocytes. Thus, it could be concluded that comminuted orange polyphenols protect the normal cells from genotoxic and carcinogenic agents, which indicates the therapeutic antioxidative role of flavonoids, or other orange comminuted compounds.

KEYWORDS: Orange comminuted, Phenolic compounds, Cytogenetic, peripheral lymphocytes, Mitotic index, Replication index, Micronucleus.

INTRODUCTION

In recent years the whole world is looking for remedies for number of serious problems that affect the environment and human health such as waste which Produce by juice processing industries, and pollute the environment and human health, like peel solids which discarded as waste or used for byproduct processing.[1,2] Several studies have demonstrated that orange peel is a rich source of flavonoids which can be used as a preventive for several chronic degenerative diseases,[3,4] therefore, orange peel is suitable for human consumption. One of the products derived from citrus is comminuted orange, that is obtained by grinding defined proportions of peel and juice, and that is mainly used as a natural basis to enhance sensory properties of soft drinks. In addition to augmenting beverages quality, production costs and wastes are reduced because orange peel is used in comminuted elaboration.[5] Fruit juice decomposition is frequently associated with fermentative yeasts such as Saccharomyces cerevisiae, causing ethanolic spoilage, carbonation, production of H2S and off-odors.[6, 7] The processing of industrial products obtained from citrus fruits, are heat pasteurization, freezing and sterilization, allows the reduction or elimination of microbial spoilage potential. Although effective, these processes can severely damage the quality of food outcome. Losses in sensorial, nutritional, and physicochemical addition to the impact on antioxidants and reduce its effectiveness as a result of thermal processing like pasteurization which can affect polyphenol contents.[8,9,10] had shown that polyphenol contents could be affected by different processing techniques. According to[11, 12] reports, clarification also could decrease the polyphenol contents of commercial fruit juices, revealed that pasteurization influence (-27% decrease) on the polyphenol contents. Thus, the aim of this work is to quantify phenolic compounds, and provide data on the effect of thermal processing on the content of the total and free polyphenol in orange comminuted and compare it with orange juice to determine the activity of comminuted orange. Then for the purpose of ascertaining the safety of the product, we have evaluate the cytogenetic effects of orange comminuted in vitro on human lymphocytes, using from mitotic Index (MI), replication index (RI)and micronucleus (MN) parameters.

MATERIALS AND METHODS

Orange comminuted preparation

Orange comminuted valencia oranges were purchased from local supermarket. Comminuted was prepared by mixing orange components in the following proportions: juice 71.6% (w/w), pericarp 12.4% (w/w) and outer layer 16.0% (w/w). All elements were liquefied for two

different one minute cycles at maximum speed in domestic mixer. Samples were stored in polyethylene bags and frozen at -80°C until used. [7, 5]

Phenolic compounds

Total phenols were determined by Folin- Ciocalteu reagent (13). 250 mg of Orange comminuted were mixed with 5 mL of methanol: water (1:1v/v) (for free phenolics) and 1.2 M HCl in (1:1 v/v) methanol/water (for total phenolics).The mixture was vortexed at 3000 rpm/1 min and then heated at 90 °C for 3 h with vortexing every 30 min. Sample was cooled to reach room temperature, diluted to 10 ml with methanol and centrifuged at 5000 rpm/10 min/4 °C (14). 50µL free or total phenolic extracts were placed in a 2 mL microcentrifuge tubes followed by 650µL of water. Then 50µL of the Folin Ciocalteau reagent (FCR) (1:1) were added. The resulting mixture was vortexed and incubated 5 min at room temperature. Finally, 250µL of 1 N sodium carbonate solution were added and the test tubes were incubated at 37 °C/2 h. and the optical density was measured colorimetrically at 765nm. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml), Concentration was expressed as mg gallic acid equivalents/100 g comminuted orange in wet basis mg GAE/100 g).[15,16]

Blood sample collection

Three fresh blood samples from the volunteers were collected, generally from the arm by venipuncture, and placed into a heparinised tube.

Peripheral Blood Culture Initiation

Few drops of whole blood (0.5mL) ware cultured in 5 mL medium RPMI 1640 with 10% foetal calf serum (FCS) and 1% penicillin / streptomycin and add phytohemagglutinin (PHA). Then Orange Comminuted was added to obtain four final concentrations (5,10 and 15 µg / ml). The orange comminuted extracts were not added to the tubes of the control groups. Peripheral blood cultures were placed in a 37°C incubator for 72 hrs.

Harvesting of the blood cultures

By administering colchicine or other colchicine-derivative medications (i.e. colcemid) you can arrest the cell cycle at this point leaving the chromosomes in their visible form. Colchicine disrupts the microtubule formation which is necessary for the spindle fibers to separate the chromosomes during anaphase. To arrest the cell division at metaphase, After 70 h of incubation, 0.1 mL of colcemid solution (1 µg/ml) was added to each tube and the

contents were mixed by shaking the tubes gently. At the end of the incubation, the tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. The pellet was resuspended using 10 mL of hypotonic solution (0.075 M KCl) and the tubes were incubated at 37°C for a further 4 min.

Fixation of blood cultures

To the pellet 5 ml of fixative solution (3:1 methanol: acetic acid) was added. The tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. This procedure was repeated three times. The fine pellet was dropped onto microscopic sterile and chilled slides.

Micronuclei assay (MN).

In order to detect the number of micronucleated lymphocytes, cytochalasin B (4.5 µg/ml, Sigma) were added to cultures at 44th hour. At the end of the 72 h incubation period, the lymphocytes were treated with 0.075 M KCl for 8 minutes at 37oC. After three repetitive fixation with methanol/acetic acid (3:1, v/v), cell suspension was dropped onto cold slides. The slides were air-dried at room temperature and then stained with 5% Giemsa for 15 minutes. All slides were coded before scoring. The criteria for scoring micronuclei were as described by[17] At least 2000 binucleated lymphocytes were examined for the presence of one, two or more micronuclei per concentration.[18]

Replicative index (RI) assay

The replicative index (RI) was determined by counting the number of cells at the first, second and the third metaphase in (100) a cell at metaphase, the RI was calculated according to the following equation:

RI= (1xM1%) + (2xM2%) + (3xM3%)/100. [19]

Mitotic Index (MI) Assayt

The slides were examined under high power (40 X) of compound light microscope and of divided and non-divided cells were counted and the mitotic index was calculated according to the following equation

Mitotic index =no. of the dividing cells/ total no. of the cells (1000) ×100

Statistical Analysis

The data are expressed as mean ± SD. Statistical comparisons were performed by One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test, and compared

the differences between the moral test averages less significant difference (LSD) probability ($P < 0.05$).[20]

RESULTS AND DISCUSSIONS

Dosage of total polyphenols

Table 1, show the concentration of free and total phenol of fresh comminuted orange in mg (GA: Gallic acid) equivalent/L. The Folin Ciocalteu colorimetric method used for phenolic determination is based in a single electron transfer reaction in which phenolic compounds are oxidized at high pH, yielding a colored product with max 765 nm. Knowledge of their concentration of these nutraceuticals is fundamental for evaluating the level in comminuted orange which is a source of these compounds, and can provide antioxidant activity. The amount of total and free phenols, is estimated at (286.5 and 180.6 mg GAE/100 g) respectively. Concentration of total phenols found in orange comminuted is similar to that reported by the U.S. Department of Agriculture for navel orange (337 mg GAE/100 g wb). then free phenols were subtracted from total phenols, and 36.6% of conjugated phenols were calculated, indicating that most phenols are in free form. This percentage is similar to that reported by[5] and similar to that obtained in orange juice by Sun *et al*.[21] (30% of bound phenols).[22] From this result we find that the heat used in the processing of this product did not affect the ratio of phenols, and then, antioxidant activity also.

Table 1: Analysis of total polyphenols in six different samples of Orange comminuted.

Orange samples	Total Polyphenols	Polyphenols Free
Orange comminuted	286.5	180.6
Orange Juice	145.5	52.2

Values are the mean of duplicate experiments and represented as mean±SD .to equivalents GAE= Gallic acid

Cytogenetic analysis

The orange comminuted is relevant to human health. Where used as a refreshing drink base to enhance nutritional and sensory characteristics, by the hundreds of millions of people across the world. It is considered a source of dietary constituents. In this study, cytogenetics effects of orange comminuted were investigated in cultured human lymphocytes. The peripheral lymphocytes are the best materials for the determination of cytogenetic effects. MI, MN and RI are used as indicators of adequate biomarkers, as a useful tool for the measurement of genotoxicity in vivo and in vitro cultures.

Mitotic index (MI)

The results of orange comminuted in concentrations (5,10 and 15) µg/ml on the mitotic index of lymphocyte cultures comparison with the negative control group (lymphocyte blood cells only without treatment) and positive control (lymphocyte blood cells with MMC treatment) was shown in table(2) 3.72, 3.86, 4.02 respectively, no significant differences were observed among the three concentrations (5,10 and 15) µg/ml of the orange comminuted, comparison with the negative control ($p<0.05$) on lymphocyte cultures. The MI assay is used to characterize proliferating cells and to identify compounds that inhibit or induce mitotic progression. The proportion in hibition of cells in the M-phase of the cell cycle is considered as cellular death or delay in the cell proliferation kinetics.[23]

Table 2: Mitotic index (MI)(%) in human lymphocyte cultures exposed to different concentrations of orange comminuted.

Test substance	Concentration µg /mL	MI%
Control	0	4.12
Positive control (MMC)	0.25	1.32**
Orange comminuted	5	3.72
	10	3.86
	15	4.02

ANOVA: ** - significantly different from control ($P<0.05$)

MI: Mitotic Index

Replication index (RI).

RI measures cell division kinetics by counting the percent of cells containing 1, 2, 3 or more nuclei per individual cell.[24] Through the results showed that all the tested concentrations of orange comminuted in cultured peripheral blood lymphocytes did not lead to a marked decrease in the RI in comparison with the control group. As shown in Table 3, increasing extract concentrations of orange comminuted had increase the RI, but these increases were not statistically significant ($P<0.05$).

Table 3: Replication index (RI) in human lymphocyte cultures exposed to different concentration of orange comminuted

Test substance	Concentration	Cell cycl Progression %			(RI)%
		1M	2M	3M	
Control	0	34	28	40	1.98
Positive con. Mitomycin-c (MMC)	0.25	6	28	66	1.40**

Orange comminuted	5	30	34	36	1.94
	10	30	32	38	1.92
	15	32	33	35	1.97

ANOVA: ** - significantly different from control (P<0.05)

RI: Replication Index = (1xM1 + 2xM2 + 3xM3)/500

M1: The number of cells in first metaphase

M2: The number of cells in second metaphase

M3: The number of cells in third or more metapzz

Micronucleus (MN).

The results of micronucleus (MN) test are given in Table 4. After treatment with different concentrations of Orange comminuted, (5,10,15) µg / ml), it did not induce a significant increase in the numbers of micronucleus in relation to the negative control (p>0,05) therefore, no clastogenic and aneugenic effect were seen. Only MMC (positive control) induced a significant increase in the amount of micronucleus (p<0,05) (Table 4).

The micronucleus (MN) test are a very sensitive index of genetic damage.[25] According to Cammerer *et al*[26] the micronucleus assay done in peripheral blood has further advantages, such as the easy preparation of the sample with small amount of blood, the speed in obtaining results and the ability to obtain repeated samples. An increase in the frequency of micronuclei is an indicator of chromosomal damage induced.[27] Micronuclei are an indicator of numerical and/or structural chromosome aberrations during cell mitosis.

Table 4: Micronucleus (MN) (%) in human lymphocyte cultures exposed to different concentration of orange comminuted.

Test substance	**Concentration µg /mL**	**Distribution of MN in BN**				**MN%**
		MN0	**MN1**	**MN2**	**MN3**	
Control	0	**998**	**0**	**0**	**6**	0.6
Positive con. (MMC)	0.25	959	8	12	21	6.1**
Orange comminuted	5	993	0	0	13	1.3
	10	996	0	0	10	1.0
	15	994	0	0	8	0.8

ANOVA ** - significantly different from control (P<0.05)

MN: Micronucleus

The ways processing industrial products, eg, enzymatic treatment, pressing, temperature, pressure and fermentation, can affect the levels of polyphenols and vitamins thus causes to effect its nutritional quality. Most of studies, focused on the effect of a thermal processing on nutritional quality through its effect on the three main families of bioactive compounds during processing industrial, such as carotenoids, phenolic compounds and vitamin C.[28] Some studies descrip the impact of technological processes on various antioxidants, like vitamin C,[8] while another studies focused on the loss of one type of micronutrient, eg, one carotenoid;[29; 30] or two types of micronutrients, such as phenolic compounds and vitamin C;[31] or flavonoids and carotenoids;[32] and phenolic compounds.[33; 34]

The results obtained by us indicate that the treatment of lymphoid blood cells with different concentrations of orange comminuted at all concentrations, from three different parameters, mitotic index (MI), replication index (RI) and micronucleus (MN), were almost similar to that of controls. As our results confirmed the safe use of this product, it could be the reason that the high content of phytochemicals in comminuted orange is due to the use of peel and juice in its formulations, it has a protective characteristies could be from the antioxidative, The antioxidant property of this product has been mainly attributed to many of the natural bioactive compounds, such as vitamin C, phenolics, and carotenoid contents,[5] and it is known that natural bioactive substances can modify redox status and interfere with basic cellular functions such as cell cycle and apoptosis.[35] Many studies had confirmed that bioactive components may act as a blocking agent, thus preventing the metabolic activation of pro mutagens. They can also form adducts or scavengers of free radicals such as the activity of phenolics and vitamin C in vitro and in vivo.[36] Studies had pointed out to the importance Vitamin C in reduceding (L-ascorbic acid, AA) and oxidizeding (dehydroascorbic acid, DHAA); thus it prevents the oxidative damage of lipids, DNA and proteins, which are associated with the development of chronic degenerative illnesses such as cardiovascular disease, cancer.[37] Phenolic compounds are classified as flavonoids and non flavonoids, flavones are exclusively found in citrus fruits, especially in the peels.[38] Phenolic compounds have strong antioxidant activity which is associated with their ability to scavenge free radicals, break radical chain reactions and chelate metal ions. Increased consumption of phenolic compounds is found to be associated with reduced risk of several diseases such as cardiovascular diseases and certain types of cancer.[39] Flavonoids from peels citrus have shown antioxidant activity, anti atherogenic, and addition to effective anticancer.[40] Some carotenoids, they also exhibit antioxidant activity ,the prevention of cancer,[41,42] These

findings supported the previous reports regarding the antioxidant effect of. The orange comminuted could protect the normal cells from the genotoxic, or carcinogenic agents This protection, Compared with other foodstuffs widely recognized as sources of antioxidants, such as blackberry, blueberry and red grape respectively,[43] antioxidant capacity of comminuted could be considered as high, due to the content of carotenoids and other lipophilic compounds in peel orange such as limonene, the latter being the most active component.[44]

CONCLUSION

It is concluded that orange comminuted a good source of natural antioxidants, have no genotoxic effects, showed considerable anticlastogenic and antigenotoxic effects as observed in vitro in human lymphocytes, therefore, can be considered orange comminuted is safe for consumption, we can use this product as a potential ingredient for developing functional beverages of low cost as compared with the orange juice. Further studies should be carried out to determine the effects of other components outside the main, which founds in beverages formulated with comminuted orange will be also essential to guaranty beneficial effects.

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