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RESEARCH ARTICLE

IN VITRO GENOTOXICITY OF THREE PLANTS FROM ASTERACEAE FAMILY IN HUMAN LYMPHOCYTES CULTURES

M. Kinan Aljaja[1]*, Adawia Kitaz[1], Raghda Lahdo[2]
[1]*Dept. Of Pharmacognosy and medicinal plants, Faculty of Pharmacy, University of Aleppo, Syria.*
[2]*Dept. Of Biochemistry and Microbiology, Faculty of Pharmacy, University of Aleppo, Syria.*

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***Address for Correspondence:**
Dr. M. Kinan Aljaja, Dept. of Pharmacognosy and medicinal plants, Faculty of Pharmacy, University of Aleppo, Syria. Tel-:+963940309008;
E-mail: *ph.kinan.aljaja@gmail.com*

Abstract

Background: *Onopordum carduiforme, Centaurea verutum,* and *Achillea santolina* are medicinal plants grown in Syria and commonly used in traditional medicine. Such as antibacterial, antioxidant and anticancer properties. However, the genotoxic effects of these plants have not been studied.
Aim and objective: the aim of this study was to evaluate the genotoxic effects of hydroethanolic extracts of these plants on human lymphocyte cultures model by evaluating the cell proliferation, determination of mitotic index (MI), and their effects on chromosomes.
Methods: the hydroethanolic extracts of the aerial parts of the three plants were extracted using an Ultrasonic bath. Then the genotoxic effects of hydroethanolic extracts of these plants on human lymphocyte cultures was conducting by determination of mitotic index (MI).
Results: the results showed that all three plants decreased non-significantly the mean of mitotic index in comparison with negative control (normal MI) (p>0.05) at concentrations (1, 3, 5 mg/ml) and the mitotic index values ranged was between (2.25±0.07 and 3.3±0.28). However, *C. verutum* showed the lowest mitotic index (3±0.14 at 1 mg/ml) and (2.25±0.07 at 5 mg/ml), and did not induce chromatid or chromosome breaks or gaps.
Conclusion: these preliminary results on cytotoxicity and mutagenicity of these plants provide valuable information about the safety of using them in alternative medicine.
Keywords*: Achillea, Centaurea*, human lymphocytes cultures, karyotype, mitotic index (MI), *Onopordum*.

INTRODUCTION

Medicinal plants have played a crucial role within the treatment of human sicknesses all over the world. The chemical components of medicinal plants are important for development of the new medicines[1]. *Onopordum carduiforme, Centaurea verutum,* and *Achillea santolina* are the plants used in this study. These plants belong to the Asteraceae family, which is one of the largest families in the plant kingdom, with an estimated number of 25,000 species spreading in wide areas. In addition, this family comes second in Syria in terms of species figures (322 species in Syria) belonging to 99 genera[2].

O. carduiforme is a biennial or perennial herb, 30-100 cm height, sparsely cobwebbed, stem erect, much-branched; wings consisting of triangular long-spiny lobes connected by a narrow green margin, leaves are almost glabrous on the upper face, often cobwebbed and greyish on the lower, florets dark purple. The heads are numerous. Involucre 2-5 cm in diameter (not including spines). Florets time: March-August[3]. *Onopordum* genus has been commonly used in folkloric treatments, as an emetic expectorant, anti-asthmatic, and diuretic to treat nervousness, tetanus, and carcinomas. Various studies have demonstrated the efficiency of antihypertensive, antibacterial, cardio tonic, and hemostatic properties, increasing the tone of smooth muscles, and having protective activity against lipid peroxidation. These therapeutic properties are related to the wide range of secondary metabolites content, such as phenolic compounds, lignans, sesquiterpene lactones, coumarins, terpenes, and steroids[3-5]. *C. verutum* is an annual plant, 50-130 cm height, stem rigid, simple, erect, or with erect branches; stem and leafy branches, lower leaves are mostly sinuate, other leaves are entire, oblong, the florets are yellow. Involucre broadly ovoid, truncate at base, 1.5-2 cm (not including spines). Flowering head 2-3 cm in diameter, Florets time: May-July[3]. This plant was used

for the treatment of infectious diseases, stomach ache, edema, swellings, arthritis, and pain[6]. Several studies have confirmed that species of Centaurea have antibacterial and antioxidant properties, and these properties are due to flavonoids and sesquiterpene lactones, lignans, alkaloids, simple phenolics, steroids, triterpenes, hydrocarbons, polyacetylenes, anthocyanins[6,7].

A. santolina is a perennial herb, 15-30 cm height, woolly, stems erect to ascending, simple or branched, leafy up to the inflorescence, leaves narrow, linear, green. Heads are radiate, in compound and corymbs. Involucre 4-5 mm. Florets time: March-April[3]. Pharmacological studies have shown that *Achillea* genus has different chemical and therapeutic properties as an anti-hyperlipidemia, Diuretic, hypotensive action, and antioxidant[5,8], and these therapeutic properties due to phenolic compounds (phenols, flavonoids) such as, rutin and luteolin, essential oils, sesquiterpenes. However, extreme usage of medicinal plants can affect poisoning and death in humans and animals. Therefore, the genotoxicity and cytotoxicity of medicinal plants were used by the population are necessary to be aware of, along with the ones which pose mutagenic and carcinogenic risks[1,5]. Cytogenetics is the area of genetics that studies chromosomes, consisting of numerical (46 chromosomes) changes, and their relationship to structural imbalances morphology, organization, and physiology. There is a bond between numerical and structural chromosomal and syndromic diseases, which include describing trisomy 21 in Down syndrome, describing monosomy X in Turner syndrome[9]. Lymphocytes cultures is a blood cell karyotyping method which gives information about chromosomal abnormalities, such as, mitotic index (MI) as a measure of cytotoxicity: the MI assay is used to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression[10]. The Mitotic index is described as the rate between the number of cells in mitosis and the total number of cells. MI can be worked out from a slide by using light microscopy. And to quantify cell division, colchicine, or other colchicine-derivative medications are used (i.e. colcemid).

Colchicine can arrest the cell cycle at this point leaving the chromosomes in their seen shape[11]. So far, no clinical evidence has been found in the literature regarding the genotoxic effects of these plants. It is of interest to determine whether the use of these plants has any genotoxic effect of the cytological and chromosomal levels. This work evaluates the genotoxic effects of *O. carduiforme, C. verutum,* and *A. santolina* grown and used in Syria as folk medicine, by treating human lymphocyte cultures with their hydroethanolic extracts, and indicated these plants as anti-tumor activity.

MATERIALS AND METHODS

Plant Material

Fresh aerial parts (stems, flowers, leaves) of *O. carduiforme, C. verutum, A. santolina* were collected from different areas of Aleppo city in Syria. Plant specimens were identified by Dr. Ahmed Jadouh, Professor and expert at the Faculty of Agricultural Engineering, Aleppo University, Syria. The aerial parts were washed under running tap water, shade dried, then powdered using a mechanical grinder and kept in an airtight glass container until use.

Chemical reagents

Ethanol GR (Eurolab, UK), KCl, chromosome medium P (EuroClone S.p.A, Italy), Colchicine 10 µg/ml, Glacial acetic acid, trypsin 1g/l, Giemsa stain.

Equipment

Sensitive balance (Sartorius TE214, Germany), rotary evaporator (Heidolph Instruments, Germany), UV-1800 spectrophotometer (Shimadzu, Japan), ultrapure TM water purification system (Lotun Co., Ltd., Taipei, Taiwan), centrifuge Germany, (Heraeus Megafuge), ultrasonic bath (POWERSONIC 405, Hwashin Technology Co., Korea), disposable syringes 5 ml (UK), disposable tub (China), heparin tub (China), autoclave, incubator, light microscopy.

Preparation of extracts

The powdered plant samples (130 g of each plant) were extracted by ultrasonication assisted extraction, using ten folds of ethanol 70% at 40ºC for one hour. The extracts solutions were then filtered through Whatman No. 1 filter papers, and the residual material was re-extracted three times using the same procedure. The combined extracts were evaporated using the rotary evaporator at 40°C to remove the solvent. The crude extracts were kept separately in sterile sample tubes and stored at 4°C for further usage. The yield percentage was then calculated using the following equation[5].

$$\text{Yield (\%)} = \frac{\text{Wex}}{\text{Wp}} \text{x100}$$

Where W_{ex} is the weight of the dried extract and W_p is the weight of the dried plant material[5].

Lymphocyte cultures and cell harvesting.

Blood sample collection

Whole venous blood was collected from healthy volunteers (3) male (all participants' volunteers were informed by the aim of the study and verbal permit was obtained from each subject to participate in this study), who were not taking any medications, and their ages ranged between 18-30 years old and placed into a heparinized tube[10].

Lymphocyte cultures and cell harvesting

Using an aseptic technique, 500 µl of blood was added to 10 ml of chromosome medium P in sterile culture tubes, then 200 µl of plants' extracts were added to obtain the concentrations (1, 3, 5 mg/ml). And one of the tubes does not contain plants' extracts as a negative control. Mixing the contents of each culture tube by shaking gently, then the culture tubes were incubated in a slanted position at 37°C for 71 h[11].

Harvesting of the lymphocyte cultures

After 71 h of incubation, 100 µl of colchicine (10 µg/ml) was added to each culture tube and mixed by shaking gently. This add is necessary for the spindle fibers to separate the chromosomes during anaphase. And to arrest the cell division at metaphase. Cells were harvested by centrifugation at 1200 r/min for 10 min. Then added hypotonic solution (0.75 M KCl) about 8

ml to the pellet pre-warmed and Incubated for 25 min at 37°C[10].

Fixation of lymphocyte cultures

Approximately, 8 ml of fixative solution (3:1 ratio of methanol and acetic acid) was added and centrifuged four times of fixative washes were given. The fine pellet was dropped on microscopic sterile and chilled slides[10].

Spreading

Chromosome spreads were prepared by gently dropping the cell suspension from a height of 40-60 cm onto a clean grease-free chilled slide and air-dried[11].

Staining

G-banding Technique: Slides were rinsed in distilled water and dried at room temperature. Then, soaked in trypsin (0.15 g/100 ml) for 7 Sec at 37°C, rinsed, and stained with 5% Giemsa solution for 10 min[11].

Examination of Mitotic Index (MI)

MI was calculated as the proportion of metaphase for 1000 cells for each donor and concentration[1].

$$\text{Mitotic Index (MI)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} X100$$

Statistical analysis

The effect of plants' extracts on MI was performed in duplicate. The results were expressed as mean values±standard deviation (SD). Statistical analysis was carried out using SPSS Version 25; where the data were subjected to ANOVA one-way test. Differences were considered significant at probability *p* value < 0.05.

RESULTS

Extraction yield

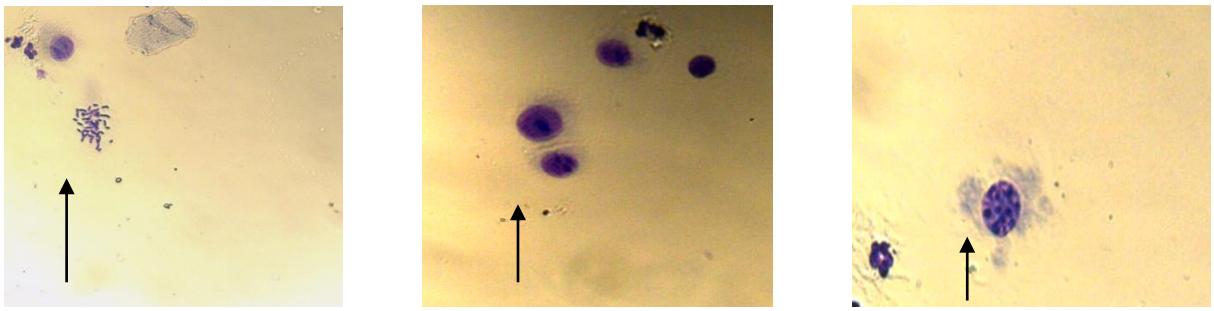
The crude dried extracts obtained were nicely aromatized, brown, and the yield percentage of the solid residue weight of *C. verutum* extract was higher than the other plants, followed by *O. carduiforme* extract and *A. santolina* extract came last in yield percentage[5], as shown in (Table 1).

Table 1: The yields percentage of solid residue of plants extracts.

Plants	Yield (%)
C. verutum	13.44
O. carduiforme	15.92
A. santolina	9.17

Determination of mitotic index (MI)

For the calculation of the mitotic index, the number of dividing cells has been counted for every 1000 cells counted, by using light microscopy. The microscopic slides sights showed three types of cells: a-Dividing cells, b-Non-Dividing cells, c-Blast cells (Figure 1). The effects of plants' extract on MI are shown in (Table 2, Figure 2). These results showed that there was a decrease in the mean of Mitotic Index values depending on the increasing plant concentrations, but not significantly in comparison with negative control (normal MI) (p>0.05). The mean value of MI of the control is 4.05±*.0.21* The mitotic index range was between (2.25±0.07 and 3.3±0.28). *C. verutum* showed the lowest Mitotic Index (3±0.14 at 1 mg/ml) and (2.25±0.07 at 5 mg/ml). While *A. santolina* has the highest Mitotic Index 2.85±0.07 at 5mg/ml. Statistically, no significant effect was found between culture tubes that were treated with the extracts compared to culture tubes without extracts (negative control) at probability *p* value >0.05 and did not have any mutagenic effects, such as inducing chromatid or chromosome breaks or gaps (Figure 3), deleted or duplicated chromosome (Figure 4).



a). Dividing cells. b). Non-Dividing cells. c). Blast cells.

Figure 1: Images of the human lymphocyte cells stained with 5% Giemsa solution under microscope (M.B. 100x).

Table 2: The Mitotic Index (mean±SD) of human lymphocyte cultures (1000 cell counted) treated with *O. carduiforme, C. verutum, A. santolina* extracts and Negative control.

Plants' extracts	Mitotic Index (Mean±SD) Concentration (mg\ml)		
	1	3	5
C. verutum	3±0.14	2.8±0.14	2.25±0.07
O. carduiforme	3.25±0.21	2.9±0.14	2.6±0.14
A. santolina	3.3±0.28	3±0.14	2.85±0.07
Negative control (Normal MI)		4.05±0.21	

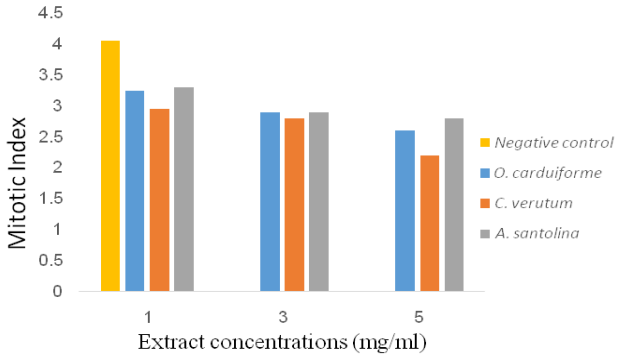
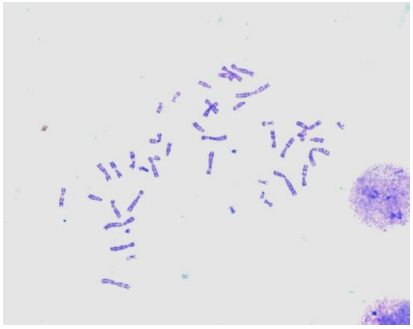


Figure 2: MI values of human lymphocyte cultures treated with different concentrations of plants' extracts.

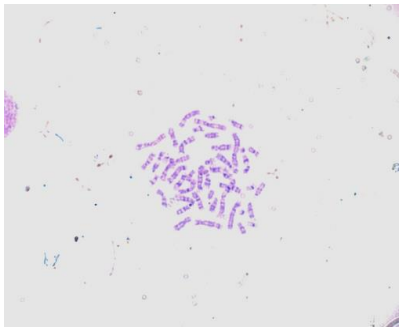
All images show that there are not any Genotoxicity effect on the chromosomes, the extracts do not change the number (46 chromosomes), and do not affect on structural imbalances morphology, organization, and physiology.

DISCUSSION

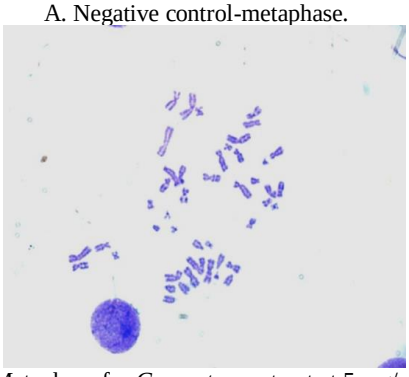
All Plants' extract decreased the mean of mitotic index (MI) and the range was between (2.25±0.07 and 3.3±0.28). However, these values are all not significant compared to the negative control. Nevertheless, *A. santolina* was the best among the studied plants because it has the highest MI values at all concentrations used (3.3±0.28 at 1 mg/ml, 3±0.14 at 3 mg/ml 2.25±0.07 at 5 mg/ml) close to the normal MI (4.05±0.21). The decrease in the mean of mitotic index may be due to the phenolic compounds: flavonoids such as, Artelasticin, Artelastin, Artelastochromene, phenols and tannins derivatives and their concentrations since they induced DNA damage, inhibits replicative synthesis and inhibit cell proliferation. This effect due to the presence of a C-2,3- double bond and the potency of inhibition was dependent on the number and position of hydroxylation of the B-ring[8,12,14-16,]. Studies showed that the flavonoid and thymol decreased MI at a higher concentration, and also the pure resveratrol from grape skin antagonized the mitotic action of PHA and acted as an anti-mitotic agent[17,18]. The extracts from three plants which are investigated in the current study contain flavonoids and tannins (Phytochemical screening), and the highest flavonoid content was observed in *C. verutum*[5]. This could explain why this plant showed the lowest Mitotic Index (3±0.14 at 1 mg/ml and 2.25±0.07 at 5 mg/ml). However, these values are not significant compared to the negative control which makes these plants nontoxic on lymphocytes within the studied concentration. A study of *Helichrysum* species from Asteraceae family showed that three genera of *Helichrysum* decreased the MI and the values were significant compared to the negative control, in order that they were genotoxic and they should not be used although their antiproliferative activity may suggest antimitotic and anticarcinogenic properties[14].



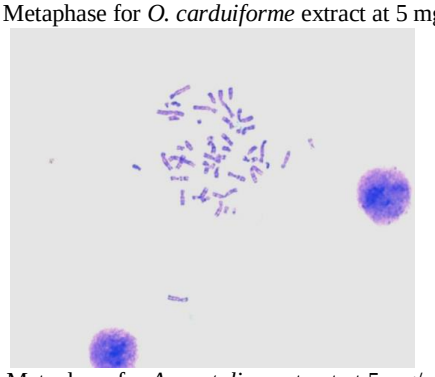
A. Negative control-metaphase.



B. Metaphase for *O. carduiforme* extract at 5 mg/ml.

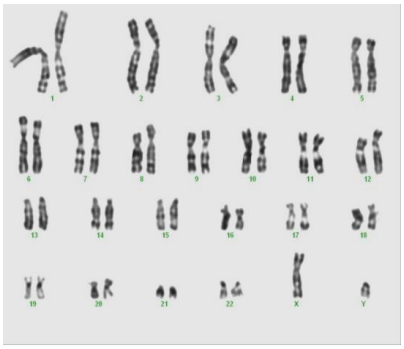


C. Metaphase for *C. verutum* extract at 5 mg/ml.

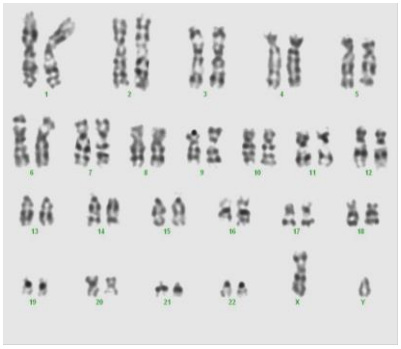


D. Metaphase for *A. santolina* extract at 5 mg/ml.

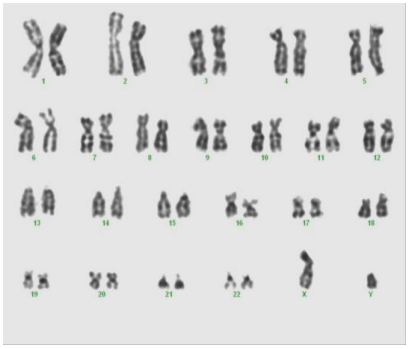
Figure 3: Metaphase of human lymphocytes cultures stained with 5% Giemsa solution and treated with plants' extracts at 5 mg/ml (M.B. 100x).



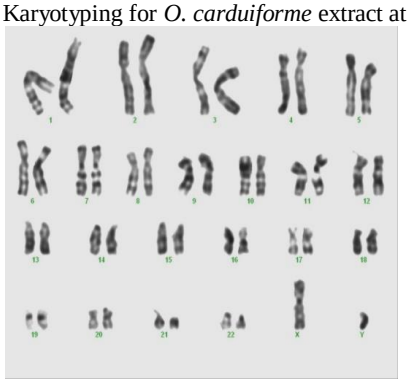
A. Karyotyping for negative control.



B. Karyotyping for *O. carduiforme* extract at 5 mg/ml.



C. Karyotyping for *C. verutum* extract at 5 mg/ml.



D. Karyotyping for *A. santolina* extract at 5 mg/ml.

Figure 4: Karyotyping of human lymphocytes cultures treated with plants' extracts at 5 mg/ml.

Other studies showed that the same genus of plants studied in the current study had antitumor activity, such as *A. millefolium* which does not have a cytotoxic effect, and it has antiproliferative activity on different human liver tumors, whereas the average inhibition of proliferation was 55.3% for the HepG2/C3A, SK-HEP-1, and HA22T/VGH lines and 20.3% for the lines Hep3B and PLC/PRF/5[19]. Accordingly, and since the decrease in cell proliferation is considered one of the mechanisms adopted as anti-tumor effects, plants in this study could be candidates as anti-tumor because they reduced the mitotic index of lymphocytes, yet not significantly. Therefore, we suggest conducting studies on the same plants but at higher concentrations to confirm the possible anti-tumor activity or not.

Table 3: Mean of mitotic index of human lymphocytes cultures treated with plants' extracts from Asteraceae family.

Plants name	Mean of Mitotic Index MI	Concentration (mg\ml)	Significant or not significant	References
O. carduiforme	2.6±0.14	5	not significant	The current study
C. verutum	2.25±0.07	5	not significant	
A. santolina	2. 85±0.07	5	not significant	
A. millefolium	2.43± 0.5	3.5	not significant	[12]
S. marianum	2.63±0.5	0.1	not significant	[13]
S. marianum	2.35±0.4	0.5	not significant	
S. marianum	1.58±0.3	1	significant	
S. oleraceus	2.49±0.5	0.1	not significant	
S. oleraceus	1.83±0.6	0.5	significant	
S. oleraceus	1.12±0.2	1	significant	
M. chamomilla	2.58±0.4	0.1	not significant	
M. chamomilla	1.99±0.5	0.5	significant	
M. chamomilla	1.49±0.3	1	significant	
H. sanguineum	4.78±1.26	0.01	not significant	[14]
H. sanguineum	3.01±1.55	0.05	significant	
H. sanguineum	1.10±0.43	0.1	significant	
H. sanguineum	0.30±0.05	0.5	significant	
H. sanguineum	0.21±0.08	1	significant	

CONCLUSIONS

The primary results of this study of plants of Asteraceae family from Syrian flora conclude that the *O. carduiforme, C. verutum*, and *A. santolina* doesn't showed toxicity effect on lympho-cytes at the concentrations (1, 3, 5 mg/ml). In addition, these plants also did not induce chromatid or chromosome breaks or gaps. Thus, there is a need to investigate the anti-

tumor activity by determining the reduction of MI at higher concentrations.

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AUTHOR'S CONTRIBUTIONS

Aljaja MK: performed the experiments; analyzed and interpreted the data; wrote the paper. **Kitaz A:** data analysis, conceptualization. **Lahdo R:** formal analysis, critical review. The final manuscript was read and approved by all authors.

DATA AVAILABILITY

The data supporting the findings of this study are not currently available in a public repository but can be made available upon request to the corresponding author.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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