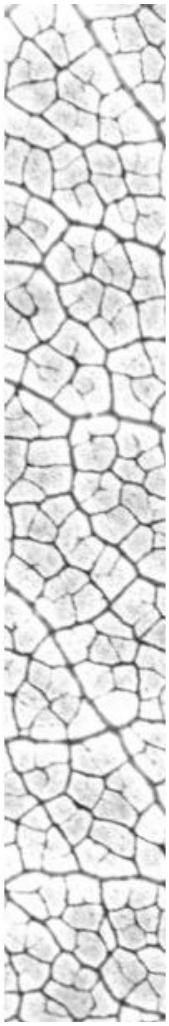

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Cytogenetic studies and cytotoxic effect of *Ruta chalepensis* extract on Swiss Albion mice and MCF-7 tumor cells

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Abstract
Ruta chalepensis is a native herb of the Mediterranean region used in the traditional medicine of many countries. Phytochemical screening has shown the presence of bioactive molecules that are responsible for its pharmacological properties. The present study aimed to investigate the effect of *R. chalepensis* methanolic extract on metaphase index and micronucleus formation in bone marrow of Swiss albino BALB/c male mice as well as *in vitro* cytotoxic effect against MCF-7 tumor cell line. The results of cytogenetic evaluations indicated that a treatment with *R. chalepensis* was associated with a significant increase in metaphase index and reduction in micronucleus formation in dose-dependent pattern comparing with blank control and vehicle control. The results of methylthiazol tetrazolium (MTT) assay on MCF-7 tumor cells indicated a significant increase in cell proliferation inhibition in dose-dependent manner after treatment with *R. chalepensis* methanolic extract with potent IC_{50} of 51.31 µg mL^{-1}, comparing with WRI68 normal cell line.

Keywords: Ruta chalepensis, metaphase index, micronucleus, MTT assay, MCF-7

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INTRODUCTION

Medicinal plants are considered as one of the most important sources, which frequently utilized by people not only for the traditional medicine but also provide a great impact in health to a large population in the world. Most of medicinal plants may relatively nontoxic or have positive effects when used by humans, while few considered very harmful with the potential of damaging certain organs in human body (Brower, 2008). The plant genus *Ruta*, commonly known as rue, has around 40 different species and members of this genus been about 20 – 60 cm tall. *Ruta* belong to the family *Rutaceae*, which cultivated in southwest Asia and Mediterranean region (Li and Vederas, 2009). The most famous species of *Ruta* are *R. angustifolia* (Egyptian rue), *R. chalepensis* (fringed rue), *R. corsica* (Corsican rue), and *R. graveolens* (Pollio et al, 2008). *R. chalepensis* contains numerous pharmacological and biological activities, it considers as the main source of important aromatic secondary metabolites that is originally found and cultivated it in the Mediterranean area and is distributed worldwide (al-Said *et al*, 1990). It is widely used for treatment of inflammation, gastric, diuretic, rheumatism disorders and headache. Chemical composition of *R. chalepensis* extracted from the the aerial parts showed many active compounds like flavonoids, phenols ,alkaloids, amino acids, furocoumarins and saponins (Eickhorst and Csaposs, 2007). Although these active components may be responsible for the plant's advantageous, the bioactivities of *R. chalepensis* extracts and its several preparations against tumor cells have involved only recently (Wessner et al, 1999). The prevalence of the cancer as a disease still remarkably high in many countries worldwide. Many factors affecting the initiation of tumor cells, nevertheless the precise cause of tumors remains enigmatic. Distributing the cellular defense mechanisms for eliminating tumor cells is mainly accepted as a background of carcinogenesis. The multidrug resistance (MDR) properties of tumor cells against chemotherapeutic drugs is one of the challenges in the clinical treatment of cancer. For this reason, the need for novel antitumoral drugs capable of targeting chemical entities and selectively induce apoptosis and/or reverse the function of MDR (Oliva *et al*, 2003). Many therapies that are now under extensive trails for cancer medicine (about 60%) are natural products or derivatives from natural sources. For this reason, plants considered as a library for chemicals with structurally diverse compounds of various compositions. Breast

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cancer is the most prevalent invasive tumor and responsible for most deaths related to cancer among women. Alkaloids, furanoacridone isolated from *R. chalepensis* showed anti-proliferative action on cell lines of female breast cancer (MCF-7, MDA-MB-361, MDA-MB-231 and T47D) (Ohnishi and Takeda, 2015). In this work, naturally occurring compounds of *R. chalepensis* with cytostatic activity towards tumor cells were detected and evaluated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plant Collection and Identification

Leaves of *R. chalepensis* were collected from different area in Baghdad during April 2019. The plant was authenticated by The Iraqi National Herbarium which revealed the taxonomical classification of the plant.

Experimental Animals

Swiss albino BALB/c male mice (*Mus musculs*) were thankfully supplied from Biotechnology Research Center/Al-Nahrain University. A total of 25 mice (their age ranged between 8-10 weeks and weighing 23-27 g) were distributed into five groups, and each group was kept in a separate plastic cage at room temperature. The mice were fed with suitable quantity of water and complete diet.

Methanolic Extraction

R. chalepensis leaves were separated and washed under running tap water. Leaves were oven-dried at a constant temperature of 50°C for 3 days. The dried leaves were then ground using a commercial blender to a fine powder. The dried powdered leaves (50 g) were soaked in 500 mL methanol and extracted used Soxhlet for 3 hrs at 65°C. The resulted extract was filtered through a Whatmann filter paper No. 3 and concentrated under pressure using a rotary evaporator (100 rpm) at 40°C (Fu *et al*, 2010).The powdered residue were transferred into vials and stored at 4°C in airtight vials for next experiments and to prepare the required doses.

Cytogenetic Analysis

Experimental Design

Three doses of *R. chalepensis* methanolic extract (50, 100 and 200 mg Kg^{-1}) were intraperitoneally administrated to mice in group I, II and III, respectively. Group IV were treated with distilled water (negative control), while group V treated with 80 mg Kg^{-1} cyclophosphamide (CYP) as a vehicle control. *R. chalepensis* methanolic extract, distilled water and cyclophosphamide were injected intraperitoneally as a single dose (0.1 mL) per a day and for 10 successive days. Groups I, II and III were subjected to intraperitoneal injection of 80 mg Kg^{-1} starting from day 4 to 6 along with extract treatment. At day 11, the mice were sacrificed for laboratory assessments.

Metaphase Index Assay

Metaphase index (MI) was determined for cells isolated from bone marrow of tested animals (Allen *et al*, 1977). Briefly, mice were injected intraperitoneally with 0.25 mL of colchicine (100 µg mL^{-1}) for 2 hours, the animals were sacrificed and the cellular contents from femur bone were collected by using normal physiological saline (5 mL). The cells were suspended in a test tube and centrifuged at 2000 rpm for 10 min. The cell deposit was suspended in 5 mL of a warm (37ºC) hypotonic solution (KCl; 0.075 M). The tubes were incubated in water bath at 37ºC for 30 minutes, and then were centrifuged at 2000 rpm for 10 min. Finally, five mL of the fixative solution, absolute methanol and glacial acetic acid 3:1 (v/v), were added and the tubes were stored in the refrigerator at 4ºC for 30 min. The cell suspension was dropped on clean slide and stained with Giemsa stain for 15 min followed by rinsing with distilled water. More than 1,000 cells were examined for each slide under oil emersion lens (100X). The percentage of metaphase cells (MI) was recorded using the following equation:

$$\text{Metaphase Index}\,(\%) = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total Count}}\right) \text{x}\,100$$

Micronucleus Formation Assay

The following procedure was conducted for micronucleus (MN) formation assessment (Schmid, 1976) was followed with minor modifications. After sacrificing the animals by cervical dislocation, they were dissected to obtain the femur bone. Both ends of the bone were cut, and the bone was grasped from the middle with a forceps in a vertical position over the edge of a test tube and then the cellular contents were collected with a heat-inactivated (56°C for 30 min) human AB plasma (2 mL) using a disposable syringe. The test tube was centrifuged (1000 rpm) for 10 min, and the supernatant was discarded after that the cellular deposit was gently mixed, and a thin smear was made on a clean slide and air-dried at room temperature. The smear was fixed with absolute methanol for 5 min and then air-dried at room temperature. Finally, the smear was stained with Giemsa stain for 15 min and rinsed with distilled water. The slides were examined under oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of MN formation. The MN index was obtained using the following equation:

$$\text{Micronucleus Index (micronucleus/cell)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}}\right) \text{x}\,100$$

Cytotoxic Activity of *R. chalepensis* Methanolic Extract Against MCF-7 Cells

Cell line and Cell Maintenance

Human breast adenocarcinoma MCF-7 and normal WRL68 cell lines were kindly provided from Biotechnology Research Center/Al-Nahrain University. Cells were cultured in 75 cm^2 flask containing RPMI-

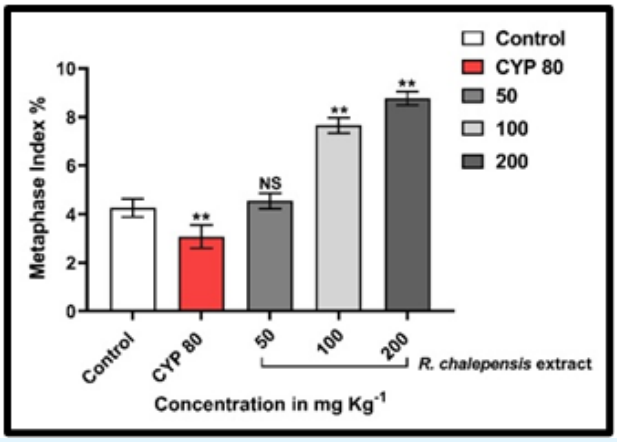

Fig. 1. Mean percentage (±SD) of metaphase index of Swiss albino BALB/c male mice bone marrow cells treated with 50, 100 and 200 mg kg^{-1} *R. chalepensis* methanolic extract. All groups were compared with untreated control group (n = 5). NS: Nonsignificant, **: $p \leq 0.01$, SD: Standard Deviation

1640 media (Sigma-Aldrich – Germany), supplemented with 10% fetal bovine serum (Sigma-Aldrich – Germany), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37ºC (Shamsee et al, 2019).

MTT Assay

The cytotoxic effect of different concentrations (6.2, 12.5, 25, 50, 100, 200, and 400 µg/mL) from *Ruta chalepensis* methanolic extract was performed using MTT ready to use kit (Intron Biotech - Korea) against MCF-7 and WRL68 cells. cells (1×10^4 to 1×10^6 cells mL^{-1}) were seeded onto 96-well micro-titer plates to reached final volume of 200 µL and then cultured at 37°C, 5% CO_2 for 24 hrs. After reaching 80% confluent cells, the medium was replaced with fresh medium containing the desired treatment. Plates were further incubated at 37ºC, 5% CO_2 for 24 hrs. Each treatment, in addition to the controls (cells treated with serum free medium), were evaluated in triplicate. After incubation, 10 µL of MTT solution was added, and then incubated at 37°C, 5% CO_2 for 4 hrs. After removing the media carefully, 100 µL of solubilization solution (DMSO) was added to each well and incubated for 5 min. Formation of formazan was determined by measuring the absorbance at 570 nm using an ELISA microplate reader (Bio-Rad, USA).

Statistical Analysis

One mode examination of variance ANOVA (Duncan) was made to test whether group alteration was important or not. Statistical significance was defined as * $p \leq 0.05$ and ** $p \leq 0.01$. Data were expressed as mean± standard deviation and statistical significances were carried out using Graph Pad Prism version 8 (Graph Pad Software Inc., La Jolla).

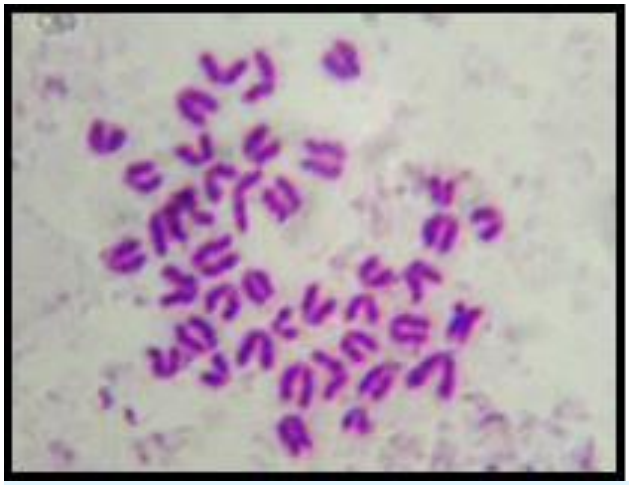

Fig. 2. Bone marrow cell of Swiss albino mouse treated with 200 mg Kg^{-1} at metaphase (100X)

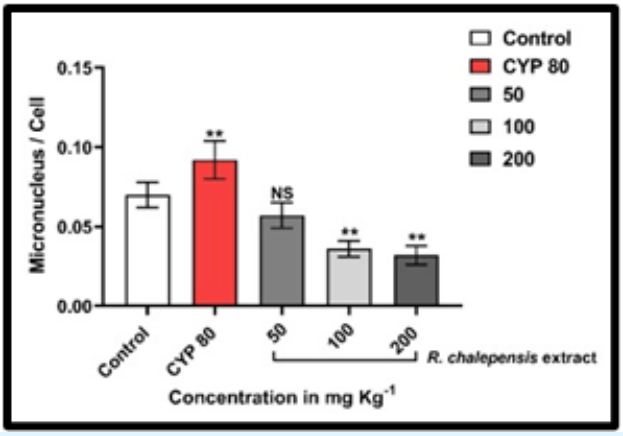

Fig. 3. Mean frequency (±SD) of micronucleus formation of Swiss albino BALB/c male mice bone marrow cells treated with 50, 100 and 200 mg kg^{-1} *R. chalepensis* methanolic extract. All groups were compared with untreated control group (n = 5). NS: Nonsignificant, **: $p \leq 0.01$, SD: Standard Deviation

RESULTS

Cytogenetic Analysis

Results of MI (**Fig. 1**) revealed that the percentage of MI was significantly increased by increasing the *R. chalepensis* methanolic extract concentration. All treatments except 50 mg Kg^{-1} were significantly different from control. As expected, the vehicle control (CYP treatment) significantly (p = 0.0002) decreased the MI%. *R. chalepensis* methanolic extract was able to modulate the reduction effect of CYP and restored MI almost to their normal percentage (**Fig. 2**). At higher doses, the MI exceeded the control significantly, in which 7.66 ± 0.32% and 8.76 ± 0.28% were recorded for 100 and 200 mg Kg^{-1}, respectively.

The spontaneous frequency of MN treatment groups was illustrated in **Figs. 3** and **4**. Results indicated that the MN frequency in control group was 0.070 ± 0.008

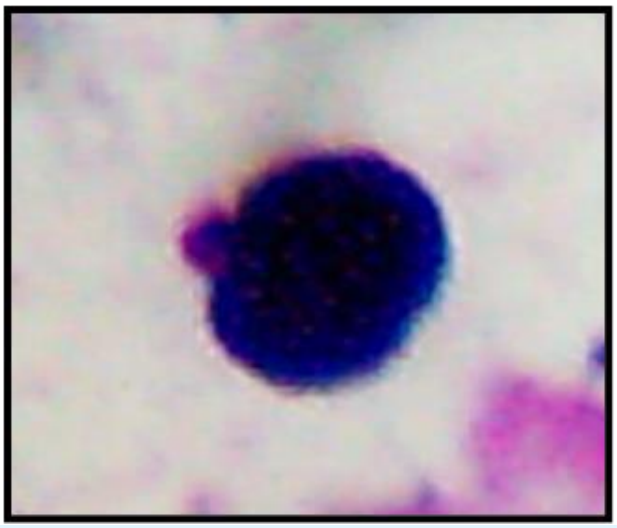

Fig. 4. Micronucleus formation in Swiss albino mouse bone marrow cell treated with 80 mg Kg^{-1} cyclophosphamide (100X)

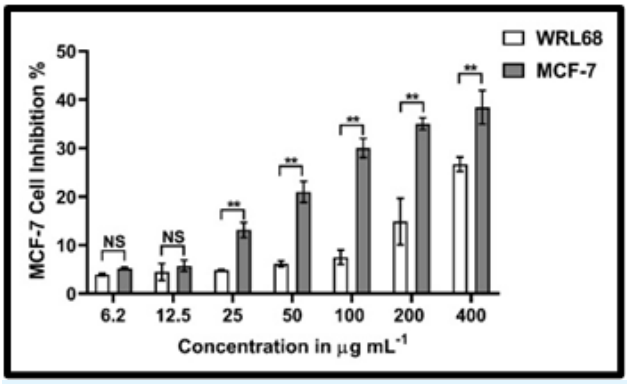

Fig. 5. Mean percentage (±SD) of growth inhibition. MTT assay for *R. chalepensis* methanolic extract against MCF-7 and WRL68 cells at 37ºC for 24 hrs (n = 3). NS: Nonsignificant, **: $p \leq 0.01$, SD: Standard Deviation

MN/cell, while in vehicle controls (CYP-treated mice), the frequency significantly (p = 0.0014) increased to 0.092 ± 0.012 MN/cell. Significant reduction of MN frequency was observed when mice treated with *R. chalepensis* methanolic extract in a dose-dependent manner mainly at 100 and 200 mg Kg^{-1} with MN frequency of 0.036 ± 0.005 (p < 0.0001) and 0.032 ± 0.006 (p < 0.0001) MN/cell, respectively. On the other hand, treatment of 50 mg Kg^{-1} showed no significant differences compared with control.

Cytotoxic Effect of *R. chalepensis* Mthanolic Extract

The test of MTT was accomplished to conclude the cytotoxic effect of *R. chalepensis* methanolic extract on MCF-7. MTT Assay was made to calculate the cell viability and inhibition rate on the tumor cell line by using different concentrations of plant extract. The percentage viability of treated cells was calculated in a comparison with normal cell line WPL68. The cytotoxic effect of *R. chalepensis* methanolic extract in concentration ranged from 6.2 to 400 µg mL on MCF-7 cells (**Fig. 5**) exhibited an increased inhibition in cell proliferation in a dose-dependent pattern. The cell viability was reduced by increasing the concentration of the plant extract. The *R. chalepensis* methanolic extract exhibited significantly the most potent cytotoxic activity with IC_{50} value of 51.31 µg mL^{-1} comparing to the extract effect against WRL68 normal cells with a recorded IC_{50} of 374.7 µg mL^{-1}.

DISCUSSION

Natural products have been a great success in our society with the usage of plant and its secondary metabolites in reducing ailments (Al-Ezzy et al, 2018). The use of plant has helped in doubling our life span in the 20th century. Since their chemical diversities are based on biological and geographical diversity (Al-Ezzy et al, 2017). One of these medicinal plants is *R. chalepensis* which originates from Southern Europe and North Africa. It is used for medicinal and culinary purposes since ancient times. *R. chalepensis* decoction is commonly used to cure cramps, flatulence fever and as anticancer agents (França Orlanda and Nascimento, 2015). All these bustles due to its chemical compounds like coumarin, alkaloid and flavonoids. Flavonoids can reduce oxidative stress by directly scavenging free-radicals, due to interfering with free-radical producing mechanisms; therefore, increasing the function of endogenous antioxidants (Alotaibi et al, 2018). The results of genetic evaluations showed that a treatment with *R. chalepensis* was associated with a significant reduction in MN formation and increase metaphase index and such effects were highly dependent on dose. These results could be attributed to these functions and thus suggesting that the administration of *R. chalepensis* which resulted in reduction of MN frequency, considered as a safe with beneficial anti-mutagenic potential and protective effect on the DNA of treated bone marrow cells (Dahija et al, 2014). The reason behind such activity that *R. chalepensis* extract contains several vitamins and polyphenolic compounds which play a vital role in the reducing the mutagenic effect. Flavonoids may have an additive effect to the endogenous scavenging compounds by increasing the activity of detoxifying enzymes such as glutathione transferase and superoxide dismutase (Akkari et al, 2015). In addition, flavonoids can stimulate immune system, inhibition of DNA adducts with carcinogens, inhibition of hormonal actions and metabolic pathway associated with the development of cancer, and inducing phase I or II detoxification enzymes (Carocho and Ferreira, 2013, Ibraheem et al, 2018).

It was proposed that isolated compounds from the methanolic extract of roots and aerial parts of *Ruta* species stimulated the inhibition of platelet aggregation property and exhibited cytotoxicity against different

cancer cell lines such as colon, lung, breast and cervical cancers (Haddouchi et al, 2013). The naturally occurring furanoacridones in *Ruta* species can be regarded as excellent starting structures for the potential development of new anticancer agents (Haddouchi et al, 2013). Other than that, leave extracts of *Rut* sp. were found to be commonly used by the Chinese community in treatment of cancer (Kacem et al, 2015). Many compounds related to *Rut* sp. including dictamine, skimmianine, psoralen, chalepensin, clausindin, graveolinine, arborinine and isopimpinellin exhibited cytotoxic activity against different tumor cell lines (Raghav et al, 2006). Several alkaloids isolated from natural herbs exhibit antiproliferative and anti-metastatic effects on various types of cancers both *in vitro* and *in vivo* (Pollio et al, 2008). Alkaloids, such as camptothecin and vinblastine, have already been successfully developed into anticancer drugs (El Sayed et al, 2000). Rutamarin and chalepin are furanocoumarins that mostly abundant in *Rutaceae* and *Umbelliferae* family, such chemicals exhibited anti-proliferative of many types of tumor cells (Ashour et al, 2011).

CONCLUSION

We can conclude that *R. chalepensis* extract exhibited potent protective effect on the genetic materials of normal cells and promising anti-proliferative potential on tumor cells *in vitro*, which needed more investigations regarding chemical composition of *R. chalepensis* extract with studying the mechanism of tumor cell inhibition.

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