RESEARCH ARTICLE

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Cytotoxic Activity of *Hypericum triquetrifolium* Turra Methanolic Extract Against Cancer Cell Lines

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Abstract

Background: Since ancient times, several people in the Mediterranean region have employed *Hypericum triquetrifolium* Turra in traditional medicine. However, only the composition of its essential oils has received extensive research. **Objective:** This study investigated the cytogenetic and cytotoxic effects of *H. triquetrifolium* methanolic leaf extract on four different cancer cell lines. **Methods:** Methanolic extract of *H. triquetrifolium* leaves was prepared. Albino male mice were grouped into five: group 1 (blank control) received water;group II (CYP) received cyclophosphoamide; groups III, IV, and V were administered 50, 100, and 200 mg/kg of *H. triquetrifolium* extracts. On the 11th day, the animals were sacrificed and bone marrowwascollected. The metaphase index (MI) of the bone marrow cells was determined to evaluate the cytogenetic effect of the extract. The cytotoxic activity of the extract was tested on four cancer cell lines (HepG2, PC3, MDA, and A594), while WRL-68 normal cells were employed as control. **Results:** The index of bone marrow cells in cyclophosphamide (CYP)-treated albino male mice shows a significant difference ($P \leq 0.05$) between concentration inhibition 50 % IC_{50} of cancer cell line compered to WRL-68 normal cells, on MDA and WRL-68 cells (IC_{50} =185.7, 200.7), HepG2 and WRL-68 (IC_{50} 104.9, 564.6), A549 and WRL-68 (IC_{50} 115, 192), PC3 and WRL-68 (IC_{50} 160.7, 298.7). The results showed that the extracts was able to increase metaphase index compared to cyclophasoamide-treated mice, which caused a drop in the percentage of metaphase index. **Conclusions:** The current study was showed that significant anti – proliferation activity of Hypericum triquetrifolium methanolic extract against MDA-MB-231 cell line is an epithelial, human breast cancer cell line,WRL-68, HepG2 and lung cancer cell lines (A594).

Keywords: Hypericum triquetrifolium- cytogenetic- cytotoxicity- inhibitory concentration

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Introduction

Hypericum triquetrifolium Turra is native to Eastern Europe and the Mediterranean area (Couladiset al., 2002). Several studies have reported the potential use of its essential oil and crude extracts as therapeutic substances, mainly in the treatment of burns, and gastroenteritis. It is also used as an antinociceptive and antioxidant agent (Alzoubi et al., 2020). Numerous studies have shown that the constituents of herbal derivatives are important in the conventional treatment of many diseases and have cytotoxic effects, which inhibit the growth of cancer cells (Ad'hiahet al., 2018). According to some studies, *H. triquetrifolium* has antinociceptive (Çiraket al., 2011), anti-inflammatory, antioxidant (Conforti et al., 2002), antibacterial, antifungal, and cytotoxic (Fraternaleet al., 2006) activities.

Numerous bioactive substances, including the naphthodianthrones hypericin and pseudohypericin, have been shown to be present in the methanolic extract of the various parts of Hypericum species. Phenolic compounds have potent antioxidant agents (Çiraket al., 2011). Clinical research has shown that flavonoids, which are considered dietary components, can help prevent cancer and a variety of cardiovascular problems. Hypericum triquetrifolium was reported previously to contain chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, kaempferol, phenolic, and flavonoid compounds (Rafieian-Kopaie, 2012; Brankiewiczet al., 2023). It has been used for centuries for its anti-inflammatory, anti-septic, and worm-killing properties (Rouiset al., 2013; Ozkan and Mat, 2013). The presence of phenolic compounds such as phenylpropane derivative, chlorogenic acid, and flavonoids like rutin, hyperoside, apigenin-7-O-glucoside, kaempferol, quercitrin, quercetin, and amentoflavone has been demonstrated in *H. triquetrifolium* (Cirak et al., 2011; Ayan and Cirak, 2008). Tumor necrosis factor-α (TNF-α) and interleukine-6 (IL-6) production and release, as well as inducible nitric oxide synthase, were assessed using THP-1 human monocytic cells (iNOS). Nitric oxide (NO) release, iNOS expression, and the levels of soluble proteins and mRNA for TNF-α and IL-6 were all evaluated in THP-1 cells by Saad et al., (2011) and Ahmadet al., (2021).

The present study wasconducted to investigate the cytogenetic and cytotoxic effects of Hypericum

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triquetrifoliummethanolic extract on four different cancer cell lines.

Materials and Methods

Source of plant materials

Leaves of Hypericum triquetrifolium were collected from Erbil City in northern Iraq in March 2022 (Fua et al., 2010). The plant was identified by a scientist at the state herbarium of Iraq. The plant samples were air-dried to a moisture content of 10%.

Source of experimental animals

Male albino mice (Mus musculus) were used in the study. They were obtained from the Biotechnology Research Centre at Al-Nahrain University. At the beginning of the experiment, the animals were 8–10 weeks old and weighed 23–27 grams. The animals were divided into groups, and every group was kept in a separate plastic box. The animals were maintained at room temperature and fed with lots of food and drink (ad libitum).

Extraction of plant materials

After drying, 0.5-1.0 g of the air-dried leaves were mechanically ground with a laboratory mill to obtain a homogeneous powder, which was then extracted for 72 hours at room temperature using 750 mL of 96% methanol. A modified method of Wagner and Bladt (1994) was used for the extraction of the powdered plant. Soxhlet extraction was performed using methanol at 80°C, utilizing 750 ml of methanol for every 100 g of crude plant. The prepared extracts were kept in the dark in a refrigerator until used (Ozturk et al., 2002; Gülçinet al., 2010).

Experimental groupings

The effect of different concentrations (50, 80, 100, and 200 mg/kg) of methanolic extract of Hypericum triquetrifoliumleaves was tested. The experimental animals were grouped into five. In group I (blank control), water was given to the mice;in group II (CYP), the mice received cyclophosphamide at a dose of 80 mg/kg; in groups III, IV, and V, the mice received 50, 100, and 200 mg/kg of Hypericum triquetrifolium extracts, respectively. Administration of the test solutions was done intraperitoneally (IP) once a day (0.1ml) for 10 days. On the 11thday, the mice were sacrificed and bone marrow collected for further analysis (Allen et al., 1977).

Cytogenetic analysis using the metaphaseindex assay

The metaphase index (MI) of bone marrow cells was determined using the methoddescribed by Allen et al., (1977). Each mouse was injected intraperitoneally with 0.25ml of colchicine solution. After 1.5–2 hours, the animals were sacrificed by cervical dislocation and then dissected to get the femur. A disposable insulin syringe (1 ml) and physiological saline were used to collect the cellular contents of the femur after it had been cut off at both ends (5 ml). The cells were gently transferred using a Pasteur pipette, and the tubes were spun at 2,000 rpm for 10 minutes. The cell deposit was placed in 5 ml of a warm (37°C) KCl-based solution (0.075 M) to prevent it from settling, and the supernatant was discarded. The tubes were then submerged for 30 minutes in a 37°C water bath while being gently shaken every five minutes. For 10 minutes, the tubes were spun at 2,000 rpm, and the liquid that emerged was discarded. The cell deposit was then gently and continuously stirred, and 5 ml of the fixative was added drop by drop, resulting in a homogenous cell suspension. After that, the tubes were placed in a fridge (4°C) for 30 minutes. The tubes were fixed twice after being spun at 2,000 rpm for 10 minutes. After thoroughly combining the cell deposit with 2 ml of fixative, 4-5 drops of the cell suspension were placed onto a clean slide from a height of about two feet. The slide was cleaned with distilled water after being stained with Giemsa stain for 15 minutes and allowed to dry at room temperature. At least 1000 cells were viewed on the slide using an oil emersion lens at a magnification of 100x. The number of cells in metaphase (also known as the metaphase index) was calculated using the following expression:

Determination of the cytotoxic activity of Hypericum triquetrifoliummethanolic extract

The cytotoxic effect of *H. triquetrifolium* methanolic extract was tested against different cancer cell lines, including liver cancer (HepG2), prostate (PC3), breast (MDA), and lung (A594) using the MTT (3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.A hemocytometer was used to count the number of cells after maintaining them in accordance with (Allen et al., 1977). Then, various extract concentrations (6.2, 12.5, 25, 50, 100, 200, and 400 µg/ml) were prepared, and a ready-to-use kit was utilized to measure the amount of MTT. Briefly, $1x10^4$–$1x10^6$ cells/ml of tumor cells were cultivated in micro-titer plates. Each well received 200 µL of culture medium, which was then covered. The plates were maintained for 24 hours at 37°C with 5% CO_2. Different extract concentrations were added after the medium had been removed, and the mixture was allowed to settle for 24 hours. The tube was then filled with 10 µL of the MTT solution and kept in the incubator for another 4 hours. The medium was removed, and 100 µL of the solubilization solution was then added. An enzyme-linked immunosorbent assay (ELISA) reader was used to measure the absorbance at a 575 nm wavelength. The values obtained were analyzed to compute the amount of the test compounds required to kill 50% of each of the cell lines.

Results

Effect of Hypericum triquetrifoliumextract on the metaphase indexof bone marrow cells

In bone marrow cells, the MI rate was 5.24+0.37% in the negative control group, but it was 3.57+0.47% in the CYP group. At the first dose of *H. triquetrifolium* methanolic extract (50 mg/kg), the MI was (5.54+0.32%), however, at higher doses, the MI was significantly higher than the percentages of the blank controls. The MI of bone marrow was 6.66+0.32 and 7.76+0.28% at 100 and 200 mg/kg, respectively (Table 1).

Table 1. Effect of *Hypericum triquetrifolium* Methanolic Extract on Metaphase Index of Bone Marrow Cells in Cyclophosphamide (CYP)-Treated Albino Male Mice

Group		Dose (mg/kg)	Metaphase index mean ± SD (%) Bone Marrow
I (Blank control)		-	5.24 ± 0.37^{D}
II (Vehicle control)		80	3.57 ± 0.47^{E}
H.triquetrifolium Methanol Extract	IV	50	5.54 ± 0.32^{D}
	V	100	6.66 ± 0.32^{B}
	VI	200	7.760.28^{A}

Different letters: Significant difference ($p \leq 0.05$) between means in each column

Table 2. Cytotoxicity Effect of *Hypericumtriquetrifoliumon* MDA and WRI-68 Cells after 24 Hours of Incubation at 37ºC

H. triquetrifoliumconcentration (µg/mL)	Viable cell count of MDA cell line (Mean±SD)	Viable cell count of WRL-68 cell line (Mean±SD)
400	61.70±3.13	76.00±2.32
200	73.23±3.17	84.80±1.20
100	81.02±1.86	93.60±2.10
50	91.51±2.65	95.33±1.18
25	94.25±3.34	95.22±0.82
12.5	96.88±0.40	95.95±1.02
6.2	96.41±1.01	95.95±0.20

Discussion

Cytotoxic effect of Hypericum triquetrifoliummethanolic extract

The MTT assay was employed to determine how well and how much the cancer cell type could live using various concentrations of plant extract. The number of treated cells that were still alive was estimated by comparing them to a regular cell type designated as WPL-68 (Sharma and Ratain, 2015). The results demonstrated a dose-dependent decrease in cell survival depending on the type of cancer cell. The proportion of MDA cells that were still alive at 400 µg/ml was 61.70+3.13, while the proportion of MCF-7 cells that were still alive at 6.2 g/ml was 96.41+1.01. Meanwhile, the IC_{50} for MCF-7 and WRI-68 normal cells were 185.7 and 200.7 µg/ml, respectively (Figure 1 and Table 2). The results (Figure 2 and Table 3) showed that the proportion of MDA cells that were still alive decreased to 52.62+2.65 at 400 µg/ml, but the maximum percentage of MCF-7 cells that were still alive was 95.68+0.57 at 6.2 µg/ml. MCF-7 cells had an IC_{50} value of 104.9 µg/ml while WRI-68 normal cells had an IC_{50} value of 564.6 µg/ml.

As shown in Figure 3 and Table 4, the effects of plant extract on lung cancer cell lines (A594) decreased cell viability as concentrations increased, with an IC_{50} value of 115.0 µg/ml. However, a normal cell line (WRI-68)

Table 3. Cytotoxic Effect of *Hypericumtriquetrifoliumon* HepG2 and WRI-68 Cells after 24 hours of Incubation at 37ºC

H. triquetrifoliumconcentration (µg/mL)	Viable cell count of HepG2 cell line	Viable cell count of WRL-68 cell line
	Mean±SD	Mean±SD
400	52.62±2.65	85.03±1.03
200	61.96±1.27	91.26±2.20
100	73.65±6.10	92.21±2.77
50	90.20±5.90	94.29±2.97
25	95.02 ±3.04	96.95 ±1.14
12.5	96.80±0.93	96.49±1.29
6.2	95.68 ±0.57	96.07 ±0.11

Table 4. Cytotoxic Effect of *Hypericumtriquetrifolium* on A549 and WRI-68 Cells after 24 hours of Incubation at37ºC

H. triquetrifoliumconcentration (µg/mL)	Viable cell count of A594 cell line Mean± SD	Viable cell count of WRL-68 cell line Mean±SD
400	29.21±3.30	65.06±1.45
200	40.32±3.35	79.17 ±1.22
100	68.02±3.87	95.12±2.80
50	90.05±2.42	96.23±2.77
25	96.22 ±0.67	96.82 ±2.01
12.5	96.64±1.36	96.42±1.33
6.2	96.37±0.81	96.13 ±0.57

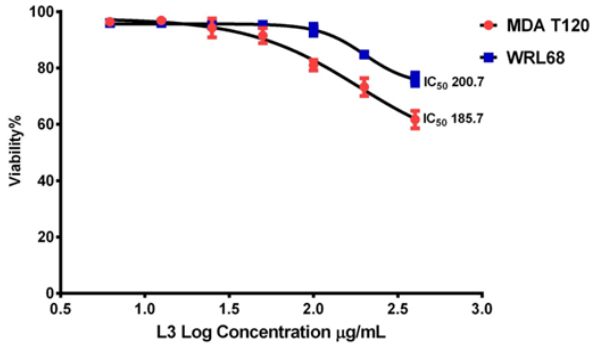

Figure 1. Cytotoxic Effect of *Hypericumtriquetrifoliumon* MDA and WRL-68 Cells after 24 hours of Incubation at 37ºC

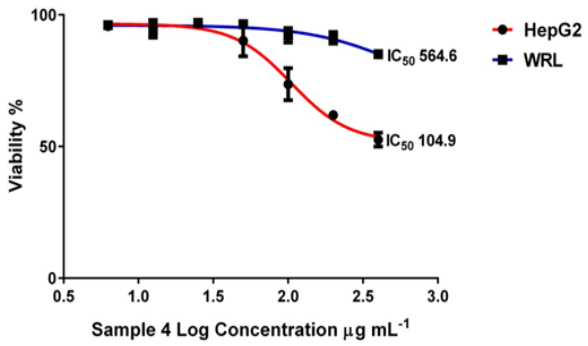

Figure 2. Cytotoxic Effect of *Hypericumtriquetrifoliumon* HepG2 and WRL-68 Cells after 24 hours of Incubation at 37ºC

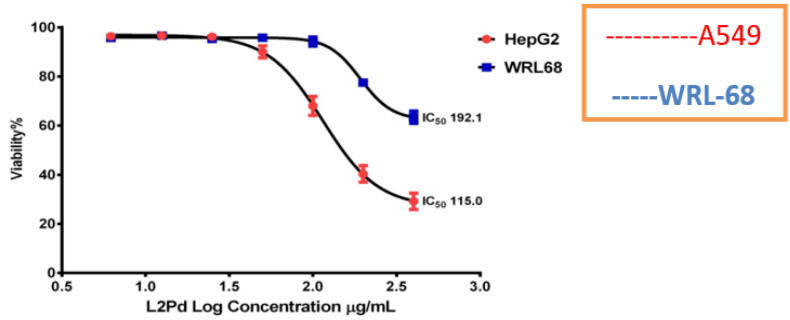

Figure 3. Cytotoxic Effect of *Hypericumtriquetrifoliumon* A549 and WRL-68 Cells after 24 Hours of Incubation at 37ºC

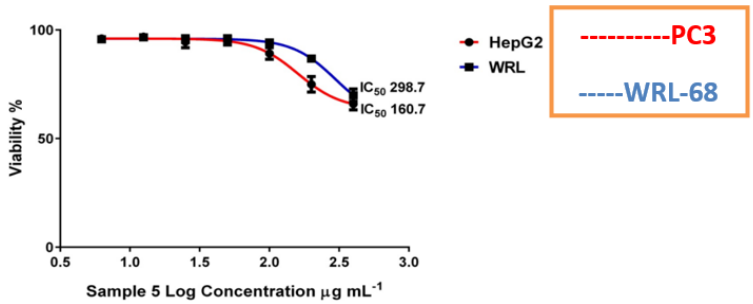

Figure 4. Cytotoxic Effect of *Hypericumtriquetrifoliumon* PC3 and WRL-68 Cells after 24 Hours of Incubation at 37ºC

Table 5. Cytotoxic Effect of *Hypericumtriquetrifoliumon* PC3 and WRI-68 Cells after 24 Hours of Incubation at 37ºC

H. triquetrifolium Concentrations (µg/mL)	Viable cell count of PC3 cell line Mean±SD	Viable cell count of WRL-68 cell line Mean±SD
400	65.93±2.97	69.98±2.90
200	75.00±3.61	86.81 ±1.51
100	89.20±2.69	94.29±1.83
50	94.56 ±2.80	95.83±0.30
25	95.22±2.15	95.37 ±0.90
12.5	96.88±0.98	96.64±0.70
6.2	96.10±1.70	96.68 ±0.40

exposed to plant extract had an IC_{50} value of 192.1 µg/ml. The results of the prostate cancer cell line (Figure 4 and Table 5) demonstrated that the IC_{50} of plant extract was 160.7 µg/ml and the IC_{50} of WRI-68 was 298.7 µg/ml. The cell viability decreased in a manner that depended on the concentration. The ability of various plant extracts to neutralize free radicals that cause diseases by lipid peroxidation, protein peroxidation, and DNA damage has long been recognized (Al-ezzy et al., 2019). There are many phenolic compounds in plants, and Franco et al., (2014) suggested that they might have a variety of molecular functions, including anticancer and protective qualities. However, El Dibet al., (2015) discovered that phenolic compounds, such as flavonoids, phenolic acids, stilbenes, lignans, and tannins, which are frequently found in leaves, flowers, and woody parts like stems and bark, have various biological effects.

The results of an in vivo and in vitro investigation by Tsuji et al., (2013) revealed that several flavonoids protect against cancer in various ways. Meanwhile, some flavonoids directly protect cells by acting as antioxidants, others indirectly protect cells by inducing enzymes that shield them from oxidative stress and other damage. Numerous phenolic acids, glycosides, diterpenoids, and flavonoids are present in *H. triquetrifolium*. According to Ad'hiahet al., (2018) and Al-Anee et al., (2015), some of these have a wide range of biological effects, including being antioxidant, anti-platelet, anti-inflammatory, anticancer, and antiviral. Kaempferol-3-O-glycoside acted similarly on the kidney cell carcinoma (ACHN) and CORL-23 cell lines. Hypericin caused little harm to the C32 cell line but had a significant impact on the MCR-5 cell line, which represents the normal human fetal lung cells (El-Nggaret al.,2017).

In conclusion, the findings of the study revealed that methanolic extract of Hypericum triquetrifolium exhibits significant antiproliferation activity against the epithelial MDA-MB-231, WRL-68, HepG2, and lung cancer cell lines (A594).

Author Contribution Statement

Rafal Shakeeb Al-Anee conceptualized the experimental design, conducted the research, collected and analyzed the data, and wrote the draft manuscript. Enas Hamed AL-Ani and Zaineb Sabeeh Omran validated the results, proofread the manuscript, and produced the final draft. All the authors agreed to submission of the manuscript for publication.

Acknowledgements

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Study Registration

There is no registration number or support for this project.

Conflict of interest

The authors declare that they have no competing interests.

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