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Evaluate the Effect of Ethanol Fraction of Iraqi Hibiscus tiliaceus L. Leaves Extract on Mitotic Index and Micronucleus Frequency in Comparison with Metformin in Male Rats

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Abstract

Hibiscus tiliaceus L. is a member of the Malvaceae family. It has been documented to exhibit antibacterial, antioxidant, anti-inflammatory, expectorant, anthelmintic, and anticancer properties. The objective of this study was to assess the impact of ethanol leaf extract from *Hibiscus tiliaceus L.* on methotrexate-induced chromosomal aberrations, specifically the Mitotic Index and Micronucleus Frequency, in spleen and bone marrow cells.The study involves the categorization of the animals into five groups, with each group consisting of six rats. The rats are then subjected to the following treatments: Group I (negative control) that were administered a daily oral dose of 1ml/kg/day of distilled water for seven consecutive days. Group II (positive control): rats were administered a solitary dose of 20mg/kg of methotrexate on the initial day. Group III consisted of six rats who were administered a daily oral dose of 100 mg/kg of metformin for a period of seven consecutive days. Group IV: rats were administered a daily oral dose of 250 mg/kg of the ethanol fraction for seven consecutive days. In Group V, six rats were administered a daily oral dose of 500 mg/kg of the ethanol fraction for seven consecutive days. The results indicate that the ethanol extract of *Hibiscus tiliaceus* leaves, at both doses, significantly reduced the mitotic index in bone marrow cells and spleen cells compared to the negative and positive control groups. Additionally, it significantly increased the appearance of micronuclei in bone marrow cells. In conclusion, the ethanol extract of *Hibiscus tiliaceus* leaves exhibited a strong effect on the mitotic index and micronucleus appearance in bone marrow and spleen cells of rats.
Keywords: *Hibiscus Tiliaceus L*, Mitotic Index, Micronucleus Appearance, Methotrexate, Metformin.

Introduction

Herbal medicine, a widely recognized alternative treatment option, is used worldwide to address toxicity and related ailments [1, 2]. Herbal medicines have the ability to act as potent antioxidants, effectively removing harmful free radicals from the body. They utilize methods to improve the regeneration and protection of organs, resulting in increased overall vitality and stability [2, 3]. The use of herb extracts in alternative medicine has become increasingly popular as more and more plants with medicinal properties are being recognized and embraced in therapeutic medicine.*Hibiscus tiliaceus L.* (from the Malvaceae family) has a rich history of being utilized as a traditional remedy for a wide range of health issues, such as infections, diarrhea, coughs, chest congestion, and dry throat [4]. According to thephytochemistry test, a wide range of chemical compounds were discovered in the leaves of *H. tiliaceus*, such as polyphenols, flavonoids, tannins, glycosides, terpenoids, and steroids, as well as proteins and carbohydrates [5].

The leaf extract of *H. tiliaceus* is well-known for its wide range of pharmacological properties, including anti-inflammatory, anthelmintic, antibacterial, and antioxidant activity. It has been used as a therapeutic and chemopreventive drug [6]. Methotrexate (MTX) is a highly effective chemotherapeutic drug that is commonly used to treat a range of cancers, such as acute lymphoblastic leukemia, lymphoma, osteosarcoma, breast cancer, and head and neck cancers. In addition, it has been shown to be effective in managing non-oncologic conditions such as rheumatic diseases and psoriasis [7]. Methotrexate effectively hinders the production of thymidylate and purine nucleotides by specifically targeting dihydrofolate reductase and, to a lesser degree, thymidylate synthetase. Cells treated with methotrexate exhibited a progressive accumulation of

strand breaks in fully formed DNA, particularly in DNA that had undergone replication. Aside from the therapeutic advantages, there are also several adverse effects to consider, including gastrointestinal, central nervous system, hepatic, and bone marrow toxicity [8,9].

Metformin effectively decreases insulin resistance and hepatic glucose output while enhancing glucose uptake. These findings demonstrate a significant reduction in fasting and blood glucose levels, along with a decrease in body weight and an increase in high-density lipoprotein [10] metformin exerted an ameliorative effect against MTX-induced chromosomal injury, as it significantly reduced the CA and SCE frequencies [11].

The mitotic index measures the proportion of cells in a population that are currently undergoing mitosis (specifically at metaphase), compared to those that are not. The mitotic index is used to measure cell growth [12]. The mitotic index is a crucial factor in determining both survival rates and treatment response in most types of cancer [13]. As an illustration, a low mitotic index does not provide significant predictive value for breast cancer patients who are over 70 years old. A heightened mitotic index indicates an increased rate of cell division, not only in cancer cells but also during crucial life-sustaining processes such as plant and animal growth and cellular injury repair [14]. A micronucleus is a third nucleus that forms unpredictably during the anaphase of mitosis or meiosis. Micronuclei, which are referred to as "small nuclei," are structures found in the cytoplasm that contain a fragment or entire chromosome that was not properly distributed to the opposing poles during the anaphase stage. Their development leads to the descendant cell having a partial or complete absence of a chromosome. Typically, these chromosome fragments or entire chromosomes undergo the process of developing nuclear membranes and become micronuclei, which serve as a third nucleus. Following cytokinesis, one daughter cell contains a single nucleus whereas the other daughter cell contains both a big and a small nucleus, known as micronuclei. Multiple micronuclei may occur when there is extensive genetic damage. The micronucleus test is employed as a method for evaluating the genotoxicity of different substances. The chromosomal aberration test is more complex and requires more steps and analysis compared to this test [15].

Micronucleus assays are utilized for toxicity screening of genotoxic substances. The genotoxic carcinogen assay is widely recognized for its high level of accuracy and success [14]. Typical in vivo tests utilize mouse bone marrow or peripheral blood. When a bone marrow erythroblast transforms into a polychromatic erythrocyte, the main nucleus is expelled, while any micronucleus may remain in the nucleated cytoplasm [16,17].

The objective of the research is to demonstrate the impact of ethanol leaf extract of *Hibiscus tiliaceus L.* on Methotrexate-induced chromosomal abnormalities, specifically focusing on the mitotic index and micronucleus appearance in cells of the bone marrow and spleen in comparison to metformin.

Materials and Methods

Chemicals and drugs

Methotrexate obtained from Sigma Aldrich**,** and Metformin obtained from Merk.

Plant material

The leaves of *H. tiliaceus L*. were gathered in Sayed Al-Awseia City, located on Karbala-Baghdad Rd, Karbala, during April 2022. The authentication of the leaves was conducted by Dr. Prof. Ibrahim Al-Jubory at the Al Razi Centre for Alternative Medicine.

Extraction method of the plant Leaves

The leaves were air-dried at room temperature, ground into a fine powder using an electric mill, and subsequently weighed. The plant leaves, weighing approximately 100 g, were individually defatted using an ample amount of hexane solvent for a duration of 24 hours. This process aimed to eliminate chlorophyll and hexane-soluble components, such as waxy substance. Subsequently, the specimens were introduced into the rotating chamber of the Soxhlet apparatus and subjected to extraction using ethanol (80% v/v) for 16 hrs. Upon undergoing filtration and subsequent drying using a high-pressure rotary evaporator, the extract underwent a transformation, resulting in a semi-solid consistency that was adhesive in nature. This modified extract was intended for use in animal administration solutions [18].

Phytochemical screening

A preliminary phytochemical screening was performed to identify the presence of bioactive components in the extract of *Hibiscus tiliaceus*. A variety of chemical agents were utilized. [19]

Table 1. Tests used for Detection of Phytochemicals

Phytochemicals	Tests
Polyphenols	distilled water and 5% ferric chloride solution were added to the extract. give dark green color
Tannins	10% ferric chloride drops were applied to the extract (blue-green colour developed)
Saponin	A test tube containing the extracts and distilled water was rapidly shaken for 5 minutes, resulting in 1 cm of foam.
Terpenoids	The Salkowski test: the extracts were mixed with 3 ml of concentrated H2SO4 and 2 ml of chloroform (create a reddish-brown layer)
Flavonoids	diluted NH_3 and concentrated H_2SO_4 were added to the extracts (develop yellow colour)
Coumarins	10% NaOH and chloroform were added to the extract (yellow colour)
Alkaloids	Dragendorff's reagent test: Dragendorff's reagent added to the extract. (result in an orange-brown precipitate)

Experimental animals and study approval

We obtained 30 Wistar albino rats from the animal house at the College of Pharmacy/University of Baghdad. The rats were between 1 - 2 months old and weighed between 100 - 125 grams. Prior to treatment, the animals were subjected to a two-week acclimatization period in the laboratory. Complimentary water and a regular food plan were given. The temperature was meticulously regulated at 25±5ºC, while the environment was upheld with 12-hour alternating periods of light and darkness and suitable levels of humidity. The study methods and ethics were granted approval by the Research Ethics Committee of the College of Pharmacy at University of Baghdad.

Study design

The animals used in this study divided into five groups, each group contains six rats; and treated as following:

Group I (negative control): six rats received an oral daily dose of 1ml /kg/day of distilled water (DW) for seven successive days

Group II (positive control): six rats received a single dose 20mg/kg of methotrexate (MTX) taken on the first day only [20].

Group III: six rats received daily dose of 100 mg/kg of metformin orally for seven successive days [21].

Group IV: six rats received daily dose of 250 mg/kg of ethanol leaf extract orally for seven successive days [22].

Group V: six rats received daily dose of 500 mg/kg of ethanol leaf extract orally for seven successive days [22].

At the end of the experiment, rats were sacrificed In the 8th day. Samples of bone marrow and spleen cells were taken, and genotoxic analyses were carried out.

Evaluation of the Mitotic Index in the Bone marrow and Spleen cells

Obtained bone marrow samples were aspirated from the femoral bone, while spleen cells were collected and processed using aseptic techniques to assess the mitotic index [23].

Mitotic index (MI)

The percentage of dividing cells was determined by counting at least 1000 cells prepared for metaphase spreads in each group.

Evaluation of Micronucleus Assay in Bone marrow cells

After the completion of the treatment, the rats were euthanized, and cells were extracted from the femoral marrow. Bone marrow slides were made. The bone marrow was meticulously rinsed with 1 ml of fetal calf serum and thereafter delicately applied onto pristine slides. The slides were air-dried and subsequently submerged in methanol for a duration of 5 minutes. The specimens were treated with Gemisa stain for a duration of 5 minutes, followed by rinsing with distilled water, and ultimately being mounted. A minimum of 2000 polychromatic erythrocytes (PCEs) per animal were carefully and systematically analyzed to detect the presence of micronuclei [24].

Statistical analysis

The data were analyzed using Graph Prism 10. The numerical values were presented as mean± standard deviation. Every group was compared to all other groups using the one-way ANOVA test, and the Tukey multiple comparison test was utilized to determine statistical significance compared to the induction group. All study findings were deemed significant if the p-value was less than 0.05 ($p<0.05$).

Results and Discussion

Phytochemical Screening Tests

The ethanol (8 gm) extract is ready for qualitative phytochemical analyses using established techniques to detect the following components [19].

Table 2. Phytochemical Screening Tests of the Ethanol Leaves Extract of the Iraqi *Hibiscus tiliaceus*

Phytochemicals	**Ethanol extract of *Hibiscus tiliaceus* leaves**
Phenols	+
Flavonoids	+
Coumarins	+
Tannins	+
Saponins	+
Alkaloids	+
Terpenoids	+

In Table 3, The administration of *Hibiscus tiliaceus* ethanol extract at doses of 250 and 500 mg/kg resulted in a substantial rise in the mitotic index of both bone marrow and spleen cells compared to the rats in the negative and positive control group ($P<0.05$), suggesting an enhanced rate of cell division. Methotrexate exhibited a substantial decrease in the mitotic index ($P<0.05$) in both bone marrow and spleen cells as compared to the rats in the negative control and extract-administering groups. The extract demonstrated similar outcomes to the metformin group.

Table 3. The mitotic index in the spleen and bone marrow cells of rats treated with two doses of the ethanolic extract of *Hibiscus tiliaceus* leaves

Groups	**cells of Bone marrow**	**cells of Spleen**
Group I (negative control: DW only)	5.55±0.44 ^{b}	3.11±0.37 ^{b}
Group II (positive control: 20mg/kg of methotrexate)	3.22±0.25 ^{c}	2.55±0.19 ^{c}
Group III (100 mg/kg metformin)	10.2±0.33 ^{a}	6.22±0.25 ^{a}
Group IV (250 mg/kg *H. tiliaceus* ethanol extract)	7.32+0.56 ^{a}	5.38±0.46 ^{a}
Group V (500 mg /kg *H. tiliaceus* ethanol extract)	8.11±0.61 ^{a}	6.45±0.55 ^{a}

Values are expressed in mean ± SDM (n=6 rats in each group); different superscripts refer to the significant differences among the groups ($P<0.05$).

In Table 4, figure 1, The ethanol extract of *Hibiscus tiliaceus* leaves, administered at both doses, resulted in a substantial increase in the occurrence of micronuclei in bone marrow cells, as compared to the negative control group ($P<0.05$). Methotrexate caused a considerable rise in the appearance of micronuclei in bone marrow cells compared to the negative control and extract. However, both metformin and the ethanol leaves extract showed a significant decrease in micronucleus appearance in bone marrow cells compared to the methotrexate group.

Finally, mitotic index and micronucleus appearance did not change across ethanol extract doses ($P<0.05$).

Table 4. The Micronucleus appearance in bone marrow of rats treated with two doses of the ethanolic extract of *Hibiscus tiliaceus* leaves

Groups	Bone marrow cells
Group I (negative control: DW only)	4.44±0.53 ^{c}
Group II (positive control: 20mg/kg of methotrexate)	23.2±3.43 *^{a}
Group III (100mg/kg metformin)	8.57±0.92 *^{b}
Group IV (250 mg/kg *H. tiliaceus* ethanol extract)	6.23±0.76 *^{b}
Group V (500 mg /kg *H. tiliaceus* ethanol extract)	8.11±0.82 *^{b}

Values were expressed in mean ± SDM; (n=6 rats in each group), * and different superscripts refer to the significant differences among the groups (*P*<0.05).

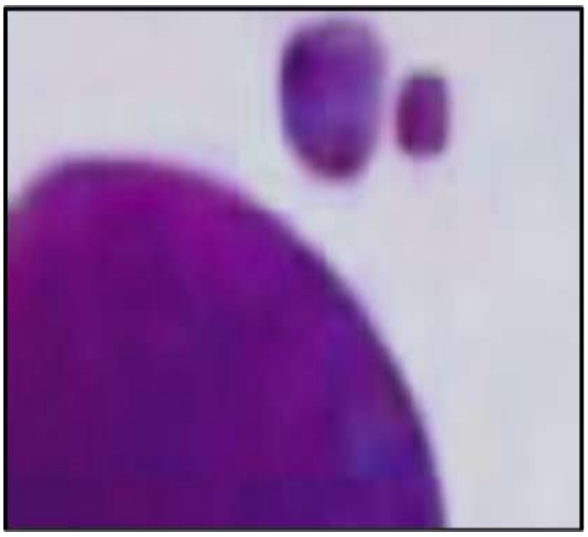

Figure 1. The Micronucleus appearance after the treatment with the ethanol leaves extract of *Hibiscus tiliaceus*

The data from Tables 3 and 4 indicate that the effect of methotrexate on the mitotic index cells was significantly diminished compared to the ethanol extract for both the 250 mg/kg and 500 mg/kg doses. Moreover, there was a significant increase in micronuclei observed in both dosage groups. The analysis of the results indicates that the ethanol extract of *Hibiscus tiliaceus* leaves includes a diverse range of chemicals, including coumarins, polyphenols, flavonoids, tannins, and terpenoids. Polyphenols encompass a wide array of secondary metabolites. All of them possess one or more phenol groups in their structure. This can be categorized into two groups: flavonoids and non-flavonoids, which consist of coumarins and simple phenols. Based on the phytochemical analysis undertaken in this study, it has been found that there are additional active ingredients present, including alkaloids and terpenoids. These components have been documented to possess antioxidant properties [25]. The current study demonstrates that a reduction in mitotic index is a very dependable marker for bone marrow and spleen injury [26]. The latest results from the injection of MTX shown a significant reduction in the mitotic index, with a statistical significance of P<0.05. These findings are consistent with research undertaken by other experts [27, 28]. Metformin is universally acknowledged as the foremost medicine for the management of type 2 diabetes mellitus. Various studies have demonstrated that metformin possesses qualities that can effectively counteract oxidative stress, inhibit cell death, and diminish inflammation [29-32]. The primary results of this study indicate that metformin successfully decreases the genotoxicity induced by MTX, as shown in the rats treated with metformin. Various investigations have demonstrated that metformin possesses antioxidant properties. It has been noted that it reduces the levels of malondialdehyde in the blood and increases the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione reductase [33-35]. The study's findings indicate that metformin successfully decreased the chromosomal damage induced by MTX. A proposal was made suggesting that metformin could potentially mitigate the detrimental impact of MTX on healthy cells through the reduction of oxidative stress and the limitation of reactive oxygen species (ROS) generation. Several studies have extensively recorded the preventive properties of metformin against substances that cause damage to DNA. Studies using HepG2 cells have shown that metformin efficiently protects against DNA damage induced by formaldehyde [36].The effect of the extract can be related to the anti-oxidant effect of this plant that have been see in many studies [5]. The mutagenic effects of methotrexate were lessened after Hibiscus tiliaceus leaf extract was administered, as assessed using the micronucleus test. In a similar investigation, the ethanol leaf extract of *H. tiliaceus* was found to inhibit the rise in lipid peroxidation and the decline in GSH content caused by oxidative stress.

This likely played a significant role in the protective effects on the cells [37].flavonoids and phenolic acids found in *H. tiliaceus* extract have antimutagenic effects and are antigenotoxic; this protection could be from the antioxidative roles of coumarins, alkaloids, flavonoids, and other active phytochemical components of this plant. These findings supported the previous reports regarding the antioxidant effect and play a significant role in anti-carcinogenesis effects ([38-40].Flavonoids are a class of polyphenolic chemicals that have a shared structure called gamma-benzo-pyrone. These molecules are known for their antioxidant properties and are widely recognized in various biological systems. Flavonoids have various applications, one of which is its ability to defend against genotoxic damage [41].Polyphenols and terpenoids were from the leaf extract of H. tiliaceus; there was a notable rise in the mitotic index and a considerable increase in the micronucleus compared to the negative control, as seen with other studies [42].Polyphenols provide protection to the body against the detrimental impact of reactive oxygen species on the integrity of DNA. So, using plant extracts offers distinct benefits in this regard [43]. Therefore, the extract contains components that have the ability to induce programmed cell death (apoptosis) and prevent DNA damage (antigenotoxic). This further supports the fact that the extract is abundant in chemicals that have important biological effects [38,44].The utilization of Hibiscus tiliaceus leaves extract as a standalone supplement or in conjunction with other natural substances holds great potential for exploring new possibilities and applications [45].Terpenoids also in the extract give several therapeutic applications, including antibacterial, antimicrobial, antitumor, and anti-inflammatory activity [46, 47].

Conclusion

The ethanol extract derived from the leaves of *Hibiscus tiliaceus L.* showed notable effects on the mitotic index and the formation of micronuclei in the spleen and bone marrow cells of the rats.

Conflicts of Interest

The author has declared that no conflict of interest.

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Ethics Statements

This study was approved by the scientific and ethical committees of the College of Pharmacy, University of Baghdad.

Author Contribution

The author's contribution to the paper was as follows: study conception and design, data collection, analysis and interpretation of results, and draft manuscript preparation: Shihab Hattab Mutlag, the author, reviewed the results and approved the final version of the manuscript.

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تقييم تأثير جزء من مستخلص الايثانول لأوراق الخطمي الساحلي العراقي على مؤشر الانقسام وتعدد النواة الصغيرة مقارنة مع الميتفورمين في ذكور الجرذان

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الخلاصة

ينتمي نبات الخطمي الساحلي العراقي إلى عائلة الخبازيات. وقد ثبت أن له تأثيرات مضادة للبكتيريا ومضادة للأكسدة ومضادة للالتهابات وطارد للبلغم ومضاد للديدان ومضاد للسرطان. صُممت هذه الدراسة لتقييم تأثير مستخلص الإيثانول لأوراق الخطمي الساحلي العراقي. ضد الاختلالات الكروموسومية التي يسببها الميثوتريكسات في خلايا الطحال ونخاع العظم. الهدف من الدراسة: فحص تأثير مستخلص الإيثانول لأوراق الخطمي الساحلي العراقي ضد الاختلالات الكروموسومية التي يسببها الميثوتريكسات في خلايا الطحال ونخاع العظام. في هذه الدراسة، تم تقسيم الحيوانات المستخدمة في هذه الدراسة إلى خمس مجموعات، كل مجموعة ستة فئران على النحو التالي: المجموعة الأولى (ضابط سلبي): تلقت ستة فئران جرعة يومية عن طريق الفم مقدارها ١ مل / كجم / يوم من الماء المقطر لمدة سبعة أيام متتالية.المجموعة الثانية (ضابط إيجابي): تلقت ستة فئران جرعة واحدة ٢٠ ملغ / كجم من الميثوتريكسات في اليوم الأول. المجموعة الثالثة: تلقت ستة فئران جرعة يومية عن طريق الفم مقدارها ١٠٠ ملغ / كجم من الميتفورمين لمدة سبعة أيام متتالية.المجموعة الرابعة: تلقت ستة فئران جرعة يومية عن طريق الفم مقدارها ٢٥٠ ملغ / كجم من مستخلص الإيثانول لأوراق الخطمي الساحلي لمدة سبعة أيام متتالية. المجموعة الخامسة: تلقت ستة فئرانجرعة يومية عن طريق الفم مقدارها ٥٠٠ ملغ / كجم من مستخلص الإيثانول لأوراق الخطمي الساحلي ا لمدة سبعة أيام متتالية. وأظهرت النتائج أن مستخلص أوراق الإيثانول من الخطمي الساحلي العراقي في كلا الجرعتين أظهر انخفاضًا كبيرًا في مؤشر الانقسام الفتيلي في خلايا نخاع العظم وخلايا الطحال مقارنة بمجموعة التحكم السلبية، في حين أظهر زيادة كبيرة في ظهور النوى الدقيقة في خلايا نخاع العظم، وفي الختام أظهر مستخلص الإيثانول من أوراق الخطمي الساحلي العراقي تأثيرًا قويًا على مؤشر الانقسام الفتيلي وظهور النوى الدقيقة في خلايا نخاع العظم وخلايا الطحال في الفئران.

الكلمات المفتاحية: نبات الخطمي الساحلي ، مؤشر الانقسام الخلوي ، ظهور النوى الدقيقة ، الميثوتريكسيت، الميتفورمين.