Iraqi Journal of Science, 2026, Vol. xx, No. x, pp: xx
DOI: 10.24996/ijs.2026.67.6.20

ISSN: 0067-2904

Cytotoxic Effect of Ethyl Acetate Fraction from the Aerial Parts of *Jatropha Integerrima* on the B16 Melanoma Cell

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Received: 15/2/2025 Accepted: 24/6/2025 Published: xx

Abstract

One plant with considerable therapeutic promise is *Jatropha integerrima (J. integerrima*), which belongs to the Euphorbiaceae family. This study aimed to assess the cytotoxic effects on the (B16) melanoma cell line of ethyl acetate fraction obtained from the aerial parts of *J. integerrima* cultivated in Iraq, with six concentrations (200, 100, 50, 25, 12.5, and 6.25 µg/ml). It has drawn interest due to its possible cytotoxic effects on several cancer cell types. The bioactive chemicals responsible for the observed cytotoxic activity were analyzed qualitatively and quantitatively using High-Performance Liquid Chromatography (HPLC) on the ethyl acetate fraction. The cytotoxicity of the fraction was evaluated with the 3- [4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Five polyphenolic components classified as phenolic acids and flavonoids were found by phytochemical study of the *J. integerrima* ethyl acetate fraction. The ethyl acetate fraction with a high concentration of polyphenolic constituents showed the highest inhibition rate of 88.00% on the skin cancer (B16) cell line at a concentration of 200 µg/ml, with a significant difference as compared to the control sample during the 72-hour exposure period. Also, the values of half-maximal inhibitory concentration (IC50) of the fraction against the B16 cell lines were 27.35 µg/ml. *J. integerrima* plant has promising effects on cancerous lines, which calls for more in vitro and in vivo research.

Keywords: *Jatropha integerrima,* Gallic acid, caffeic acid, vitexin, MTT assay.

التأثير السام لخلاصة إيثيل أسيتات من الأجزاء الهوائية لنبات الجاتروفا إنتيجيريما على خط خلايا الورم الميلانيني

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الخلاصة

من النباتات التي تحمل علاجا واعدا كبيرًا هي جاتروفا إنتيجيريميا، و التي تنتمي إلى العائلة الفربيونية. تقيّم هذه الدراسة التأثيرات السامة للخلايا على خط خلايا الميلانوما (B16) لمستخلص الأسيتات الإيثيلي المستخرج من الأجزاء الهوائية لنبات جاتروفا إنتيجيريميا و المستزرع في العراق و بالتراكيز (200، 100، 50، 25، 12.5، 6.25 ميكروغرام/مل). لقد أثار هذا النبات هتمامًا بسبب تأثيراته السامة المحتملة على عدة أنواع من خلايا السرطان.و تم تحليل المواد الكيميائية النشطة بيولوجيًا و المسؤولة عن النشاط السام للخلايا الملحوظ بشكل نوعي وكمّي باستخدام كروماتوغرافيا السائل عالية الأداء (HPLC) على جزء الأسيتات الإيثيلي. تم تقييم

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السمية الخلوية للمركب باستخدام اختبار 3- [4,5- ثنائي ميثيل ثيازول-2-يل]-2,5-ثنائي فينيل تيترازوليوم بروميد (MTT). أظهرت التحليلات الكيميائية النباتية لجزء الأسيتات الإيثيلي من نبات الجاتروفا إنتيجريما خمسة مكونات بوليفينولية و تم تصنيفها كأحماض فينولية وفلافونويدات.و وفقًا للنتيجة، أظهر جزء الأسيتات الإيثيلي الذي يحتوي على تركيز عالٍ من المركبات الفينولية أعلى معدل تثبيط بنسبة 88.00% على خط خلايا سرطان الجلد (B16) بتركيز 200 ميكروغرام/مل مع فرق كبير مقارنة بعينة السيطرة خلال فترة التعرض لمدة 72 ساعة. كما كانت قيم التركيز المثبط للنصف الأقصى(IC50) للمركب ضد خطوط خلايا 27.35 (B16) ميكروغرام/مل. إن نبات الجاتروفا إنتيجيريميا له تأثيرات واعدة على السلالات السرطانية،و هذا يستوجب اجراء المزيد من الأبحاث المختبرية و الحيوية.

1. Introduction

One of the principal causes of death worldwide, skin cancer, especially melanoma, remains a serious global health issue [1]. Melanoma's worldwide impact has grown significantly during the last several decades. From about 107,380 to 289,950, the global count of new melanoma cases increased by 170% between 1990 and 2019 [2]. An anticipated 325,000 new melanoma cases worldwide in 2020 will cause about 57,000 fatalities. Forecasts show these figures could reach 510,000 new cases and 96,000 deaths yearly by 2040 [3]. This increasing trend emphasizes the public health problem that melanoma presents. Novel plant-based cytotoxic compounds have attracted a lot of interest recently. Historically used in many civilizations for its therapeutic qualities, especially in treating skin-related diseases. *J. integerrima*, sometimes referred to as "peregrina" or "spicy jatropha" [4], has long been used for its therapeutic qualities, especially in treating skin conditions, throughout many different civilizations. Folk uses of this plant cover warts, tumors, rheumatism, herpes, pruritus (itching), toothaches, scabies, eczema, and ringworm [5]. This plant contains several bioactive compounds that may possess anticancer properties. Previous studies have highlighted its antibacterial and antioxidant effects [6]. Contemporary research on *J. integerrima* has looked at its anti-inflammatory qualities. Both orally and topically, an ethanol extract from the leaves has shown notable anti-inflammatory action in rat studies. Many of which are well-known for their anti-inflammatory action, the study found 133 metabolites in the extract, including diterpenoids, flavonoids, phytosterols, phenolic acid conjugates, sesquiterpenes, cyclic peptides, and coumarins [7]. *J. integerrima,* originally from tropical America, and Jatropha species are now found throughout the tropics and subtropics of Asia and Africa [4], and are currently being cultivated in Iraq for their ability to withstand extremes in temperature. However, little is known about its cytotoxic properties against melanoma cells. This work intends to explore, on B16 melanoma cells, the cytotoxic effects of the ethyl acetate fraction of *J. integerrima* aerial parts cultivated in Iraq. It also looks for the bioactive elements causing these effects. Such studies emphasize the need to investigate well-known and underexplored plant species for their medicinal potential, especially when grown in different geographical areas, since environmental conditions can greatly affect their phytochemical profiles.

2. Materials and Methods

Plant material collection

In April and May of 2024, the aerial parts of *J. integerrima* plants were gathered from the centre of Baghdad. The plant was authenticated by the Herbarium of the Department of Biology, College of Science at the University of Baghdad, registered under BUH No.870 as *J. integerrima* Jacq. (family: Euphorbiaceae).

Preparation of plant material

The gathered plant parts were cleansed and rinsed to remove foreign objects, left to dry at room temperature in the shade to eliminate any moisture, and then ground into a fine powder.

Preparation of Jatropha integerrima extract

One hundred g of *J. integerrima* dried powdered aerial plant material was macerated for 48 hours in n-hexane solvent to defeat it. After that, the defatted plant powder material is fully exhausted using a Soxhlet device running with 85% ethanol. Then dried by a rotary evaporator to form a dark green crude extract. After dissolving the crude extract in water, it was run through a separatory funnel using n-butanol solvents, ethyl acetate, and chloroform. Every fraction is dried using a rotary evaporator; the ethyl acetate fraction is then weighed and stored for further analysis.

Reverse phase high-performance liquid chromatography (RP-HPLC) qualitative study of ethyl acetate extract

A popular method for qualitative analysis and mixture ingredient separation is high-performance liquid chromatography (HPLC). The HPLC analysis was performed by the Ministry of Industry and Minerals in Al-Jadriyah, Baghdad. To get a phytochemical profile of the investigated fraction sample, phenolic chemicals selected among phenolic classes were separated using a reversed-phase C18 HPLC column., HPLC analysis was carried out Using a liquid chromatograph (SYKAM-German), fitted with a binary pump, vacuum degasser, diode array detector (DAD), autosampler, and thermostatted column compartment [8].

HPLC conditions for ethyl acetate fraction

The column is ODS C18 (5 µm, 250 mm x 4.6 mm). With a 1 mL/min flow rate, the mobile phase comprises a gradient elution employing the proportions of solvent A (1% formic acid in water) to solvent B (acetonitrile). Initially 10% B; 0-4 min., 15% B; 5-8 min., 30% B; 9-15 min. The injection volume was 100 µL of samples and standards: vitexin, caffeic acid, gallic acid, quercetin, and rutin. the column temperature was maintained at 40°C. Plotting for chromatograms at 254 and 277 nm by injection of the relevant standard separately, it was feasible to verify the retention times of phenolic compounds under these chromatographic circumstances [9,10].

Quantification of the polyphenol compounds detected by HPLC

For vitexin, caffeic acid, and gallic acid quantification by calibration (standardizing) of the HPLC method, the calibration curve was plotted using the area under the curve (AUC) versus four concentration levels of standards. A straight-line equation was used to determine the concentration.

B16 cell line

The B16 cell line was purchased from Sigma-Aldrich (Merck), Germany, Catalogue Number: 94042254. The cells are a murine melanoma model extensively utilized in cancer research, particularly for studying tumor formation, metastasis, and immunotherapy approaches. Originating from a spontaneous skin tumor in a C57BL/6 mouse at Jackson Laboratories in 1954, B16 cells have become a cornerstone in oncological studies due to their reproducible behavior and relevance to human melanoma [11].

Maintenance of cell culture

The B16 cell line was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged utilizing trypsin-EDTA, reseeded at 80% confluence biweekly, and kept at 37 °C in a humidified 5% CO_2 incubator [12,13].

Cytotoxicity assay

The cytotoxic effect was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 96-well plates. The cell line was planted at 1

$\times 10^4$ cells/well. After twenty-four hours, or once a confluent monolayer was obtained, B16 cells were treated with the ethyl acetate fraction at various concentrations (200, 100, 50, 25, 12.5, and 6.25 µg/ml). Cell viability was assessed after 72 hours by withdrawing the medium, adding 100 µL of a 2 mg/mL solution of MTT, and incubating the cells for 2.5 hours at 37 °C. Following incubation, the MTT solution was removed, and to solubilize the crystals remaining in the wells, 130 µL of dimethyl sulfoxide (DMSO) was added to each well, followed by incubation for 15 min with shaking [14]. Absorbance was measured with a microplate reader at 492 nm. The cell growth inhibition rate (% of cytotoxicity) was determined using the following equation [15,16].

Inhibition rate = A- B/A*100

(A) represents the optical density of the control, while (B) denotes the optical density of the samples.

The IC50 value is the material concentration required to block 50% of a biological or biochemical activity. It's a standard gauge of a compound's potency [17]. Within the framework of our cytotoxicity assay, it is the concentration of the ethyl acetate fraction of *J. integerrima* that results in 50% less cell viability by comparison to untreated cells.

Morphological changes study

Under an inverted microscope with 40x magnification (Optika, Italy), the morphological alterations after 72 hours of incubation were observed and recorded for B16 cells.

Statistical analysis

The obtained data were statistically analyzed using an unpaired t-test with GraphPad Prism 6 [18]. The values were presented as the mean ± SD of triplicate measurements [19].

3. Results and Discussion

The extraction

The yield of the concentrated ethyl acetate fraction was two grams.

Qualitative determination of phenolic compounds using RP-HPLC

HPLC is a sensitive, adaptable, and stable tool that can reveal a great deal about the extract's composition [20,21]. Whether it is purification, quantification, or another aim, HPLC technique validation mostly aims to evaluate the analysis approach and ensure it corresponds with the purpose of the experiment. Thus, in line with Flandez *et al.* [22], pre-validated HPLC qualitative quantification of flavonoids and phenolic acids in the *J. integerrima* ethyl acetate extract was performed in comparison to authentic samples, including vitexin, gallic acid, caffeic acid, quercetin, and rutin. Under the same chromatographic conditions, the sample components retention durations were contrasted with those of the real ones. The findings of the HPLC showed the presence of phenolic acids (gallic acid and caffeic acid) and flavonoids (vitexin, quercetin, and rutin), whose retention times corresponded with those of their standards (Figures 1, 2, 3, 4, 5, and 6).

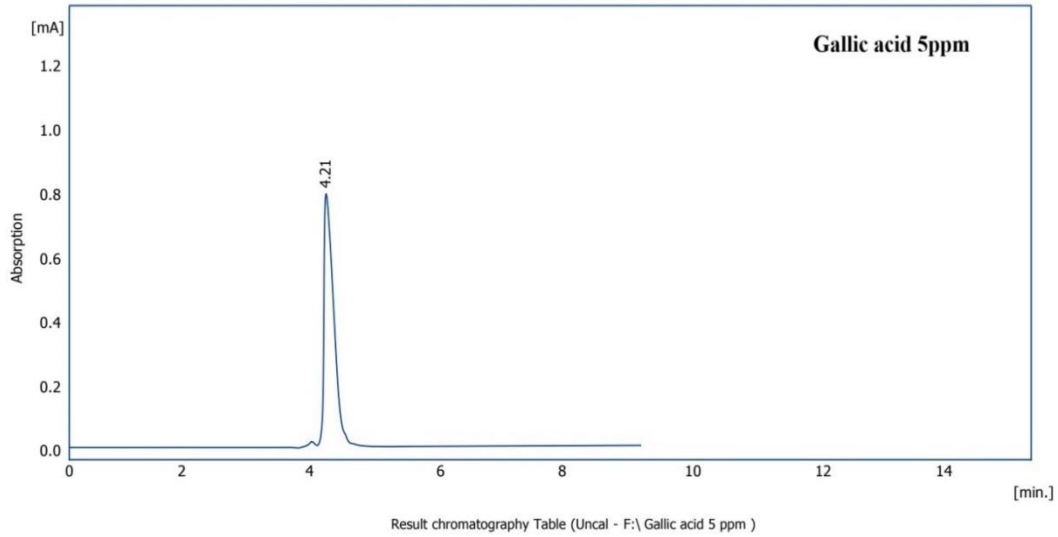

Figure 1: HPLC chromatograms of gallic acid standard.

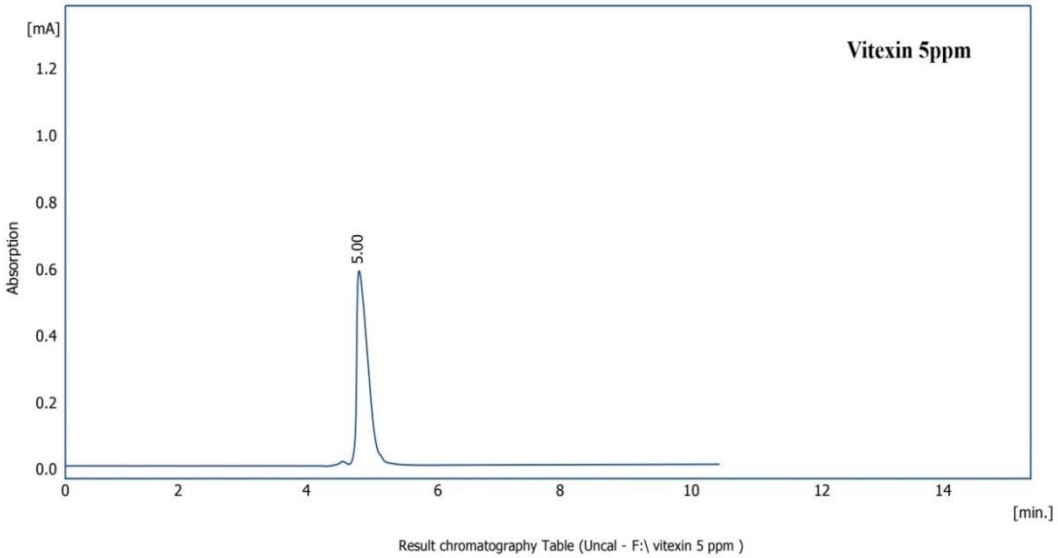

Figure 2: HPLC chromatograms of vitexin standard.

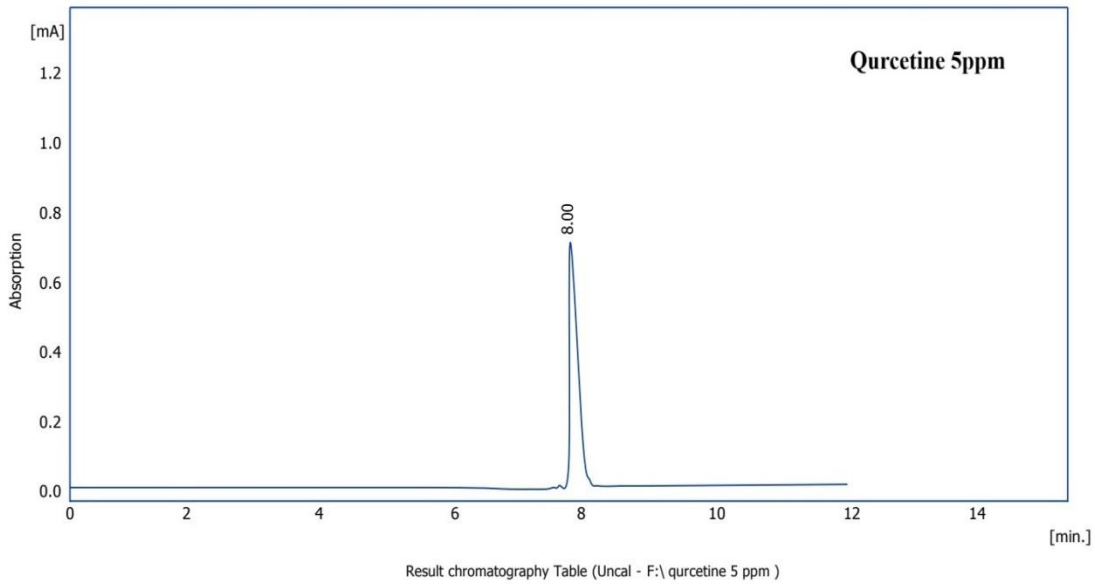

Figure 3: HPLC chromatograms of quercetin standard.

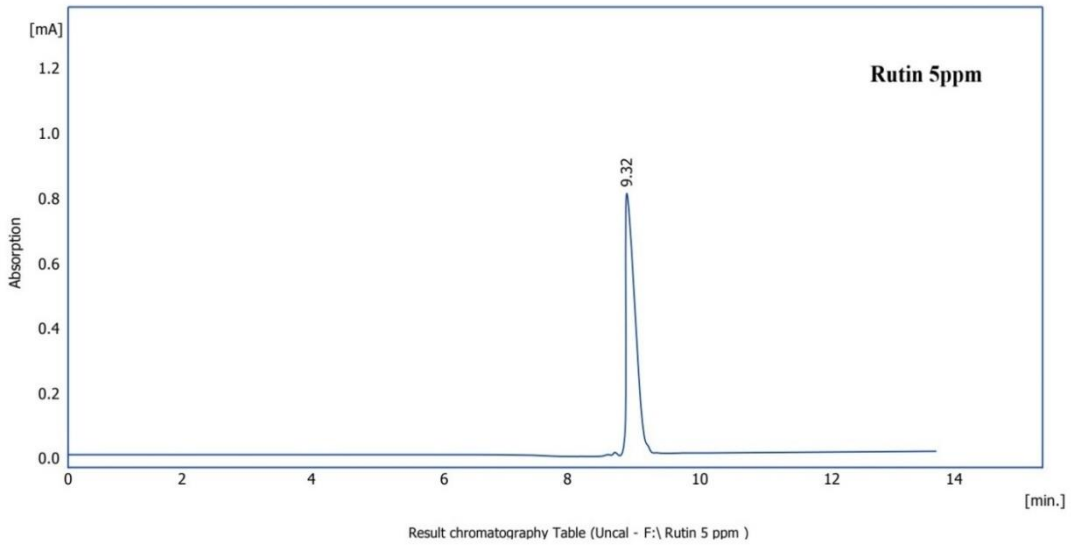

Figure 4: HPLC chromatograms of rutin standard.

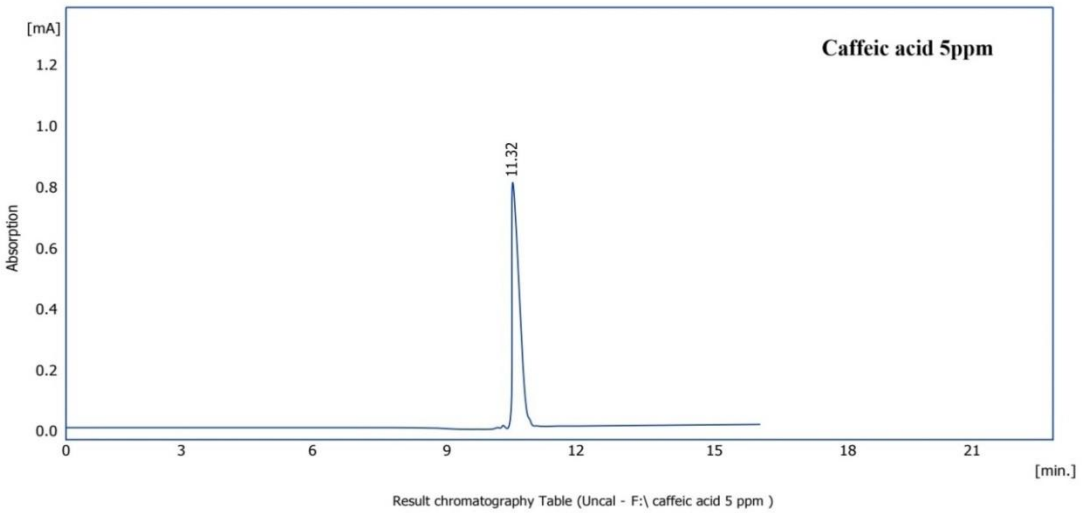

Figure 5: HPLC chromatograms of caffeic acid standard.

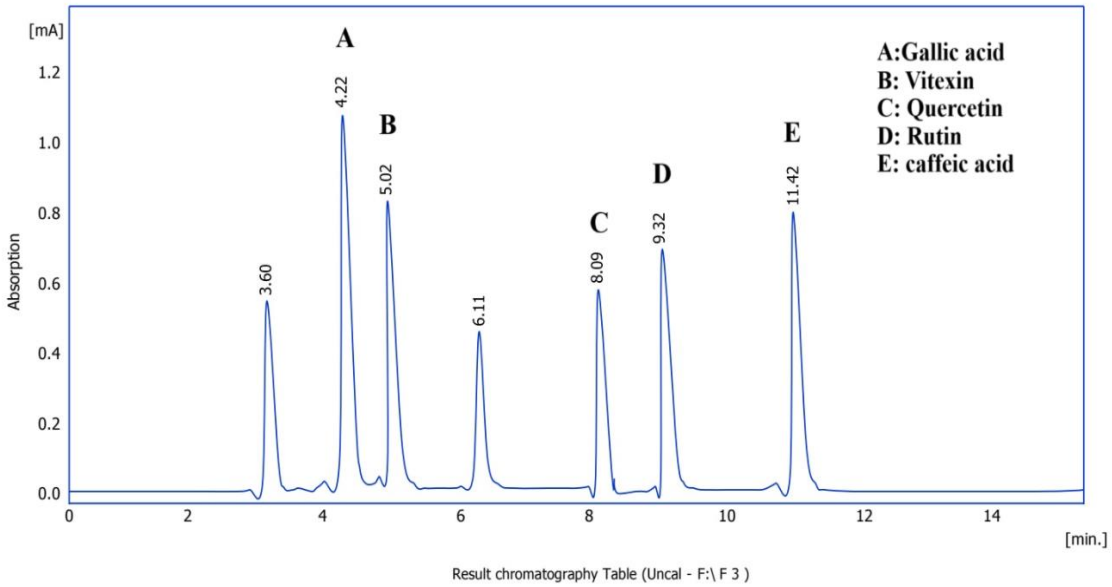

Figure 6: HPLC chromatograms of the ethyl acetate fraction of the aerial parts of *Jatropha integerrima* cultivated in Iraq using SKYAM HPLC by linear gradient elution. Mobile phase: A (0.1% formic acid), B (acetonitrile).

Quantification of the polyphenol compounds detected by HPLC

The concentrations of vitexin, gallic acid, and caffeic acid compounds in the ethyl acetate fraction of *J. integerrima* were calculated using a straight-line equation derived from graphing the area under the curve against the concentration of successive dilutions of every standard (Figures 7, 8, and 9).

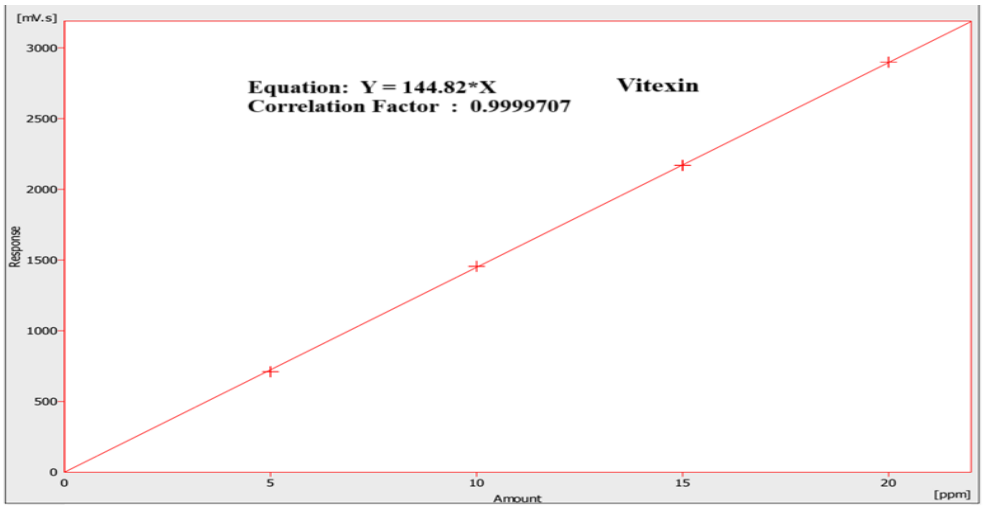

Figure 7: Calibration curve of vitexin standard.

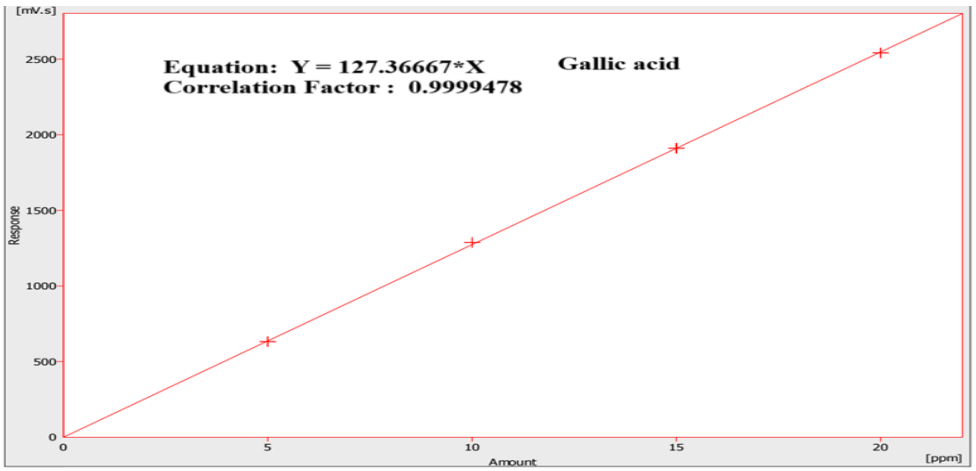

Figure 8: Calibration curve of gallic acid standard.

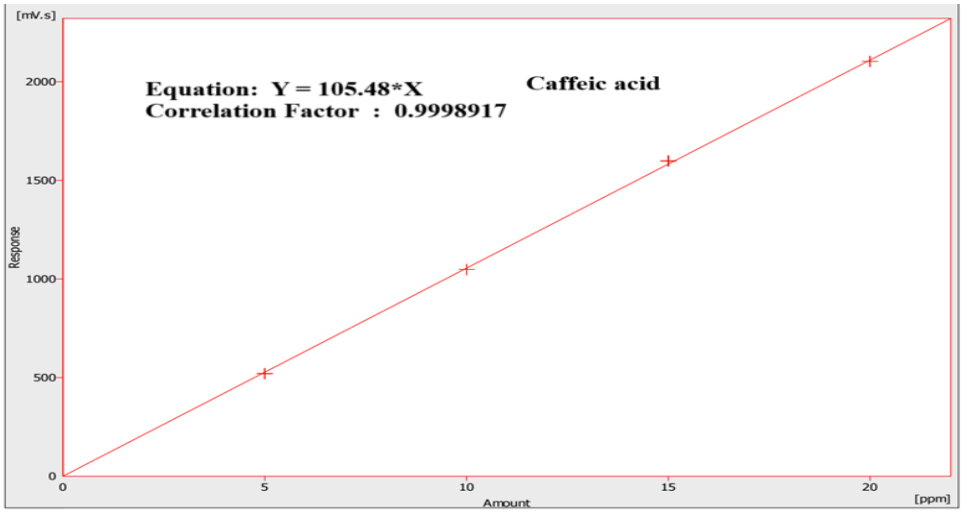

Figure 9: Calibration curve of caffeic acid standard.

Table 1 shows that in the aerial parts of the ethyl acetate fraction of *J. integerrima*, the concentration of gallic acid was higher than that of other constituents in this fraction, followed by caffeic acid.

Table 1: Concentration (ppm) of one flavonoid and two phenolic acids in the ethyl acetate fraction of *Jatropha integerrima.*

Active constituent's	peak area	Concentration (ppm)
Gallic acid	9745.01	1156.29
vitexin	7412.32	783.57
Caffeic acid	6521.99	940.42

Cytotoxic effect of Jatropha integerrima against skin cancer cells B16

In the MTT assay, the ethyl acetate fraction exhibited significant cytotoxic effects on the B16 cell line. The results were represented as the mean ± SD of three technical replicates. The highest inhibition rate of 88.00±2.49 % on the skin cancer B16 cell line at 200 µg/ml concentration indicated potent anti-cancer activity, as shown in Figure 10. The IC50 was 27.35 µg/ml.

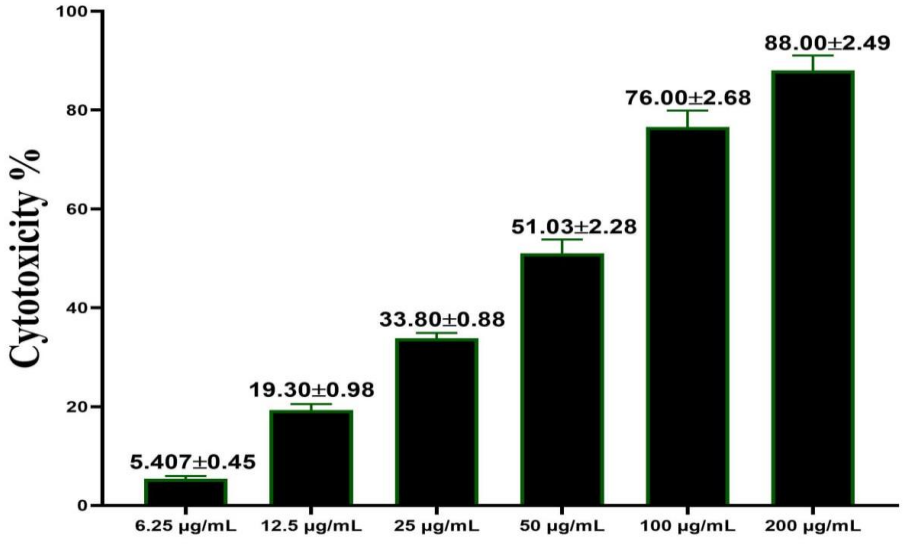

Figure 10: Cytotoxicity effects after 72 hours of incubation with different concentrations (200, 100, 50, 25, 12.5, and 6.25 µg/mL) of the ethyl acetate fraction of *Jatropha integerrima* against the B16 cell line. The results were represented as the mean ± SD of three technical replicates.

Morphological changes

The morphological comparison of control untreated B16 cells and B16 cells after being treated with ethyl acetate *J. integerrima* fraction appears as detachment or shrinkage, as shown in Figure 11.

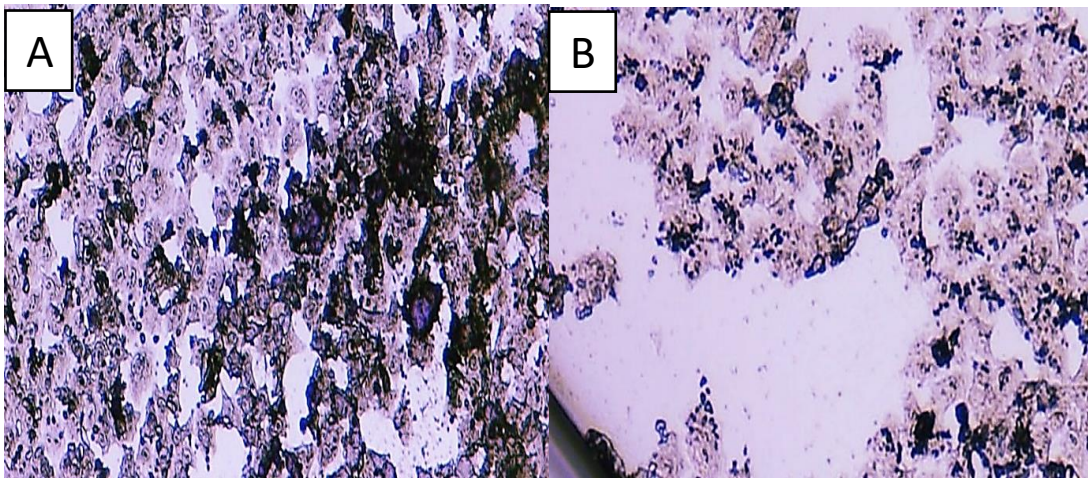

Figure 11: Effect of aerial parts ethyl acetate fraction of *Jatropha integerrima* on the morphology of skin cancer cells (B16 cells). A: Before treatment, B: After treatment with *Jatropha integerrima* ethyl acetate fraction 200 µg/ml and incubated for 72 hours.

With an 88% inhibition rate at the highest concentration investigated (200 µg/ml), the findings of this study reveal that the ethyl acetate fraction of *J. integerrima* has cytotoxic effects on B16 melanoma cells. In the future, more studies, such as apoptosis, migration, and colony formation assays, should be included to enhance the MTT assay in evaluating cytotoxicity. Important polyphenolic substances, including gallic acid, caffeic acid, vitexin, quercetin, and rutin known for their great biological activity, particularly their anti-cancer potential were found by the phytochemical examination. Gallic acid is a well-known phenolic acid compound with demonstrated anti-cancer effects through several mechanisms, such as inducing apoptosis in melanoma cells; it has been demonstrated to cause programmed cell death. It resulted in apoptotic morphological alterations such as chromatin condensation and cell shrinkage [23]. Another study shows inhibition of cell migration and invasion of human melanoma cells of the A375.S2 strain. Mediating this effect includes the downregulation of matrix metalloproteinase-2 (MMP-2) and the Ras signalling pathway, both of which are necessary for the dissemination of cancer cells [24]. One hydroxycinnamic acid (caffeic acid): Research indicates that caffeic acid induces G/M phase arrest. And also decreased tyrosinase activity [25]. Another study shows that treatment with caffeic acid decreases cell viability, suppresses colony development, cell cycle control, and changes caspase production, all significant apoptotic mediators [26]. Strong anti-cancer potential has been shown by vitexin, a flavonoid glycoside, especially concerning its capacity to target the STAT3 signalling system, thereby preventing the invasion and metastases of human melanoma cells. This suppression leads to diminished expression of epithelial-mesenchymal transition (EMT)-related proteins and matrix metalloproteinases (MMP-2 and MMP-9), therefore lowering the invasive potential of melanoma cells [27]. It has also been found to cause G2/M cell cycle arrest and death; reactive oxygen species (ROS) are generated, which destroy DNA and set off the DNA damage response system. Upregulated pro-apoptotic proteins are more likely to cause cell death; downregulated anti-apoptotic proteins help to further enable cell death [28]. A studied flavonoid having anticancer, anti-inflammatory, and antioxidant properties is quercetin, which exerts its lethal effects on B16 melanoma cells via several channels, including control of melanin synthesis and induction of apoptosis [29], as quercetin works by raising interferon-α and interferon-β expression, reducing melanoma cell proliferation, invasion, and migration according to in vitro and in vivo studies [30]. Another flavonoid, rutin, has been found to be a potent antioxidant with possible anti-melanoma properties. A study demonstrated that rutin decreased the viability of human melanoma cells and induced cellular senescence [31]. The cytotoxicity of the ethyl acetate fraction of *J. integerrima* against B16 melanoma cells results from the combined activities of vitexin, caffeic

acid, quercetin, and rutin. Every one of these bioactive compounds has been demonstrated to have antiproliferative and pro-apoptotic effects on melanoma cells by means of several but complementary approaches. Their presence in the same extract increases the likelihood of a synergistic interaction raising cytotoxicity.

Conclusion

This study indicates that the ethyl acetate fraction of the aerial parts of *J. integerrima* cultivated in Iraq has significant dose-dependent cytotoxic effects on the B16 melanoma cell line. This result has promising effects on cancerous lines, potentially starting the development of new, strong anticancer medications with minimal side effects, therefore providing new hope for cancer therapy approaches derived from natural sources. However, more research is required to confirm its effect on normal cells and guarantee its selectivity towards malignant cells. Additionally, further study is needed to isolate and identify more bioactive molecules that are responsible for the cytotoxic activity and analyze the in vitro and in vivo modes of action of these compounds.

Conflict of interest: no conflict of interest.

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