processes

MDPI

Article

Computational and *In Vitro* Assessment of a Natural Triterpenoid Compound Gedunin against Breast Cancer via Caspase 3 and Janus Kinase/STAT Modulation

Talib Hussain [1], Muteb Alanazi [2], Jowaher Alanazi [1], Tareq Nafea Alharby [2], Aziz Unnisa [3], Amir Mahgoub Awadelkareem [4], AbdElmoneim O. Elkhalifa [4], Mohammad M. Algahtani [5], SMA Shahid [6] and Syed Mohd Danish Rizvi [7,*]

1 Department of Pharmacology and Toxicology, College of Pharmacy, University of Ha'il, Ha'il 81442, Saudi Arabia; mdth_ah@yahoo.com (T.H.); js.alanzi@uoh.edu.sa (J.A.)
2 Department of Clinical Pharmacy, College of Pharmacy, University of Ha'il, Ha'il 81442, Saudi Arabia; ms.alanazi@uoh.edu.sa (M.A.); tn.alharby@uoh.edu.sa (T.N.A.)
3 Department of Pharmaceutical Chemistry, College of Pharmacy, University of Ha'il, Ha'il 81442, Saudi Arabia; khushiazeez@yahoo.co.in
4 Department of Clinical Nutrition, College of Applied Medical Sciences, University of Hail, Ha'il 81442, Saudi Arabia; mahgoubamir22@gmail.com (A.M.A.); ao.abdalla@uoh.edu.sa (A.O.E.)
5 Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia; mohgahtani@ksu.edu.sa
6 Department of Biochemistry, College of Medicine, University of Ha'il, Ha'il 81442, Saudi Arabia; sm.shahid@uoh.edu.sa
7 Department of Pharmaceutics, College of Pharmacy, University of Ha'il, Ha'il 81442, Saudi Arabia
* Correspondence: sm.danish@uoh.edu.sa

check for updates

Citation: Hussain, T.; Alanazi, M.; Alanazi, J.; Alharby, T.N.; Unnisa, A.; Awadelkareem, A.M.; Elkhalifa, A.O.; Algahtani, M.M.; Shahid, S.; Rizvi, S.M.D. Computational and *In Vitro* Assessment of a Natural Triterpenoid Compound Gedunin against Breast Cancer via Caspase 3 and Janus Kinase/STAT Modulation. *Processes* **2023**, *11*, 1452. https://doi.org/10.3390/pr11051452

Academic Editors: Alina Bora, Luminita Crisan and Elwira Sieniawska

Received: 26 March 2023
Revised: 27 April 2023
Accepted: 5 May 2023
Published: 11 May 2023

Abstract: Breast cancer is the most prevalent type of malignancy among females as per the report of the World Health Organization. There are several established chemotherapeutic regimes for the clinical management of different solid cancers; however, the after-effects of these therapeutics serve as a significant limiting factor. The natural triterpenoid compound, gedunin is one of the principal phytoconstituent found in *Azadirachta indica*. In this study, we have investigated the anticancer potential of gedunin against human breast cancer MDA-MB-231 and MCF-7 cells. Based on computational studies, gedunin exhibited significantly higher binding affinity of −7.1 and −6.2 Kcal/mol towards Janus kinase (JAK) and STAT proteins, respectively. Further, the anticancer potential of gedunin against human breast cancer was studied using hormone-independent and -dependent MCF-7 and MDA-MB-231 cell lines, respectively. The results indicated that gedunin inhibited the growth and multiplication of both MCF-7 and MDA-MB-231 cells. The nuclear fragmentation and ROS were qualitatively enhanced in the treated MCF-7 and MDA-MB-231 cells in comparison to untreated cells. The caspase-3 level was significantly enhanced with a concomitant decline in JAK1 and STAT3 mRNA expression. Based on these results, gedunin might be considered as a potential therapeutic lead against hormone-dependent and -independent breast cancer MCF-7 and MDA-MB-231 cells, respectively. However, further detailed mechanistic studies are warranted to conclusively establish the anti-breast cancer effects.

Keywords: natural compound; anticancer; breast cancer; caspase-3; Janus kinase1/STAT3

1. Introduction

Breast cancer, as per the World Health Organization, represents the most common carcinoma among females globally. The latest report by Global Cancer Observatory found that 2,261,419 new cases of breast carcinomas were reported during 2020, constituting nearly 11.7% of all the diagnosed 19,292,789 cases of various cancers globally. Subsequently, the report also stated that breast cancer was the reason behind 6.9% of deaths from

9,958,133 cancer-associated demises globally in 2020 [1]. At the molecular biology level, breast cancer exhibits considerable heterogeneity and is thus differentiated into various subtypes based on the presence and absence of various receptors. Exhaustive investigations costing billions of dollars have resulted in a better understanding of breast carcinoma's proliferation, invasiveness, and metastasis. These have further led to the development of frequently used adjuvant chemotherapeutic regimes, including docetaxel/cyclophosphamide, adriamycin/cyclophosphamide, and adriamycin/cyclophosphamide with combinatorial administration of paclitaxel. However, these regimes also have adverse clinical side effects [2]. Apart from the chemotherapy, endocrine therapy had long been recognized as a powerful and important adjuvant treatment for women with hormone receptor–positive, early-stage breast cancer. Many randomized clinical trial data demonstrated the benefit of ovarian function suppression, tamoxifen, and aromatase inhibitors alone, in combination, and sequentially, of different durations, and according to menopausal state and risk. Moreover, recent data provided evidence of the role of adjuvant CDK-4/-6 inhibitor/endocrine therapy combinations for those with high- and intermediate-risk disease [3]. Indeed, it has previously been reported that a combination of anticancer chemotherapeutics exerts superior therapeutical efficacy by improving the survival rate and reducing treatment-associated pain compared to a single chemotherapeutic [4]. Nevertheless, the adverse effects of these combinatorial chemotherapeutics cannot be overlooked since these, apart from cancer cells, also adversely affects normal healthy cells [5]. Intriguingly, breast cancer chemotherapy was recently found to be associated with the instigation of several complications, including cardiopathy, in patients [6].

Since ancient times, plants are not only a source of nutrition but have also been extensively used in the treatment of various ailments. The plants and their various isolated secondary metabolites have been substantially investigated for their anticancer potential apart from various other pharmacological characteristics [7]. One such natural compound is gedunin belonging to limonoid falling in various genera of the *Meliaceae* family, such as *Carapa*, *Cabralea*, *Azadirachta*, and *Guarea* [8]. In the epicarp of the fruit *Azadirachta indica A. Juss*, gedunin was abundantly reported [9]. Indeed, various pharmacological attributes have been previously associated with gedunin, which include anti-inflammatory, anti-allergic, anticancer, and antimalarial properties, among others [10]. Concerning breast cancer, previously anticancer effects of plants and their parts, along with purified compounds usually considered nutritive, are reported to be biologically active and possess pharmacological properties [10,11]. Limonoid is a particularly important bioactive phytocompound found abundantly in several member genera of the family *Meliaceae*. Among several other bioactive compounds extracted from *Azadirachta indica* or Indian neem, as presented in Figure 1, gedunin ($C_{28}H_{34}O_7$) is a member of the tetranotriterpenoid family, which has been explored for its anticancer effects against different cancer cell lines from colon, prostate, and ovarian origin [12–14]. Gedunin chemically acts as a reactive-thiol electrophile that activates the heat shock response by a mechanism closely similar to celastrol. Gedunin is readily soluble in organic solvents such as ethanol and DMSO; however, it is known for its poor solubility in water. Gedunin has also been previously documented for several pharmacological properties, including anti-allergic, neuroprotective effects, anti-inflammatory, antimalarial, and anticancer attributes [15]. Multiple studies have demonstrated that gedunin holds the therapeutic potential for the treatment of various cancers and its underlying mechanistic action is extensively studied. Furthermore, previous preclinical studies have shown that gedunin is capable of treating carcinomas of various organs such as lung, brain, colon, pancreas, stomach, ovary, prostate, and stem cells [9]. However, subsequent studies are warranted to provide a better understanding relating to the specific mechanism behind anticancer effects of gedunin. Gedunin is frequently used in the Indian medicine system for treating infectious diseases such as malaria [16].

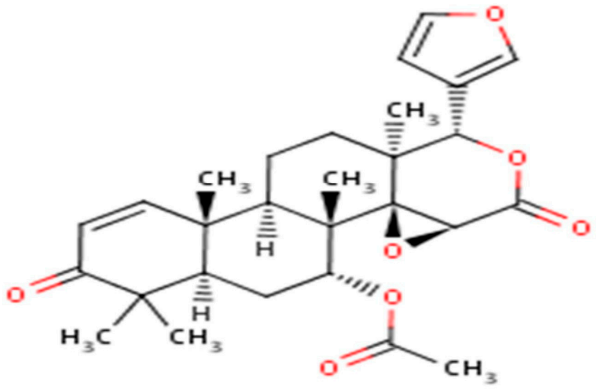

Figure 1. Chemical structure of gedunin. (Adopted from ChEBI; CHEBI:65954).

On the molecular basis, several altered signaling pathways have been associated with nearly all reported cancers. Janus kinase (JAK) signal transducer and activator of transcription (STAT) signaling play a pivotal role during embryo development by regulating various homeostatic cellular functions, including hematopoiesis, maintenance of cells, and inflammatory response. The pathway is responsible for transducing signals from growth factors, interleukins, and cytokines, which are functionally active through various transmembrane receptors. Previously, it was demonstrated that JAK1 serves to be an essential kinase required for cytokine-based activation of STAT protein in breast cancer cells [17]. Furthermore, recently, it has also been demonstrated that hyper- and constitutive-expression of STAT3 is involved in developing chemo-resistance, progression, and metastasis of breast cancer. Intriguingly, upstream and downstream STAT3 target-associated novel pathways have also been recently deciphered in breast carcinoma [18]. However, the anticancer effects of gedunin against human breast adenocarcinoma MCF-7 cells remain unexplored. Therefore, based on present evidence, the authors hypothesize that gedunin may exert an antiproliferative effect on human breast cancer MCF-7 cells by altering the expression of the JAK/STAT signaling pathway.

2. Results

2.1. Molecular Docking Results

3D structures of gedunin and gemcitabine were downloaded from the Pub Chem database. These compounds were docked to the JAK1 and STAT3 proteins to predict the mechanism of breast cancer cell suppression, as shown in Figure 2a–d. This report selected the commercial anticancer drug gemcitabine as a reference compound. Our docking studies have predicted that the binding energy of gedunin towards JAK1 and STAT3 are −7.1 and −6.2 kcal/mol, respectively, which are nearer to the binding energies of gemcitabine to JAK1 and STAT3 (−6.6 and −5.0 kcal/mol, respectively) as shown in Figures 3 and 4. More negative binding energy correlates with the better stability of the protein–ligand complex. Thus, it can be inferred from the results that gedunin showed better interactions with the targeted JAK1 and STAT3 proteins than gemcitabine. Moreover, further analysis showed the details of amino acids of JAK1 and STAT3 involved in hydrophobic interactions and hydrogen bonding with gedunin and gemcitabine.

The residues involved in hydrophobic interaction of JAK1 with gemcitabine are Phe^{1046} (A), Arg^{1041} (A), and Phe^{1044} (A). However, Gln^{1098} (B), Asp^{1042} (A), His^{885} (A), Ser^{1043} (A), and Val^{1045} (A) are engaged in hydrogen bonding (bond lengths 2.93, 3.05, 2.80, 3.01, and 3.30). Furthermore, the amino acids involved in hydrophobic interactions of STAT3 with gemcitabine are Tyr^{446} (A), Gln^{361} (A), Leu^{358} (B), Gln^{361} (B), His^{447} (B), and Tyr^{446} (B). However, Glu^{357} (B) and Gln^{448} (B) are engaged in hydrogen bond interactions (bond lengths 2.98 Å, 3.01 Å, and 3.25 Å) (Figure 3a,b).

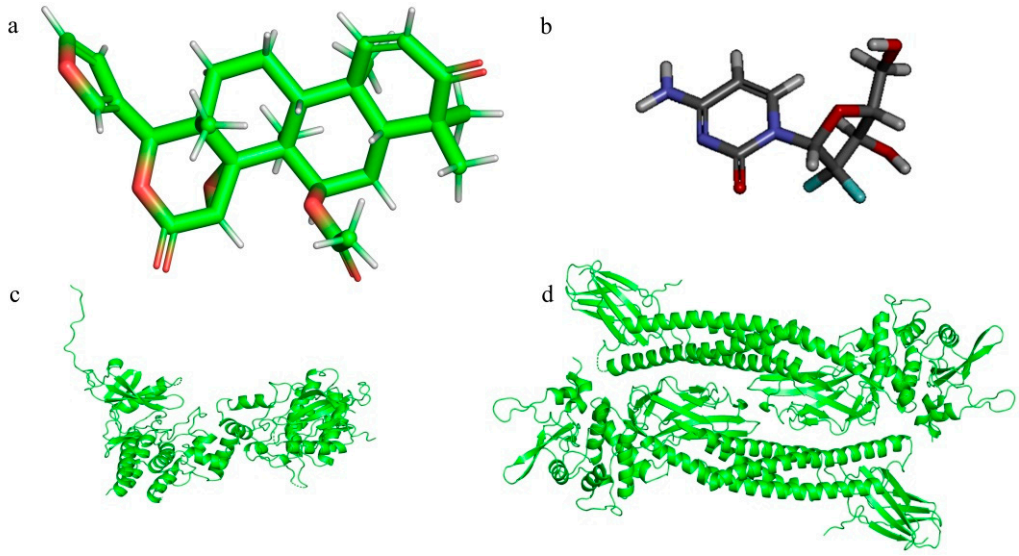

Figure 2. 3D chemical structures of (**a**) gedunin, (**b**) gemcitabine, (**c**) JAK1, and (**d**) STAT3.

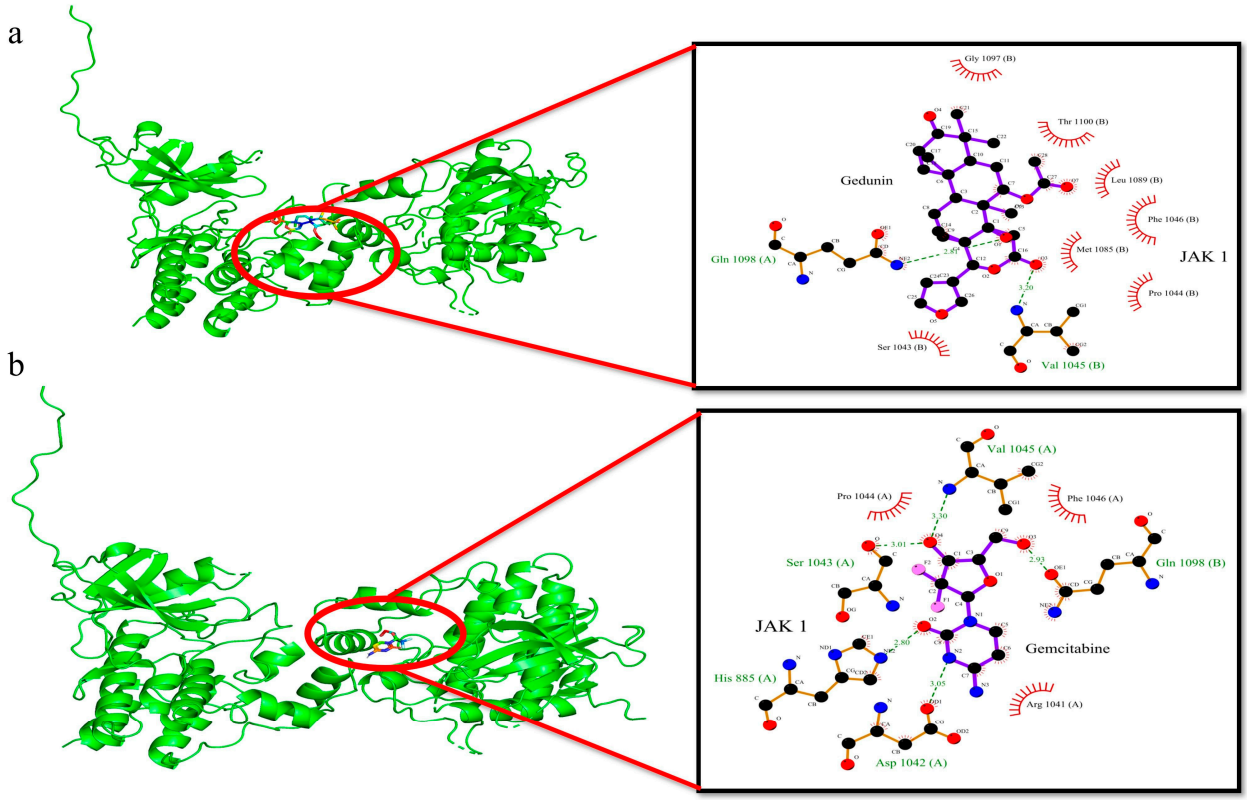

Figure 3. 2D interaction complex of (**a**) gedunin and (**b**) gemcitabine with JAK1 protein; where red shows the target protein, purple shows the ligand molecule, and red circle shows the enlarged view of the residues of gedunin with respective target proteins.

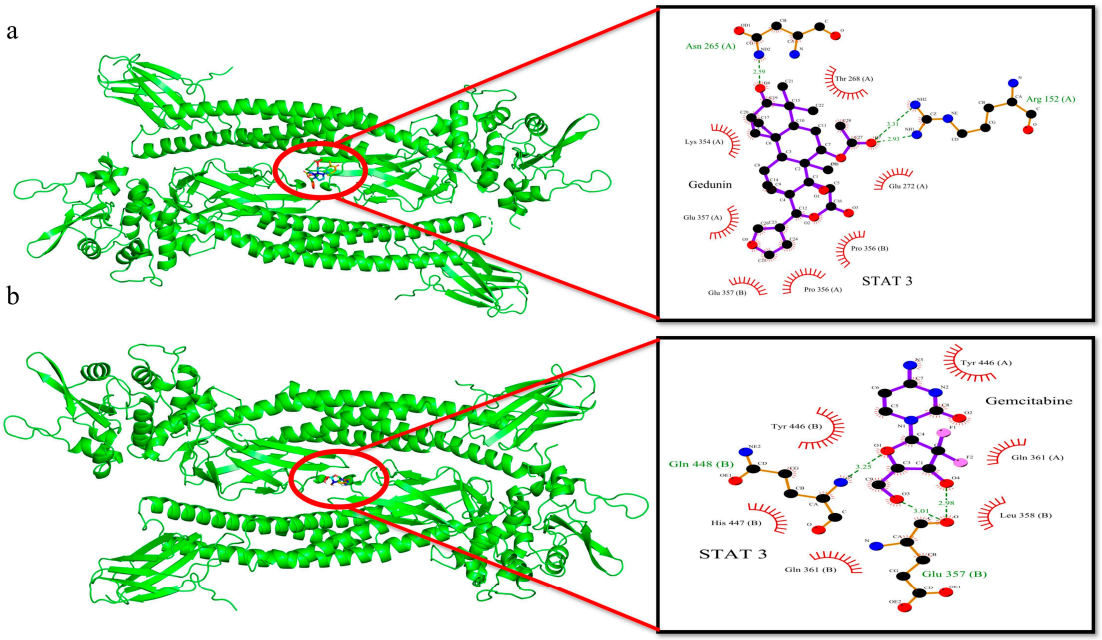

Figure 4. 2D interaction complex of (**a**) gedunin and (**b**) gemcitabine with STAT3 protein; where red shows the target protein, purple shows the ligand molecule, and red circle shows the enlarged view of the residues of gedunin with respective target proteins.

Gedunin has been hypothesized to interact with JAK1 and STAT3 proteins. It was found that the JAK1 residues involved in hydrophobic interaction with gedunin are Gly^{1097}, Thr^{1100}, Leu^{1089}, Phe^{1046}, Met^{1085}, Pro^{1044}, Ser^{1043}, and Val^{1045}, Gln^{1098}, which are engaged in hydrogen bonding (bond lengths 3.20 Å and 2.81 Å), whereas the amino acids of STAT3 involved in hydrophobic interactions with gedunin are Thr^{268} (A), Glu^{272} (A), Pro^{356} (B), Pro^{356} (A), Gln^{357} (B), Gln^{357} (A), and Lys^{354} (A). However, Arg^{152} (A) and Asn^{265} (A) engage in hydrogen bond interactions (bond lengths 3.31 Å, 2.93 Å, and 2.59 Å), as shown in Figure 3a,b and Figure 4a,b. The binding energies of gedunin and gemcitabine with breast cancer targets (JAK1 and STAT3) and the interacting amino acids have been summarized in Table 1.

Table 1. Binding energies of gedunin and gemcitabine with breast cancer targets (JAK1 and STAT3) and the interacting amino acids.

Compound	Binding Energy (Kcal/mol)	Hydrogen Bonds (Bond Length in Å)	Hydrophobic Interactions
JAK1–gedunin	−7.1	Gln^{1098} engage in hydrogen bonding (bond lengths 3.20 and 2.81)	Gly^{1097}, Thr^{1100}, Leu^{1089}, Phe^{1046}, Met^{1085}, Pro^{1044}, Ser^{1043}, and Val^{1045}
JAK1–gemcitabine	−6.6	Gln^{1098} (B), Asp^{1042} (A), His^{885} (A), Ser^{1043} (A), and Val^{1045} (A) engage in hydrogen bonding (bond lengths 2.93, 3.05, 2.80, 3.01, and 3.30).	Phe^{1046} (A), Arg^{1041} (A), and Phe^{1044} (A)
STAT3–gedunin	−6.2	Arg^{152} (A) and Asn265 (A) engage in hydrogen bond interactions (bond lengths 3.31, 2.93, and 2.59).	Thr^{268} (A), Glu^{272} (A), Pro^{356} (B), Pro^{356} (A), Gln^{357} (B), Gln^{357} (A), and Lys^{354} (A).
STAT3–gemcitabine	−5.0	Glu357 (B), and Gln448 (B) engage in hydrogen bond interactions (bond lengths 2.98, 3.01, and 3.25).	Tyr446 (A), Gln361 (A), Leu358 (B), Gln361 (B), His447 (B), and Tyr446 (B).

2.2. Gedunin Impeded the Growth of MCF-7 and MDA-MB-231 Cells

To investigate the plausible cytotoxic effects of gedunin on MCF-7 cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide or MTT assay was performed after incubating MCF-7 cells with various doses of gedunin (5, 15, and 20 µM) for 24 h. The results showed that gedunin exerted substantial cytotoxic effects by suppressing the growth of MCF-7 cells by 83.13 ± 3.70%, 61.09 ± 3.87%, and 33.06 ± 4.23% at the indicated concentration of 5µM, 10µM, and 15µM, respectively (Figure 5a). Furthermore, the viability of MCF-7 was reduced to 70.13 ± 4.03% (5 µM), 46.75 ± 4.56% (10 µM), and 27.40 ± 2.03% (15 µM) after 48 h of incubation with gedunin (Figure 5c). Gedunin further reduced the viability of hormone independent MDA-MB-231 cells to 88.98 ± 3.14%, 54.01 ± 3.17%, and 29.75 ± 3.81% (Figure 5b) at above stated concentrations. The viability of MDA-MB-231 cells was also further reduced to 79.65 ± 4.56% (5 µM), 48.35 ± 5.55% (10 µM), and 24.75 ± 4.72% (15 µM) after 48 h of incubation with gedunin (Figure 5d). Gemcitabine used as a positive control in our study also significantly impeded the viability of both the stated human-derived breast cancer cell lines. The IC_{50} of gedunin was found to be 11.80 ± 1.047 µM and 10.67 ± 1.02 µM % for breast cancer MCF-7 and MDA-MB-231 cells, respectively (Figure 6a,b). Furthermore, we also inspected the toxicity of gedunin against human normal cell line HEK-293. As shown in Figure 6c, gedunin exerts insignificant cytotoxic effects on HEK-293 cells, which confirm its non-toxic nature.

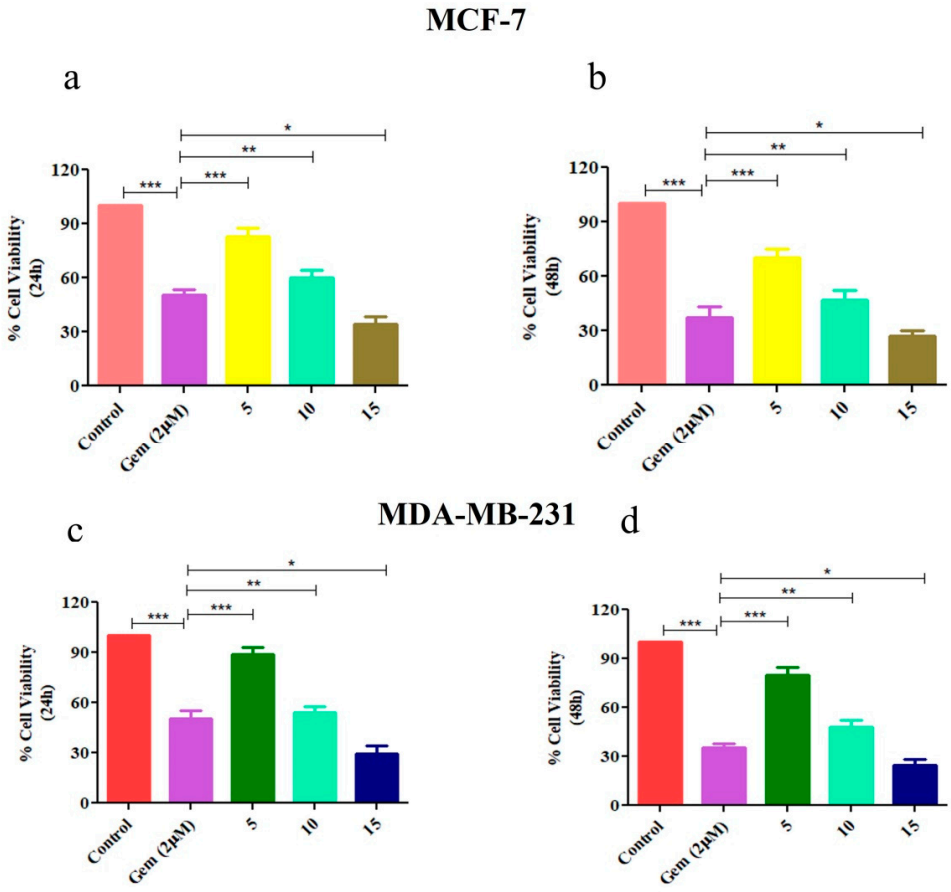

Figure 5. Gedunin suppressed the proliferation of breast cancer cells. Cell viability percentage of gedunin-treated after (**a**) 24 h and (**b**) 48 h in MCF-7 cells. The viability of MDA-MB-231 cells after treatment with gedunin after (**c**) 24 h and (**d**) 48 h. A significant difference determined by the *p* value < 0.05 was labeled with asterisk (*); *p* value < 0.01 was labeled with double asterisks (**); and *p* value < 0.001 was labeled with double asterisks (***).

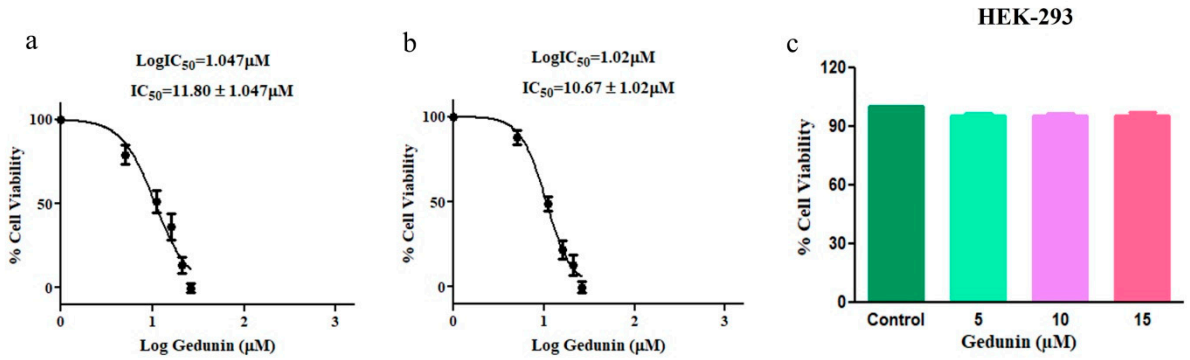

Figure 6. IC_{50} calculation for gedunin against (**a**) MCF-7 cells, (**b**) MDA-MB-231 cells, and (**c**) non-significant cytotoxicity of gedunin on HEK-293 cells.

2.3. Gedunin-Induced Nuclear Condensation and Fragmentation

4′,6-Diamidino-2-phenylindole also known as DAPI staining was performed to determine whether gedunin-mediated cell growth inhibition in breast cancer cells resulted from the induction of apoptosis. After 24 h treatment with gedunin at different concentrations (5, 10, and 15 µM), changes in nuclear morphology for both the cell lines (MCF-7 and MDA-MB-231) were observed. Fluorescence micrographs presented in Figure 7 showed bright-blue fluorescence and condensed nuclei with the increasing concentration of gedunin in both MCF-7 and MDA-MB-231 cells, indicating the onset of apoptosis. Gemcitabine-treated MCF-7 and MDA-MB-231 cells also exhibited increased nuclear condensation as seen in Figure 7. However, no substantial condensation was observed in untreated hormone-dependent and independent breast cancer cells demonstrating diffusely stained intact nuclei. The results, finally, suggested that gedunin-induced apoptosis in MCF-7 cells in a dose-dependent manner.

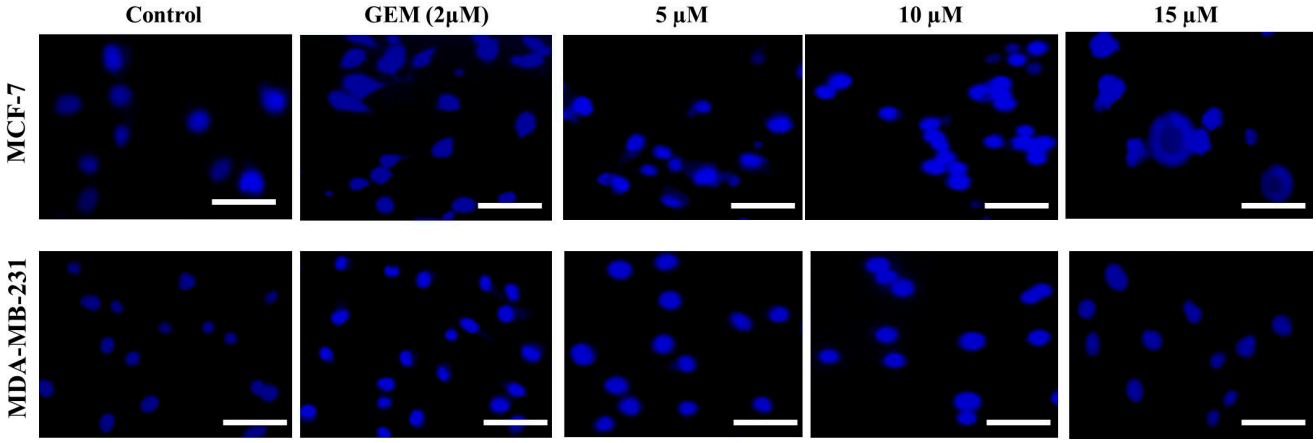

Figure 7. Efficacy of gedunin in altering the homeostatic nuclear morphology in MCF-7 and MDA-MB-231 cells as evaluated through DAPI stain. Scale bar = 20 µm; magnification: 30×.

Furthermore, the level of nuclear fragmentation and condensation induced by gedunin at different concentrations was quantified using ImageJ software, NIH, Maryland, USA. Gedunin succeeded in enhancing the levels of nuclear fragmentation and condensation by 33.38 ± 3.74 (5 µM), 54.35 ± 3.05 (10 µM), and 78.42 ± 4.61 (15 µM) after 24 h of incubation with MCF-7 cells (Figure 8a). Furthermore, in MDA-MB-231 cells, gedunin-mediated nuclear condensation and fragmentation was found to be escalated by 35.38 ± 4.32 (5 µM), 57.68 ± 4.68 (10 µM), and 73.42 ± 3.24 (15 µM) after 24 h (Figure 8b). Furthermore, gemcitabine also showed its competence in elevating the levels of nuclear fragmentation and condensation in both human-derived breast cancer cell lines.

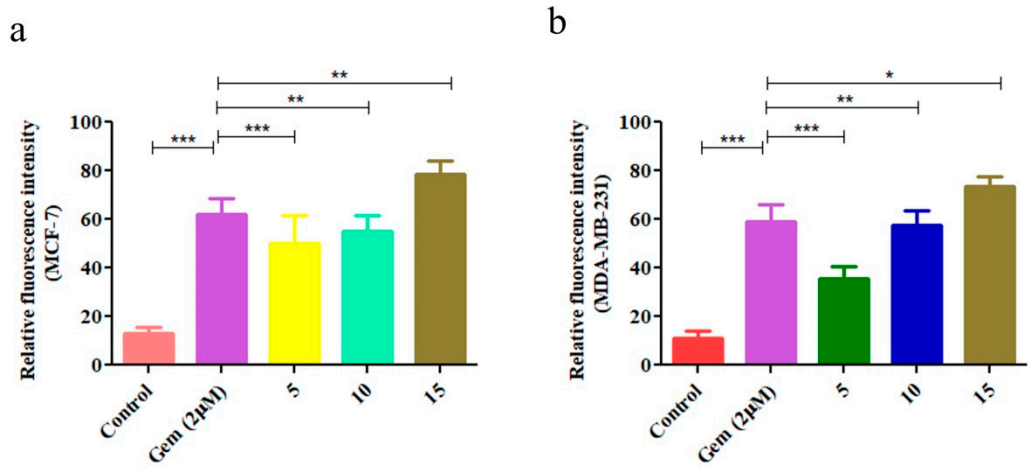

Figure 8. Quantification of gedunin-mediated nuclear condensation and fragmentation. (**a**) MCF-7 and (**b**) MDA-MB-231 cells as evaluated through DAPI stain. A significant difference determined by the p value < 0.05 was labeled with asterisk (*); p value < 0.01 was labeled with double asterisks (**), and p value < 0.001 was labeled with double asterisks (***).

2.4. Gedunin Treatment Elevated Caspase-3 Activity

Caspases are essential for transmitting the signals during apoptosis, and these are represented by a group of proteases mediating the essential functions of proteolytic cleavage of various proteins. The investigators tried to substantiate whether gedunin-instigated apoptosis altered the expression of caspase-3 comparatively with untreated control MCF-7 cells. The activity of caspase-3 was elevated by 37.40 ± 4.66% (at 5 μM gedunin concentration), 55.79 ± 5.46% (at 10 μM gedunin concentration), and 113.42 ± 3.77% (at 15 μM gedunin concentration) within the MCF-7 cells (Figure 9a). In case of MDA-MB-231 breast cancer cell lines, caspase-3 activity was found to be increased by 41.69 ± 4.45%, 59.12 ± 4.62%, and 107.25 ± 5.29% at 5 μM, 10 μM, and 15 μM concentrations of gedunin, respectively (Figure 9c). Thus, it can be concluded that the treatment of gedunin significantly increases the activity of caspase-3 in both MCF-7 and MDA-MB-231 cells. Furthermore, gemcitabine acted as a positive control and also elevated caspase-3 activity within MCF-7 (79.91 ± 3.662) and MDA-MB-231 cells (67.25 ± 3.42) in comparison with respective untreated control.

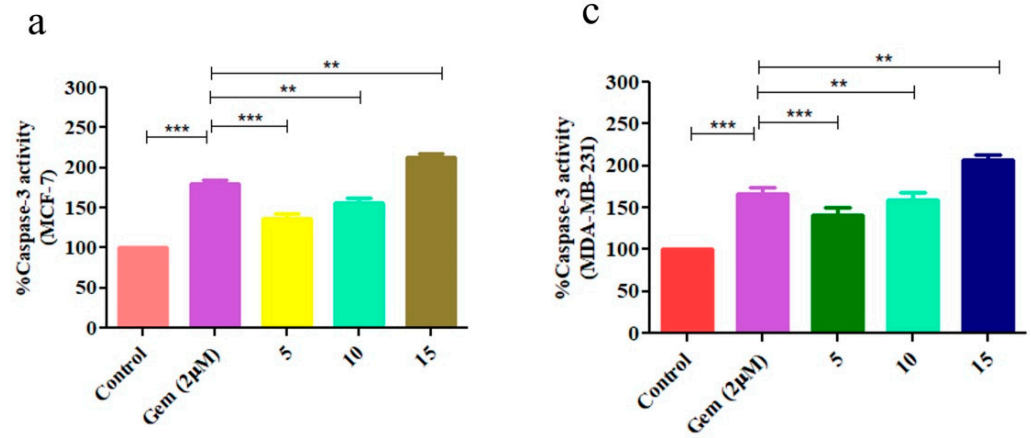

Figure 9. *Cont.*

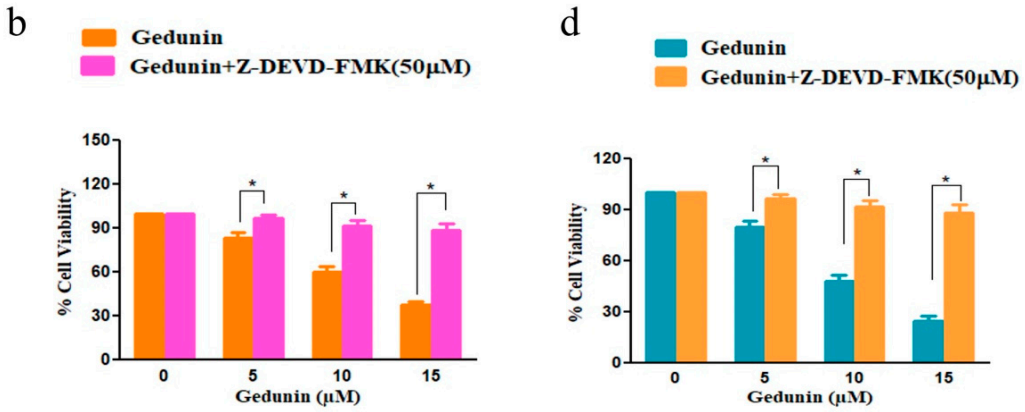

Figure 9. Gedunin mediated the activation of caspase-3 in (**a**) MCF-7 (**c**) MDA-MB-231 cells. Pre-treatment with caspase-3 inhibitor significantly ameliorated the cytotoxic effects induced by gedunin in both MCF-7 and MDA-MB-231 cells (**b**,**d**). A significant difference determined by the p value < 0.05 was labeled with asterisk (*); p value < 0.01 was labeled with double asterisks (**), and p value < 0.001 was labeled with double asterisks (***).

2.5. Caspase-3 Inhibitor Alleviated Gedunin-Mediated Cytotoxicity

To confirm the activation of caspase-3 in MCF-7 and MDA-MB-231 breast cancer cells by gedunin and its role in cytotoxicity, the cells pre-treated with caspase-3 inhibitor (Z-DEVD-FMK) were further treated with 5, 10, and 15 μM concentrations of gedunin. It was observed that caspase-3 inhibitor pre-treatment substantially impeded the gedunin-mediated cytotoxicity against both MCF-7 and MDA-MB-231 cells (Figure 9b,d), indicating a key role of caspase-3 activation during gedunin-induced effect. However, pretreatment of caspase inhibitors did not completely attenuate the cell viability in both MCF-7 and MDA-MB-231 cells, which indicated plausible role of the caspase-independent pathways as well. Thus, it is reasonable to say that gedunin might induce effects on breast cancer cells via both caspase-dependent and independent manner.

2.6. Gedunin-Instigated Intracellular ROS

Enhanced ROS production levels could be linked with the activation of apoptotic pathways in the cancer cells [19]. Thus, we investigated the effect of ROS generation after treatment with different concentrations of gedunin for 24 h in breast cancer cells. As shown in Figure 10a,b, a substantial increase in ROS level by 17.32 ± 3.12% was seen as compared to control cells following treatment with 5 μM of gedunin. Intriguingly, ROS generation was further enhanced by 54.01 ± 3.14% and 101.08 ± 4.79% in MCF-7 cells at the concentrations of 10 μM and 15 μM, respectively. Similarly, ROS levels increased by 26.38 ± 2.89% (5 μM) in comparison with untreated MDA-MB-201 control cells. ROS levels further escalated by 58.68 ± 4.84% (10 μM) and 117.75 ± 3.53% (15 μM) (Figure 10c,d). Importantly, gedunin also succeeded in instigating the production of intracellular ROS. As shown in Figure 10c,d, gemcitabine also increased intracellular ROS level by 71.42 ± 4.90% in MCF-7 cells whereas in MDA-MB-231 cells the levels of ROS increased by 77.75 ± 3.53% in comparison with respective untreated control. The stated observations indicated that gedunin treatment augmented ROS production in hormone-dependent and independent breast cancer cells.

2.7. NAC Pretreatment Abrogated Gedunin-Instigated Intracellular ROS

N-acetyl-l-cysteine (NAC; a potent ROS inhibitor) was used to ascertain the role of gedunin in augmenting ROS within MCF-7 and MDA-MB-231 cells. Initially, both the cells were treated with NAC for 15 min, followed by the treatment with various concentrations of gedunin (5, 10, and 15 μM) for another 24 h, using MTT assay. It was demonstrated that pretreatment with NAC significantly decreased the amount of gedunin-induced ROS in breast cancer cells. Moreover, NAC pre-treatment also subdued ROS production in both MCF-7 and MDA-MB-231 cells treated with gemcitabine. Thus, our results suggested that

increased generation of intracellular ROS is crucial for apoptosis induced by the treatment of gedunin (Figure 10e,f). However, it was intriguing to note that NAC did not entirely ameliorate the inhibitory action of gedunin on the growth of MCF-7 and MDA-MB-231 cells, which suggested the involvement of various other ROS-independent pathways in gedunin-treated breast cancer cells.

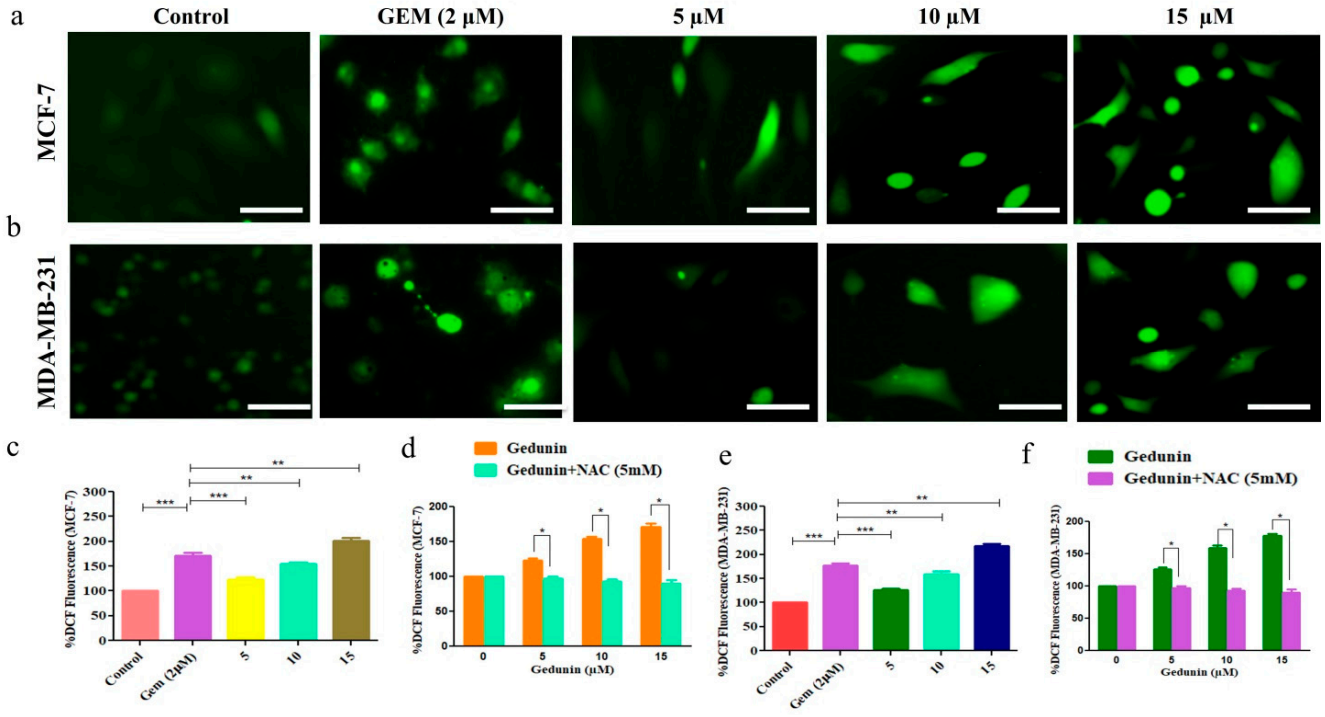

Figure 10. Gedunin-mediated effects on (**a**,**b**) instigation of intracellular ROS within human breast adenocarcinoma MCF-7 and MDA-MB-231 cells, (**c**,**d**) percent DCHF-DA fluorescence in gedunin-treated MCF-7 and MDA-MB-231 cells, and (**e**,**f**) the effects of NAC pretreatment in ameliorating gedunin-mediated ROS. Scale bar = 20 µm; magnification: 30×. A significant difference determined by the p value < 0.05 was labeled with asterisk (*); p value < 0.01 was labeled with double asterisks (**) and p value < 0.001 was labeled with double asterisks (***).

2.8. Gedunin Reduced JAK1/STAT3 Expression

Among different cellular pathways that reported their abrupt regulation, the JAK/STAT pathway was found to be crucial for proliferation, cellular differentiation, and immune system responsiveness [20]. Gedunin-mediated modulation of the JAK1/STAT3 signaling pathway in both the breast cancer cells was investigated. The observations indicated that gedunin exposure impeded JAK1/STAT3 signaling by reducing JAK1 and STAT3 mRNA expression. Gedunin suppressed JAK1 mRNA expression to 0.88 ± 0.04% (5 µM), 0.50 ± 0.04% (10 µM), and 0.39 ± 0.05% (15 µM) ($p < 0.05$) and mRNA level of STAT3 to 0.92 ± 0.02%, 0.71 ± 0.05%, and 0.36 ± 0.03% ($p < 0.05$), respectively (Figure 11a,b), in MCF-7 cells. Furthermore, gedunin succeeded in reducing the expression of JAK1 mRNA to 0.78 ± 0.07%, 0.57 ± 0.06%, and 0.35 ± 0.09% at 5 µM, 10 µM, and 15 µM concentration of gedunin in MDA-MB-231 cells, respectively. Similarly, STAT3 mRNA expression was reduced to 0.76 ± 0.07%, 0.49 ± 0.05%, and 0.25 ± 0.05% at 5 µM, 10 µM, and 15 µM concentration of gedunin in MDA-MB-231 cells, respectively (Figure 11c,d).

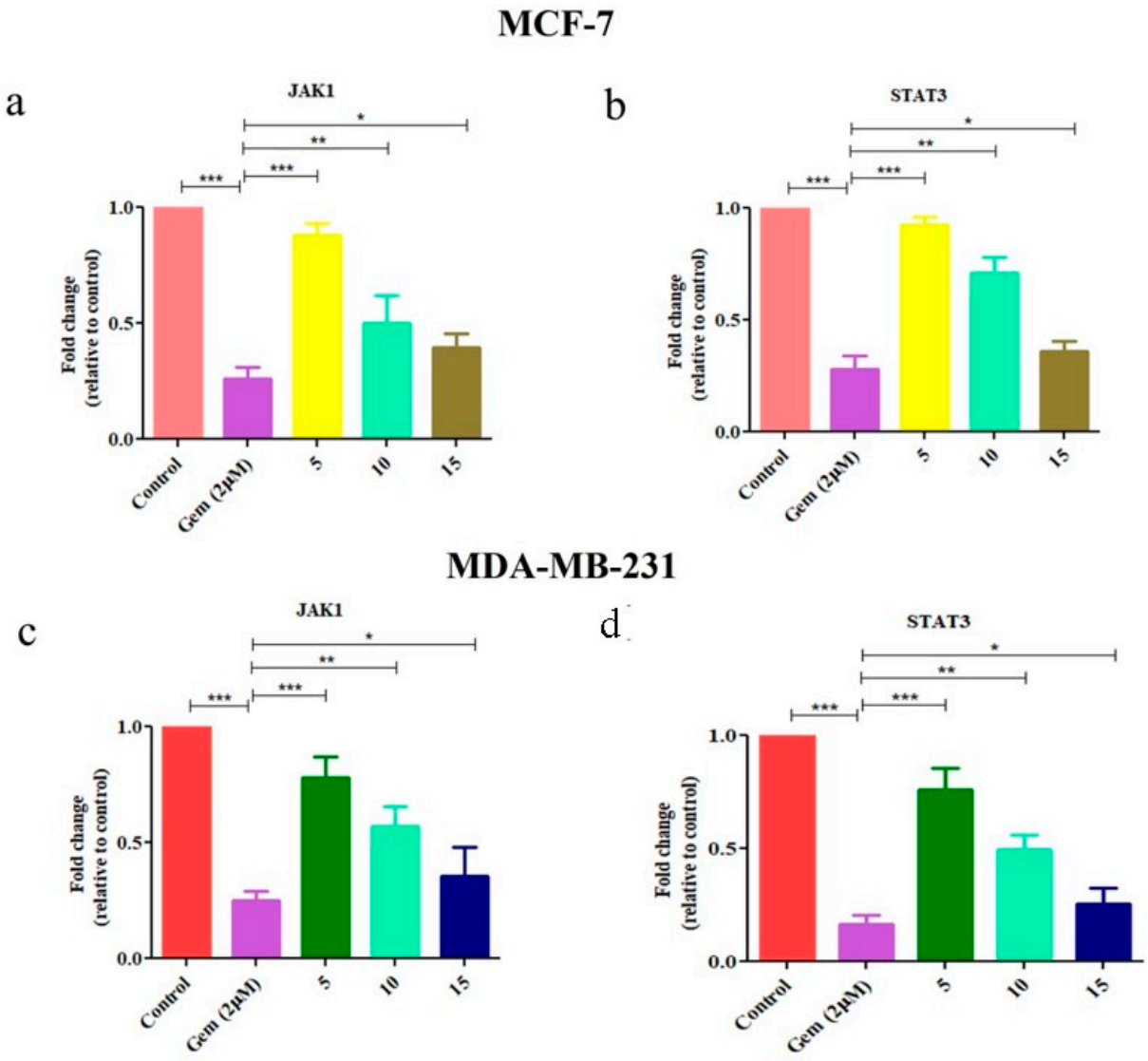

Figure 11. The effect of gedunin in modulating the expression of JAK1 and STAT3 genes in (**a**,**b**) MCF-7 and (**c**,**d**) MDA-MB-231 cells. A significant difference determined by the p value < 0.05 was labeled with asterisk (*); p value < 0.01 was labeled with double asterisks (**); and p value < 0.001 was labeled with double asterisks (***).

2.9. Gedunin Regulated the Gene Expression of JAK1/STAT3-Associated Genes

Intriguingly, constitutive STAT3 gene expression has been reported to promote the proliferation, survival, and migration of cancer cells. Furthermore, an increase in STAT3 also plays a crucial role in limiting the responsiveness of cancer cells to the individual's immune response [21]. An earlier published report has demonstrated that docetaxel can bind to Bcl-2, overexpressed in several different cancer cells, including breast and prostate cancer cells. Bcl-X_L is also downregulated by docetaxel [22]. Anti-apoptotic proteins, including Bcl-2, Bcl-X_L, and Mcl-1, are often elevated after activation of STAT3, which subsequently assist cancer cell survival [23,24]. We observed that there was a significant reduction in the level of expression of these stated anti-apoptotic markers (Bcl-X_L and Bcl-2) after gedunin exposure at the indicated concentrations of 5, 10, and 15 μM to 0.81 ± 0.04%, 0.66 ± 0.03%, and 0.44 ± 0.07%; 0.90 ± 0.02%, 0.74 ± 0.06%, and 0.57 ± 0.03% (Figure 12a–c), whereas the same in case of MDA-MB-231 cells was found to be 0.87 ± 0.04%, 0.61 ± 0.04%, and 0.37 ± 0.05%; 0.83 ± 0.05%, 0.71 ± 0.05%, and 0.41 ± 0.05%, respectively (Figure 12d–f). Concomitantly, gedunin enhanced the expression of Bax to 1.25 ± 0.03, 1.65 ± 0.05, and 1.99 ± 0.08 folds and was also seen in MCF-7 cells,

whereas the same in case of MDA-MB-231 cells was found to be 1.32 ± 0.06, 1.53 ± 0.07, and 2.08 ± 0.06 folds, respectively (Figure 12d).

Enhanced proliferation of cancer cells is usually associated with enhanced c-myc and cyclin D1 expression [25]. The qPCR results revealed that cyclin D1 and c-myc mRNA expression was lowered to 0.82 ± 0.03%, 0.64 ± 0.04%, and 0.33 ± 0.03% ($p < 0.05$) and 0.84 ± 0.02%, 0.61 ± 0.05%, and 0.23 ± 0.04% ($p < 0.01$), respectively, in MCF-7 cells (Figure 13). In case of MDA-MB-231, the reduction was calculated to be 0.79 ± 0.07%, 0.59 ± 0.06%, and 0.38 ± 0.08%; 0.84 ± 0.06%, 0.62 ± 0.05%, and 0.37 ± 0.08%, respectively. Gedunin-mediated modulation of cell cycle regulatory genes within MCF-7 cells were also investigated. mRNA expression of $p21^{Cip1}$ was significantly increased by 1.22 ± 0.04, 1.54 ± 0.02, and 1.76 ± 0.03 ($p < 0.001$) folds post-gedunin exposure whereas the same in case of MDA-MB-231 cells was found to be 1.64 ± 0.05, 1.94 ± 0.06, and 2.53 ± 0.11 folds, respectively. STAT3 downregulation is also associated with regulating protein expression such as p53, which is important for apoptosis induction [26,27]. The results indicated that gedunin declined p53 expression by 1.14 ± 0.02, 1.37 ± 0.04, and 2.16 ± 0.05 ($p < 0.001$) folds in MCF-7 cells. In case of MDA-MB-231, the reduction in p53 expression was calculated to be 1.32 ± 0.03, 1.60 ± 0.09 and 2.06 ± 0.04 folds, respectively (Figure 13e,f).

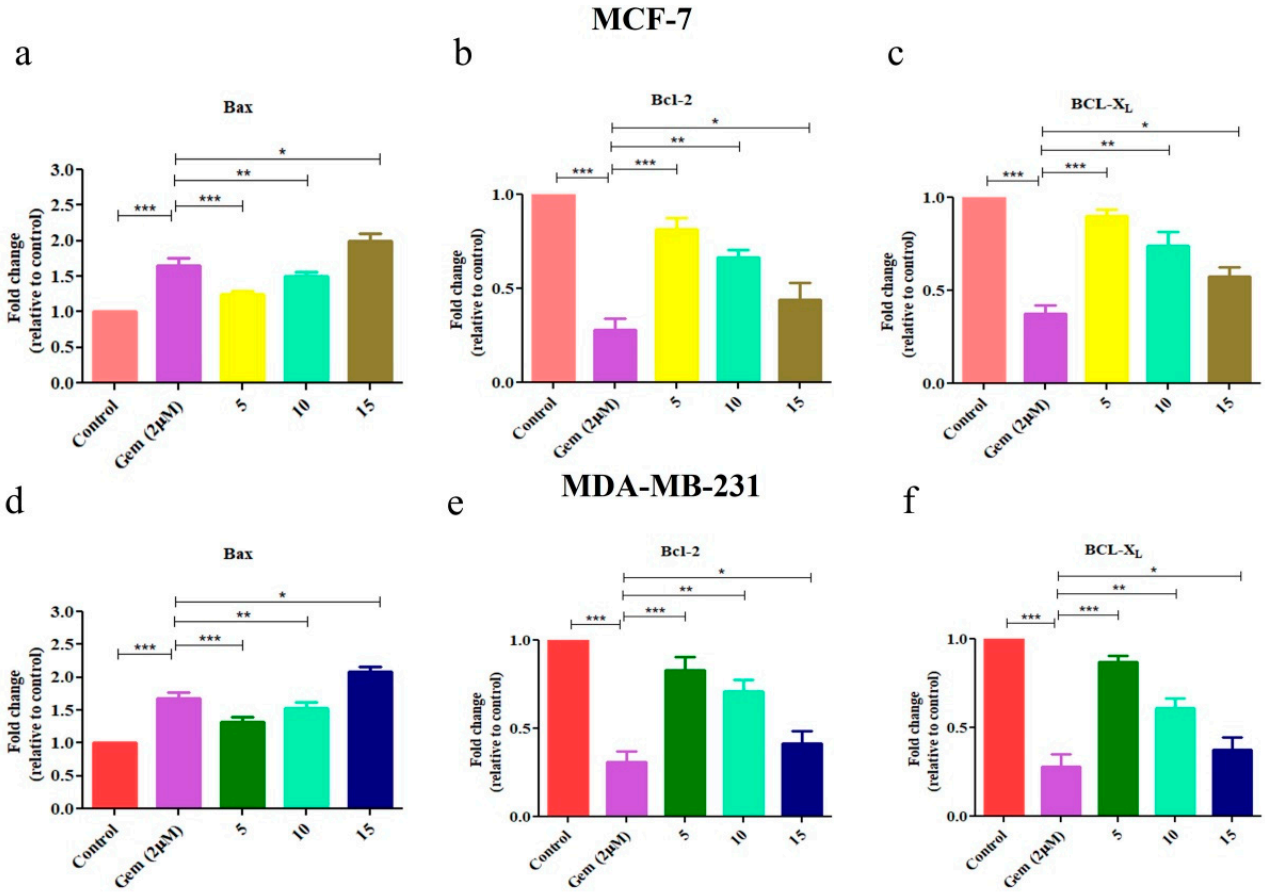

Figure 12. The modulation of JAK/STAT-associated gene expression in gedunin-treated human breast adenocarcinoma (**a**–**c**) MCF-7 and (**d**–**f**) MDA-MB-231 cells. A significant difference determined by the p value < 0.05 was labeled with asterisk (*); p value < 0.01 was labeled with double asterisks (**); and p value < 0.001 was labeled with double asterisks (***).

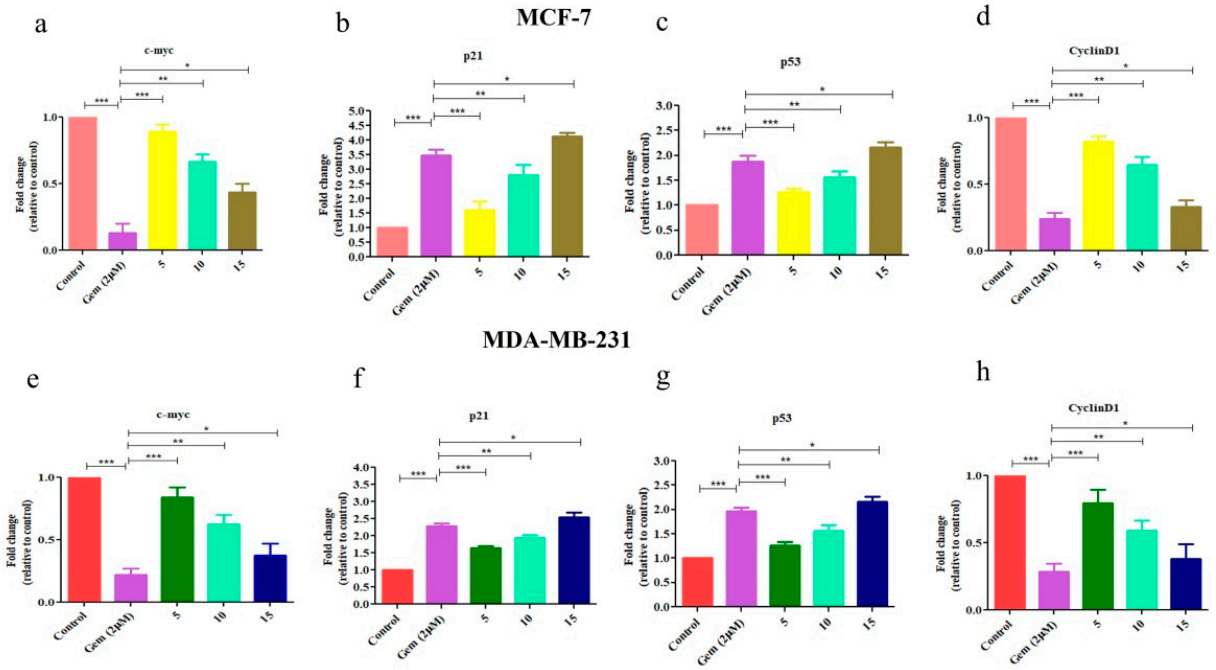

Figure 13. The modulation of JAK/STAT-associated gene expression in gedunin-treated human breast adenocarcinoma (**a**–**d**) MCF-7 and (**e**–**h**) MDA-MB-231 cells. A significant difference determined by the p value < 0.05 was labeled with asterisk (*); p value < 0.01 was labeled with double asterisks (**) and p value < 0.001 was labeled with double asterisks (***).

3. Discussion

Several natural compounds are usually associated with considerable anticancer efficacy because their intrinsic capabilities restrain several carcinomas' proliferation, angiogenesis, growth and metastasis. Furthermore, these natural compounds usually exhibit reduced cytotoxic effects against normal cells, reducing the chances of adverse side effects upon treatment. These attributes have compelled the exhaustive investigation of natural products for their anticancer potential because of their competence in inhibiting growth and metastasis despite instigating considerable side effects [26]. Despite the critical pharmacological relevance of gedunin, its anticancer efficacy against human breast adenocarcinoma MCF-7 cells and triple negative MDA-MB-231 cells remains unexplored. Thus, the authors hypothesized that in light of the pharmacological activities, gedunin might impede the proliferation of MCF-7 and MDA-MB-231 cells by modulating the expression of the JAK/STAT pathway.

In the present investigation, the efficacy of gedunin in modulating JAK1 and STAT3 protein was evaluated through molecular docking studies to elucidate the modulation of JAK/STAT signaling during breast cancer. The observation during the in silico studies indicated that gedunin could be an effective inhibitor of the JAK/STAT pathway due to the considerable binding efficiency of gedunin against JAK1 and STAT3 proteins. The binding efficiency of gedunin was comparable with the standard breast cancer chemotherapeutic gemcitabine. Thus, this observation served to be an initial indicator that gedunin may be involved in altering the expression of the JAK/STAT pathway, which in turn was implicated generally with cancer development and progression of breast cancer. Our preliminary in vitro observation also indicated that gedunin exerted a significant cytotoxic effect against MCF-7 and MDA-MB-231 cells exhibiting a dose and time-dependent effect. Notably, the onset of cellular death in response to several chemotherapeutics may be through autophagy, apoptosis, and necrosis. However, instigating apoptosis or cell death through either of the other stated pathway is plausibly considered a therapeutical intervention against several carcinomas [28]. An essential attribute during the instigation of apoptotic pathways was the condensation of the nucleus and the generation of apoptotic bodies [29]. Moreover,

the qualitative data of DAPI staining was also supported by quantitative data suggesting that these findings were in line with the reported observation. They elucidated that gedunin instigated the condensation of the nucleus upon exposure, with gedunin exhibiting a dose-dependent effect against breast adenocarcinoma MCF-7 and triple negative MDA-MB-231 cells.

Activating caspases during apoptosis is a peculiar characteristic defining the site-specific cleavage of aspartate residues [30]. Thus, the level of **Caspase-3 activity was estimated post-gedunin treatment in MCF-7 and MDA-MB-231 cells. The activation of caspase-3 was evident by its higher activity post-gedunin treatment, indicating apoptosis instigation by gedunin. This was further reaffirmed by the ameliorative effect of caspase-3 inhibitor in alleviating the induction of apoptosis after MCF-7 and MDA-MB-231 cells were exposed to gedunin. Cancer cells are also accredited with enhanced levels of basal ROS, which act as a dual-edged sword by supporting pro-oxidants' actions.

Nevertheless, ROS instigation resulting in the onset of apoptosis is regarded as a productive strategy for the clinical management of different carcinomas. The results presented in the report also explicitly indicated that gedunin exposure was competent in inducing ROS generation within the MCF-7 cells. Importantly, gedunin-induced ROS augmentation was significantly ameliorated in NAC-pretreated MCF-7 and MDA-MB-231 cells. Also, NAC pretreatment considerably ameliorated gedunin-mediated cytotoxicity. These results indicated that ROS instigation was essential in gedunin-mediated cytotoxicity against MCF-7 and MDA-MB-231 cells.

As previously stated, JAK/STAT **signaling is reported to be closely associated with the progression and development of metastatic carcinomas. Due to its substantial involvement in the progression of breast cancer, several antagonistic or inhibitor molecules targeting JAK/STAT pathways have been developed [29]. Indeed, the constitutive expression of JAK/STAT signaling is implicated with the downstream activation of several genes involved in modulating apoptotic pathways, including Bcl-XL, Bax, and Bcl-2 [31]. The gene expression analysis observations established that gedunin reduced the expression of anti-apoptotic genes with a concomitant increase in their apoptotic counterparts. Significantly, consecutive expression of STAT3 is further associated with enhanced proliferation, metastasis, and drug resistance of several carcinomas [32]. Thus, the elucidation of STAT3 inhibitor is a valued strategy for preventing different associated carcinomas. During our investigation, it was also found that gedunin reduced the mRNA expression of STAT3 protein, which could be attributed to reduced proliferation and instigation of apoptosis in breast adenocarcinoma MCF-7 and MDA-MB-231 cells in the present study.

In summary, our present article has demonstrated the anticancer efficacy of gedunin against MCF-7 and MDA-MB-231 breast cancer cells via attenuating the JAK1/STAT3 signaling pathway. It was observed that gedunin substantially instigated apoptotic cell death by modulating the mRNA expression of BcL-2, BclXL, and Bax genes involved in apoptosis. Therefore, it is concluded that gedunin could be an adjunct therapeutic for the treatment and management of breast cancer.

Although gedunin holds the potential to treat various human cancers and its anticancer efficacy is associated with the alteration of various signaling pathways, the low hydrophobicity of gedunin reduces its bioavailability and pharmacokinetic profile [33]. Moreover, the employment of advanced research techniques such as liposomal drug delivery and nanoformulation could ameliorate the efficacy of gedunin as a cancer therapeutic along with the combination with various chemotherapeutic drugs. Therefore, subsequent studies are needed to better understand the underlying mechanism and also substantiate the safety of gedunin for human consumption by conducting pre-clinical and clinical trials. Furthermore, it is difficult to produce gedunin in larger amounts and also cannot be easily chemically synthesized, which poses a limitation on its usage. Thus, it is recommended to encourage the production of gedunin at commercial levels for research analysis [34]. The molecular mechanistic action of gedunin against breast cancer cells has been summarized in Figure 14.

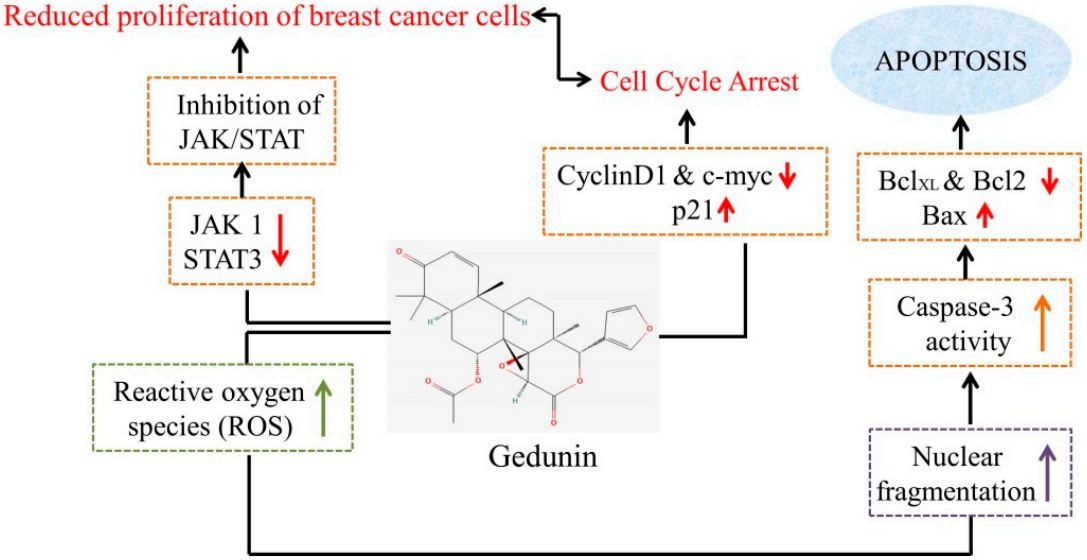

Figure 14. Schematic flowchart representation of gedunin against breast cancer cells.

4. Materials and Methods

4.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (catalogue no: RM1131) was commercially obtained from Himedia, India. 2′, 7′-Dichlorofluorescin diacetate (DCF-DA) (catalogue no: 21884), acridine orange (catalogue no: MB071), ethidium bromide (catalogue no: TC262-5G), and Z-DEVD-FMK; ** caspase-3 inhibitor (catalogue no: 264156-M), gemcitabine hydrochloride (cas no. 122111-03-9) were procured from Sigma. Gedunin (purity > 95%) was obtained from Santa Cruz Biotechnology, USA. Minimum essential medium **Eagle (MEM) with Earle's salt (catalogue no: AT154], fetal bovine serum (FBS) (catalogue no: 26140087), and the antibiotic–antimycotic solution were commercially obtained from Gibco. The caspase-3 colorimetric assay kit used was purchased from BioVision (San Francisco, CA, USA).

4.2. Cell Culture Maintenance

Human-derived estrogen, progesterone, and glucocorticoid receptor positive MCF-7 cells, MDA-MB-231, and human normal cell line HEK-293 were procured from the cell repository of the National Center of Cell Sciences, Pune. They were allowed to proliferate in minimum essential medium (MEM) with Earle's modification and DMEM-high glucose media under humidified atmosphere constituted by 5% CO_2 maintained at 37 °C. The media was supplemented with 10% fetal bovine serum (FBS) and 1% **antibiotic-antimycotic solution. Cells were monitored regularly and were passaged once the flask attained <85% confluence.

4.3. Methods

4.3.1. In Silico Investigations

Retrieval of 3D Protein Structure

The crystal structure of JAK1 and STAT3 with PDB ID: 4I5C and 6TLC, respectively, used during the present study were taken from Brookhaven Protein Data Bank (www.rcsb.org/pdb; accessed on 28 January 2023). The structures of JAK1 and STAT3 (x = 0.26, y = 32.3, z = 33.52) transcription factors employed for docking were devoid of any heteroatoms, including the non-receptor atoms, namely water, ions, etc. The binding pocket coordinates for JAK1 (x = 9.844, y = 31.334, z = 9.614) and STAT3 (x = 0.26, y = 32.3, z = 33.52) were set as default, and the grid box of proteins was put within a cubic box of magnitudes 40 × 40 × 40 Å [35].

Retrieval of 3D Structure Ligands

Required ligands were searched on a database of Pub Chem (http://pubchem.ncbi.nlm.nih.gov). The structural and functional details of the different organic compounds were retrieved from the database of PubChem, having a unique CID or compound identification number. The structural details about the desired ligands were collected using the Simplified Molecular Input Line Entry Specification string, which was further deposited in CORINA (http://www.molecular-networks.com/products/Corina) software. This software utilizes the SMILES string to create a 3D structure of the desired molecules, which can then be retrieved in PDB format for AutoDock Vina 4.0. We have utilized the Linux subsystem command line and used MMFF94 force filed for energy minimization (EM); after EM, we prepared the ligand molecules by using mgltools 1.5.6.

Docking Complex Visualization

The visualization of best-docked position was selected out of nine possible confirmations on the basis of interacting residues including hydrogen bonds with large binding energy (kcal/mol). Thereafter, LigPlot was employed to visualize the protein–ligand interaction of docked complexes in two dimensions [36], and PyMol was utilized to generate all the binding pockets [37,38]. The interactions among protein and ligand are mediated by hydrogen bonds or may arise due to hydrophobic interactions. Hydrogen bonds are represented by dashed lines within the interacting atoms. Contrastingly, hydrophobic interactions in these regions are shown by an arc with spokes radiating towards the atoms within the ligand interacting with the protein whereas the atoms contacted are represented with spokes radiating backwards.

4.3.2. In Vitro Assessments

Cytotoxic Effects of Gedunin against Breast Cancer

To investigate the cytotoxic potential of gedunin against breast cancer MCF-7 cells and HEK-293 cells, an MTT-based colorimetric assay was undertaken, as described previously [39]. Concisely, 5×10^3 MCF-7 and MDA-MB-231 cells were exposed to varying gedunin concentrations (5, 10, and 15 µM) for 24 h and 48 h under standard conditions of tissue culture. Subsequently, the treated cells were exposed to MTT dye (5 mg/mL) for an additional 4 h. Finally, the formazan crystals were read for their absorbance intensity at 570 nm by solubilizing them with tissue grade DMSO (100 µL) through a microplate reader BioRad (Hercules, CA, USA). The results were elucidated in terms of cell viability percentage (%) in contrast with untreated using the formula

Cellular viability % = (Absorbance of treated MCF-7 and MDA-MB-231 cells)/(Absorbance of untreated MCF-7 and MDA-MB-231 control cells) × 100

Assessment of Nuclear Morphology

Changes within the nuclear morphology, such as condensation of the nucleus in breast adenocarcinoma MCF-7 and MDA-MB-231 cells, were assessed using DAPI staining, as earlier reported [40]. Precisely 5×10^4 cells/well was exposed to varying stated concentrations of gedunin for 12 h. Subsequently, after washing the cells with 1× PBS, these were fixed using chilled methanol for around 10 min. The treated and untreated cells were exposed to permeabilizing buffer constituted by TritonX100 (0.25% v/v) and stained with DAPI. Eventually, DAPI-associated blue fluorescence was visualized and recorded using Carl Zeiss GmbH microscope (Model: LSM780NLO, Oberkochen, Baden-Württemberg, Germany).

Evaluation of Gedunin-Induced ROS Production

ROS-mediated oxidative stress was assessed qualitatively and quantitatively through DCF-DA stain, as reported previously [41]. A total of 5×10^4 MCF-7 and MDA-MB-231 cells were initially allowed to adhere in each well of a 96-well plate under optimum culture conditions. Subsequently, the cells were exposed to varying concentrations of gedunin for

12 h and, after that, stained with DCFH-DA (10 μM) for 30 min at dark 37 °C. The cells were then washed cautiously using PBS, and finally, the cells were visualized through Carl Zeiss GmbH microscope (Model: LSM780NLO).

To quantify the generation of intracellular ROS, 1×10^4 cells were allowed to adhere in each 96-well black bottom plate well under optimum culture conditions. Cells were treated and incubated with gedunin for 12 h, as stated above. Eventually, the fluorescence of treated and untreated control MCF-7 cells was quantified in terms of fluorescence intensity percentage in comparison with untreated control at excitation: emission wavelength of 485:528 nm.

Effects of ROS Inhibitor

To ascertain the involvement of gedunin in augmenting ROS within human breast adenocarcinoma MCF-7 cells, a potent ROS inhibitor, N-acetyl cysteine (NAC), was used. Briefly, post-adherence 5×10^4 MCF-7 and MDA-MB-231 cells in each well of a 96-well plate were pretreated with NAC (10 mM) and incubated under standard conditions for 2 h. Subsequently, the cells were re-exposed to the above-stated concentrations of gedunin and incubated for 12 h. This was followed by treated and untreated control treatment with DCFH-DA (10 μM; 37 °C for 30 min) in the dark as described previously [42]. Finally, the fluorescence intensity of different treated and untreated cells was quantified, as stated above in evaluation of Gedunin-induced ROS production.

Assessment of Caspase-3 Activation

Caspase-3 activation in gedunin-treated human breast adenocarcinoma MCF-7 and triple negative MDA-MB-231 cells was assessed colorimetrically using a commercially available kit (BioVision). Around 3×10^6 gedunin-treated and/or untreated cells were lysed using lysis buffer (50 μL). The lysate was centrifuged at 10,000× *g*, and the resulting supernatant was collected and immediately transferred on ice. The lysate belonging to various treated and control group (50 μL) was aliquot in each well of the 96-well plate and mixed with reaction buffer (50 μL; 10 mM DTT). Subsequently, DEVD-pNA substrate was also supplemented in each well at a volume of 50 μL for one h** at 37 °C. Finally, the absorbance of each well was recorded at 405 nm. Compared with the untreated control, the observations were interpolated as caspase-3 activity percentage (%).

Effects of Caspase-3 Inhibitor

To further characterize the cytotoxic effects of gedunin, MCF-7 and MDA-MB-231 cells with pretreated for 2 h with caspase-3 specific inhibitor and subsequently were re-exposed to varying concentrations of gedunin as stated above for 24 h. Finally, the cell viability of cells belonging to different groups was estimated through MTT assay, as stated above in Cytotoxic Effects of Gedunin against Breast Cancer.

Quantitative Real-Time PCR (qRT-PCR)

A total of 1×10^6 MCF-7 and MDA-MB-231 cells, after adherence, were subjected to varying stated concentrations of gedunin for 24 h. Subsequently, the mRNA content of both treated and untreated control cells was isolated and **two μg of this was used for cDNA synthesis using commercially available cDNA synthesis kits. The primers involved in the present investigation were synthesized using the NCBI pick primer designing tool, which is mentioned in Table 2. GAPDH was used as a housekeeping gene for normalization. The results were interpolated by the $^{2\Delta\Delta}$CT method [42].

Table 2. List of the primer sequences used in the study.

Genes	Forward Primer	Reverse Primer	NCBI Gene Number
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA	2597
Bcl2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC	596
Bcl_{XL}	GCCACTTACCTGAATGACCACC	AACCAGCGGTTGAAGCGTTCCT	598
Bax	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC	581
Cyclin D1	TGAACTACCTGGACCGCT	GCCTCTGGCATTTTGGAG	595
c-myc	AGCGACTCTGAGGAGGAACAAG	GTGGCACCTCTTGAGGACCA	26,292
$p21^{Cip1}$	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG	5058
p53	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC	7157
JAK1	GAGACAGGTCTCCCACAAACAC	GTGGTAAGGACATCGCTTTTCCG	3716
STAT3	CTTTGAGACCGAGGTGTATCACC	GGTCAGCATGTTGTACCACAGG	6774

4.4. Statistical Inferences

Data of the present investigation are the average + SEM of discrete experiments performed thrice at least in triplicate. Differences in comparison with control and different groups were considered statistically significant in the case of $p < 0.05$ and were analyzed using one-way ANOVA followed by Dunnett's post hoc and Student *t*-test using GraphPad Prism Ver. 5 software as per their applicability. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

5. Conclusions

Conclusively, these findings demonstrated the anticancer efficacy of gedunin against MCF-7 and MDA-MB-231 breast cancer cells by inhibiting the JAK1/STAT3 signaling pathway. Gedunin competently instigated apoptotic cell death by altering the expression of BcL-2, Bcl_{XL}, and Bax genes involved in apoptosis. Significantly, the other target genes of the JAK1/STAT3 pathway, such as c-Myc, cyclin D1, and $p21^{Cip1}$, were modulated upon treatment with gedunin. Therefore, it may be affirmed that gedunin could be a plausible therapeutic against breast cancer, owing to its capability of regulating the JAK1/STAT3 pathway, which warrants a further detailed mechanistic study of gedunin in preclinical disease models.

Author Contributions: Conceptualization, T.H. and S.M.D.R.; Formal analysis, M.A., J.A. and A.M.A.; Funding acquisition, T.H. and A.O.E.; Investigation, A.M.A. and S.M.D.R.; Methodology, M.A., J.A. and T.N.A.; Project administration, T.H. and A.O.E.; Resources, S.S.; Software, M.M.A. and S.S.; Supervision, A.O.E. and S.M.D.R.; Validation, M.A., J.A., A.U. and T.N.A.; Visualization, A.M.A., M.M.A. and S.S.; Writing—original draft, A.U., T.N.A. and M.M.A.; Writing—review & editing, A.U. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Research Deanship, University of Hai'l, Hai'l, Kingdom of Saudi Arabia, under Project grant no. RG-20159.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Global Cancer Observatory Factsheet on Cancers. Last Updated: December 2020. Available online: https://gco.iarc.fr/today/data/factsheets/cancers/20-Breast-fact-sheet.pdf (accessed on 8 August 2022).
2. Waks, A.G.; Winer, E.P. Breast Cancer Treatment: A Review. *JAMA* **2019**, *321*, 288–300. [CrossRef] [PubMed]
3. Pistilli, B.; Lohrisch, C.; Sheade, J.; Fleming, G.F. Personalizing Adjuvant Endocrine Therapy for Early-Stage Hormone Receptor-Positive Breast Cancer. *Am. Soc. Clin. Oncol. Educ. Book* **2022**, *42*, 60–72. [CrossRef] [PubMed]
4. Shyam, M.M.; Moin, A.; Medishetti, R.; Rajendra, K.M.; Raichur, A.; Prashantha, K.B.R. Dual drug conjugate loaded nanoparticles for the treatment of cancer. *Curr. Drug Deliv.* **2015**, *12*, 782–794. [CrossRef]

5. Baudino, T.A. Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Curr. Drug Discov. Technol.* **2015**, *12*, 3–20. [CrossRef]
6. Ibraheem, A.; Stankowski-Drengler, T.J.; Gbolahan, O.B.; Engel, J.M.; Onitilo, A.A. Chemotherapy-induced cardiotoxicity in breast cancer patients. *Breast Cancer Manag.* **2016**, *5*, 31–41. [CrossRef]
7. Kooti, W.; Servatyari, K.; Behzadifar, M.; Asadi-Samani, M.; Sadeghi, F.; Nouri, B.; Zare Marzouni, H. Effective Medicinal Plant in Cancer Treatment, Part 2: Review Study. *J. Evid. Based Complement. Altern. Med.* **2017**, *22*, 982–995. [CrossRef]
8. Jeon, H.J.; Kim, K.; Kim, C.; Kim, M.J.; Kim, T.O.; Lee, S.E. Molecular mechanisms of anti-melanogenic gedunin derived from neem tree (*Azadirachta indica*) using B16F10 mouse melanoma cells and early-stage zebrafish. *Plants* **2021**, *10*, 330. [CrossRef]
9. Braga, T.M.; Rocha, L.; Chung, T.Y.; Oliveira, R.F.; Pinho, C.; Oliveira, A.I.; Morgado, J.; Cruz, A. Biological Activities of Gedunin-A Limonoid from the Meliaceae Family. *Molecules* **2020**, *25*, 493. [CrossRef]
10. Kapinova, A.; Kubatka, P.; Golubnitschaja, O.; Kello, M.; Zubor, P.; Solar, P.; Pec, M. Dietary phytochemicals in breast cancer research: Anticancer effects and potential utility for effective chemoprevention. *Environ. Health Prev. Med.* **2018**, *23*, 36. [CrossRef]
11. Moin, A.; Wani, S.U.D.; Osmani, R.A.; Abu Lila, A.S.; Khafagy, E.S.; Arab, H.H.; Gangadharappa, H.V.; Allam, A.N. Formulation, characterization, and cellular toxicity assessment of tamoxifen-loaded silk fibroin nanoparticles in breast cancer. *Drug Deliv.* **2021**, *28*, 1626–1636. [CrossRef]
12. Wylie, M.R.; Merrell, D.S. The Antimicrobial Potential of the Neem Tree *Azadirachta indica*. *Front. Pharmacol.* **2022**, *13*, 1535. [CrossRef] [PubMed]
13. Aarthy, T.; Mulani, F.A.; Pandreka, A.; Kumar, A.; Nandikol, S.S.; Haldar, S.; Thulasiram, H.V. Tracing the biosynthetic origin of limonoids and their functional groups through stable isotope labeling and inhibition in neem tree (*Azadirachta indica*) cell suspension. *BMC Plant Biol.* **2018**, *18*, 230. [CrossRef] [PubMed]
14. Gupta, A.; Ansari, S.; Gupta, S.; Narwani, M.; Gupta, M.; Singh, M. Therapeutics role of neem and its bioactive constituents in disease prevention and treatment. *J. Pharmacogn. Phytochem.* **2019**, *8*, 680–691.
15. Tan, Q.G.; Luo, X.D. Meliaceous limonoids: Chemistry and biological activities. *Chem. Rev.* **2011**, *111*, 7437–7522. [CrossRef]
16. Patwardhan, C.A.; Fauq, A.; Peterson, L.B.; Miller, C.; Blagg, B.S.; Chadli, A. Gedunin inactivates the co-chaperone p23 protein causing cancer cell death by apoptosis. *J. Biol. Chem.* **2013**, *288*, 7313–7325. [CrossRef]
17. Wehde, B.L.; Rädler, P.D.; Shrestha, H.; Johnson, S.J.; Triplett, A.A.; Wagner, K.U. Janus Kinase 1 Plays a Critical Role in Mammary Cancer Progression. *Cell Rep.* **2018**, *25*, 2192–2207.e5. [CrossRef]
18. Ma, J.H.; Qin, L.; Li, X. Role of STAT3 signaling pathway in breast cancer. *Cell Commun. Signal.* **2020**, *18*, 33. [CrossRef]
19. Aggarwal, V.; Tuli, H.S.; Varol, A.; Thakral, F.; Yerer, M.B.; Sak, K.; Varol, M.; Jain, A.; Khan, M.A.; Sethi, G. Role of Reactive Oxygen Species in Cancer Progression: Molecular Mechanisms and Recent Advancements. *Biomolecules* **2019**, *9*, 735. [CrossRef]
20. Bao, L.; Zhang, H.; Chan, L.S. The involvement of the JAK-STAT signaling pathway in chronic inflammatory skin disease atopic dermatitis. *JAK-STAT* **2013**, *2*, e24137. [CrossRef]
21. Yu, H.; Lee, H.; Herrmann, A.; Buettner, R.; Jove, R. Revisiting STAT3 signalling in cancer: New and unexpected biological functions. Nature reviews. *Cancer* **2014**, *14*, 736–746. [CrossRef]
22. Ioannidou, E.; Moschetta, M.; Shah, S.; Parker, J.S.; Ozturk, M.A.; Pappas-Gogos, G.; Sheriff, M.; Rassy, E.; Boussios, S. Angiogenesis and Anti-Angiogenic Treatment in Prostate Cancer: Mechanisms of Action and Molecular Targets. *Int. J. Mol. Sci.* **2021**, *22*, 9926. [CrossRef] [PubMed]
23. Thomas, S.J.; Snowden, J.A.; Zeidler, M.P.; Danson, S.J. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. *Br. J. Cancer* **2015**, *113*, 365–371. [CrossRef]
24. Verma, N.K.; Davies, A.M.; Long, A.; Kelleher, D.; Volkov, Y. STAT3 knockdown by siRNA induces apoptosis in human cutaneous T-cell lymphoma line Hut78 via downregulation of Bcl-xL. *Cell Mol. Biol. Lett.* **2010**, *15*, 342–355. [CrossRef] [PubMed]
25. Brooks, A.J.; Putoczki, T. JAK-STAT Signalling Pathway in Cancer. *Cancers* **2020**, *12*, 1971. [CrossRef] [PubMed]
26. Hussain, T.; Bajpai, S.; Saeed, M.; Moin, A.; Alafnan, A.; Khan, M.; Kamal, M.A.; Ganash, M.; Ashraf, G.M. Potentiating effect of ethnomedicinal plants against proliferation on different cancer cell lines. *Curr. Drug Metab.* **2018**, *19*, 584–795. [CrossRef]
27. Teiten, M.H.; Gaascht, F.; Dicato, M.; Diederich, M. Anticancer bioactivity of compounds from medicinal plants used in European medieval traditions. *Biochem. Pharm.* **2013**, *86*, 1239–1247. [CrossRef]
28. Razak, N.A.; Abu, N.; Ho, W.Y.; Zamberi, N.R.; Tan, S.W.; Alitheen, N.B.; Long, K.; Yeap, S.K. Cytotoxicity of eupatorin in MCF-7 and MDA-MB-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and induction of apoptosis. *Sci. Rep.* **2019**, *9*, 1514. [CrossRef]
29. Brentnall, M.; Rodriguez-Menocal, L.; De Guevara, R.L.; Cepero, E.; Boise, L.H. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol.* **2013**, *14*, 32. [CrossRef]
30. Rah, B.; Rather, R.A.; Bhat, G.R.; Baba, A.B.; Mushtaq, I.; Farooq, M.; Yousuf, T.; Dar, S.B.; Parveen, S.; Hassan, R.; et al. JAK/STAT Signaling: Molecular Targets, Therapeutic Opportunities, and Limitations of Targeted Inhibitions in Solid Malignancies. *Front. Pharmacol.* **2022**, *13*, 821344. [CrossRef]
31. Lall, R.K.; Syed, D.N.; Adhami, V.M.; Khan, M.I.; Mukhtar, H. Dietary polyphenols in prevention and treatment of prostate cancer. *Int. J. Mol. Sci.* **2015**, *16*, 3350–3376. [CrossRef]
32. Bolomsky, A.; Vogler, M.; Köse, M.C.; Heckman, C.A.; Ehx, G.; Ludwig, H.; Caers, J. MCL-1 inhibitors, fast-lane development of a new class of anti-cancer agents. *J. Hematol. Oncol.* **2020**, *13*, 173. [CrossRef] [PubMed]

33. Rana, M.S.; Ediriweera, M.K.; Rajagopalan, U.; Karunaratne, D.N.; Tennekoon, K.H.; Samarakoon, S.R. A new liposomal nanocarrier for co-delivery of gedunin and p-glycoprotein siRNA to target breast cancer stem cells. *Nat. Prod. Res.* **2022**, *36*, 6389–6392. [CrossRef] [PubMed]
34. Khan, M.K.; Ansari, I.A.; Khan, M.S.; Arif, J.M. Dietary phytochemicals as potent chemotherapeutic agents against breast cancer: Inhibition of NF-κB pathway via molecular interactions in rel homology domain of its precursor protein p105. *Pharmacogn. Mag.* **2013**, *9*, 51–57. [CrossRef] [PubMed]
35. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. [CrossRef]
36. Zev, S.; Raz, K.; Schwartz, R.; Tarabeh, R.; Gupta, P.K.; Major, D.T. Benchmarking the Ability of Common Docking Programs to Correctly Reproduce and Score Binding Modes in SARS-CoV-2 Protease Mpro. *J. Chem. Inf. Model.* **2021**, *61*, 2957–2966. [CrossRef]
37. Lill, M.A.; Danielson, M.L. Computer-aided drug design platform using PyMOL. *J. Comput. Aided Mol. Des.* **2011**, *25*, 13–19. [CrossRef]
38. Alafnan, A.; Alamri, A.; Alanazi, J.; Hussain, T. Farnesiferol C Exerts Antiproliferative Effects on Hepatocellular Carcinoma HepG2 Cells by Instigating ROS-Dependent Apoptotic Pathway. *Pharmaceuticals* **2022**, *15*, 1070. [CrossRef]
39. Husain, I.; Bala, K.; Wani, A.; Makhdoomi, U.; Malik, F.; Sharma, A. Arginase purified from endophytic Pseudomonas aeruginosa IH2: Induce apoptosis through both cell cycle arrest and MMP loss in human leukemic HL-60 cells. *Chem.-Biol. Interact.* **2017**, *274*, 35–49. [CrossRef]
40. Husain, I.; Sharma, A.; Kumar, S.; Malik, F. Purification and Characterization of Glutaminase Free Asparaginase from Enterobacter cloacae: In-Vitro Evaluation of Cytotoxic Potential against Human Myeloid Leukemia HL-60 Cells. *PLoS ONE* **2016**, *11*, e0148877. [CrossRef]
41. Ahmad, A.; Tiwari, R.K.; Almeleebia, T.M.; Al Fayi, M.S.; Alshahrani, M.Y.; Ahmad, I.; Abohassan, M.S.; Saeed, M.; Ansari, I.A. Swertia chirayita suppresses the growth of non-small cell lung cancer A549 cells and concomitantly induces apoptosis via downregulation of JAK1/STAT3 pathway. *Saudi J. Biol. Sci.* **2021**, *28*, 6279–6288. [CrossRef]
42. Ahmad, A.; Ansari, I.A. Carvacrol Exhibits Chemopreventive Potential against Cervical Cancer Cells via Caspase-Dependent Apoptosis and Abrogation of Cell Cycle Progression. *Anti-Cancer Agents Med. Chem.* **2021**, *21*, 2224–2235. [CrossRef] [PubMed]