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Cytotoxic and Apoptotic Effects of a Novel Indole Derivative on MCF-7 Cells

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ABSTRACT

The Indole ring is a Fundamental unit that contributes significantly to the biological activity of many natural and synthetic compounds

This study is aimed to evaluate the cytotoxic and genotoxic effects of 2-(5 chloro-3 ,3-dimethylindolin-2-Ylidene)-3-((4-hydroxyphenyl) imino) propanal on (MCF7) breast cancer cell line and (REF) normal cell line.
Methods: The new compound was synthesized and Characterized using Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance Spectroscopy (^{1}HNMR and ^{13}CNMR). Cytotoxic activity was evaluated at different concentrations and exposure times, while genotoxicity was assessed using the comet assay.

The results show low cytotoxic activity on normal cells with an inhibition rate of 13% for 24 hrs. and 15% at 48 hrs. with low concentration; Moreover, the antitumor activity toward MCF7 displayed a higher inhibition rate with 62% for the concentration(60μg/ml) at 48hr. and 59% at48*h*r. for (40 µg/ml). Also, the results show that the new compound induces genotoxicity on MCF7 with severe comet tail, and it did not display signs of DNA fragmentation on normal cell lines (without significant DNA damage) after 24 hrs. of exposure time. After 48 hrs. the DNA damage is shown by the mild comet tail in REF and the moderate comet tail in MCF7 cells.

The new indole derivative demonstrates promising selective cytotoxic and genotoxic effects against breast cancer cells, with minimal effect on normal cells. These findings suggest its potential as a candidate for anticancer drug development.

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1. INTRODUCTION

Cancer extends all over the world has become the main cause of significant deaths. Therefore, the most difficult task for researchers is to develop anticancer treatments with minimal side effects [1]. Heterocyclic chemistry is an essential class of medicinal chemistry, as several drugs being used in chemotherapy have a heterocyclic ring in their basic structure [2].

Heterocyclic compounds are cyclic rings that contain carbon atoms and at least one heteroatom in their structure. The heteroatom gives heterocyclic compound distinctive chemical properties compared to all carbon ring analogs [3].

The most common heterocyclic compounds have five or six rings with nitrogen, oxygen, and *S*ulphur. Also, Heterocyclic ring may contain other Atoms phosphorus, iron and magnesium. According to data, heterocyclic compounds exist in more than 85% of all physiologically active chemical compounds, which confirms their importance in modern drug design [4].

Heterocyclic compounds with nitrogen atoms such Indoles, quinoline, pyrroles, and pyrrolidines are the most important class of chemicals. They have gained importance in many research fields, including organic synthesis and medicine [2].

Indole or benzopyrrole is an organic chemical compound, with formula C_8H_7N, including a benzene ring fused to the pyrrole ring. It is associated with several biological effects such as antimicrobial, antiviral, anticancer, and antimalarial properties [5], and is also very efficient as an antioxidants, protecting lipids and proteins from peroxidation [6].

Indole derivatives have been approved as drugs, such as pindolol, indomethacin, indapamide [7], and indole-3-carbinol as antitumor agents. Due to these pharmacological properties chemists and biologists continue to focus on the synthesis of biologically active indole analogues [8].

Schiff bases are compounds with imine groups (-CH=N-), characterized by a variety of biological effects such as antifungal, anticancer, antibacterial, and antiviral agents [9]. Schiff bases were synthesized in 1864 by Hugo Schiff in the nineteenth century by using aldehyde or ketone and primary amine [10]. The most common method for the synthesis involves condensation reaction of primary amines and carbonyl groups under acidic conditions [11].

In 2022, Kunj et al.[12]. prepared novel indole Schiff base derivatives and evaluated their anticancer activity against A549 cell line results, which exhibited high anticancer activity This study aims to synthesize a new Indole Schiff Base by reacting indole moiety with 4-hydroxy-phenylamino to form 2-(5-chloro–3,3–dimethylindolin–2–ylidene)-3-((4-hydroxyphenyl)imino) propanal compound under isotropic distillation then evaluating the cytotoxic and genotoxic activity on MCF7 breast cancer *c*ells

2. METHOD

2.1. Chemical part:

The study was conducted at The Iraqi Centre For cancer and Medical Genetics Research / Mustansiriyah University / Molecular Biology also at the college of Science /Diyala university/Chemistry Department. Thin-layer Chromatography was performed and the melting point was measured using a melting point device. The spot was detected using Silica gel sheets and the UVT-2000 fluorescence analysis cabinet. A Perkin-Elmer spectrometer recorded IR spectra, while an Agilent Technologies 400 MHz spectrometer recorded 1H and 13C Nuclear Magnetic Resonance (NMR) Spectroscopy. spectra in DMSO at 499.19 MHz and 125.54 MHz, respectively.

Chemicals and solvent:

Chemicals and solvents were optained from different companies and were used without purification such as 4-hydroxy-phenylamino. Hopkin and Williams supplied it, BDH provided glacial acetic acid, and Scharlau supplied all organic solvents. Baradarani and his group (2006) method was modified to synthesize malonaldehyde [13].

Synthetic methods:

Synthesis of 2-(5-Chloro-3,3-Dimethylindolin-2-Ylidene)-3-((4 hydroxyphenyl)imino) propanal by the reaction of (0.5g, 2 mmol) Malonaldehyde with 4-hydroxy-phenylimino (0.261 g, 2 mmol) in 15ml methanol with 1ml sulfuric acid., the mixture was refluxed at 78°C for 5 hrs. shown in Figure 1. Golden crystals was precipitous, purified, and oven at 78°C. One spot in TLC with pre-coated silica gel measures this unique compound's purity.

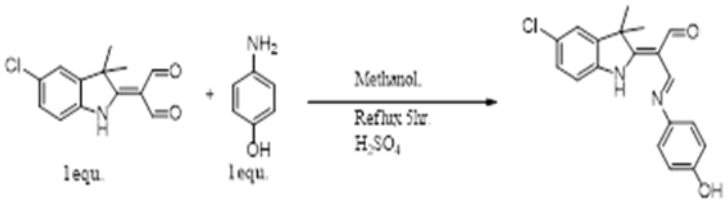

Figure 1. The synthetic pathway of 2-(5–Chloro- 3, 3–Dimethylindolin–2-Ylidene)-3-((4-hydroxyphenyl)imino)propanal.

2.2. Biological part

Cytotoxic Activity

Michigan cancer Foundation (MCF-7) as a breast Cancer cells and fibroblastic and epithelial cells (REF) as murine cell line [14] were used in this study. The Department of Experimental Therapy donated both to the Iraqi Centre For cancer and me*d*ical genetics research / mustansiriyah university which maintains them.

Solubility

The new compound was dissolved in 0.5% dimethyl sulfoxide (DMSO) then dilute in free serum nutrition medium (RPMI_1640) to (10, 20, 40 and 60)μg/ml [15].

Cell lines culture

Cell lines REF and MCF7 were maintained in RPMI-1640 media, which contains glutamine (2 mmol/L), 10% (FBS) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml) [16]. All cells were kept at 37 °C and 5% CO_2 atmosphere, the media was changed daily. Cells were subcultured by drawing off the medium and treating the cells with 0.5 ml of trypsin/versine for (3-5) min. Then, 10 ml of a prepared media was added, incubated with 5% CO2 at 37ºC [17].

Cell viability

The Trypan Blue exclusion test was used to count live cells. A mixture of 100 µl of cells and 100 µl of trypan blue was prepared followed by adding 0.5ml of PBS. The mixture was gently mixed and incubated at room temperature for three minutes [18]. Counting was performed after (3-5) minutes to prevent overestimation of dead cells [19]. From this suspension, 20µl was dropped on the edge of the coverslip that was fixed on a clean hemocytometer slide chamber, Count Cells in (40-X) Light Microscope. Calculate the number of cells per cells/ml: $\mathcal{C}=\mathcal{N}*\mathcal{D}*104$

$\mathcal{C}$: means Cell suspension /ml, : Number of Viable Cells, and $\mathcal{D}$: The Dilution Factor.(=2) [20].

Cell Line seeding

About 200 µl (10^4 cells/ well) were seeded in a 96 well micro titer plate, incubated At 24hr. with 5% CO2 at 37ºC. Treated with Indole Schiff base compound [21].

Exposure

Disposed of the media from the microtiter plate, then added a triplicate of 200 µl from the dilutions of new indole Schiff base derivative (10, 20, 40 and 60)μg/ml with leave wells contains serum-free media as a control. Then, the plates were incubated with 5% CO_2 at 37ºC for two different exposure times 24 and 48hr. [22].

Viability Assay for culture cells by crystal violate

Crystal Violet dye was used to highlight DNA in the nucleus. The medium was removed, and cells were washed with phosphate buffer saline (PBS). Then, 100µl of 0.05% crystal violet solution was added and incubated for 20 minutes. Plates were washed with Distilled water and left to air-dry [23].

A bsorbance was measured at 495nm, and inhibition was calculated using the Equation:

Growth inhibition%= (Control Absorbance–Sample absorbance)/Control Absorbance ×100 [24].

Statistical Analysis

A T-test was used to compare concentrations in each cell line and two cells during each exposure duration. Graph Pad Prism V6 calculated this statistical test, and Excel 2010 drew the tables.

Apoptosis activity

The procedure was done according to Alok Dhawan et al. (2007) with modifications by using an alkaline comet assay to measure DNA strand damage [25].

Preparation of Reagents

Lysing Solution: per 1000 mL:

146.1 gm. of NaCl, 37.2 gm. EDTA, and 1.2 gm of Trizma base were added to about 600 mL of distilled water (dH_2O) and stir the mixture. Then about 6 gm. of NaOH was added to dissolve the mixture (approximately30 min) pH must be 10. The volume was completed to 890mL with dH_2O. Store The mixture at room temperature. Finally, fresh 1% Octylphenol ethoxylate and 10% DMSO were added, and then it was refrigerated for 30 minutes before slide addition [26].

Electrophoresis Buffer:

50gm. NaOH in 125ml dH_2O (solution A), 3.72g EDTA in 50ml dH_2O, pH 10 (solution B): For 1X Buffer: per one litre, 30mL solution (A) was added to 5mL of solution (B), dH_2O was added until the total volume reached 1000ml mixed the solution well.; PH>13 [27].

Neutralization Buffer :

48.5 gm Trizma Base in 700mL H2O (pH 7.5) with Concentrated HCl. The volume was adjusted to 1000ml with H2O and then kept at room temperature [28].

Staining Solution:

Ethidium Bromide (Et Br; 10X Stock - 20 µg/mL): 5 mg in 25mL dH2O was added at room temperature. For 1X stock - mix 1 mL with 9 mL dH2O [29].

Slides Preparation for the Comet Assay

125mg low melting point Agars (LMPA) 0.5% was put in 25 ml phosphate-buffered saline (PBS); heat the mixture, then put it in water bath at 37 °C to prevent it from solidifying.

250-mg Normal Melting Agarose (NMA) 1% was put in 25ml of double-distilled water (ddH2O), and it was microwaved to dissolve the agarose. It was stored at 37°C to avoid solidification.

Put the slide in absolute ethanol, burn it by a lame. Then, the slides are dipped into the 50ml tube containing hot NMA agarose one to three times. They are gently removed and placed on a flat surface to dry and then stored at room temperature until needed [30].

Cell isolation

Cell culture flasks were treated with 0.5ml of Trypsin for 3 min At 37^{0}C to detach cells, 10 ml of culture medium was added to quench Trypsin [31]. Then, 2 ml of cell suspension were seeded on six Petri dishes (3.5cm) for two cell lines and incubated for 24 hrs. at 37ºC to create a single monolayer. Petri dishes were exposed with 60µg/ml of 2- (5–Chloro- 3, 3-Dimethylindolin–2-ylidene)-3-((4-hydroxyphenyl)imino)propanal compound, the control was treated with free serum media and then incubated for 24, 48, and 72 hours. After each different time, cells were trypsinised to release from the Petri dishes. Free serum media neutralized the trypsin, and then the cells were transferred to the micro-centrifuge tube at 12000 rpm(10min). Remove Supernatant. 1ml PBS was added to the cell pellet ,the Pellet was stored on ice in the dark. [32].

Comet Assay

10µl of cell suspension was Mixed with 150 µl of LMPA at 37 ^{0}C, 100µl of mixture was pipetted into a slide coated with (NMA)to hold the cells. Slide was covered with coverslip, left it at 4°C for 5minutes. Coverslip was removed smoothly, and then the slides were ready to dip in lysis solution for 1:30 hour in the refrigerator [33]. After 20 min in fresh electrophoresis buffer pH>13, the slides were electrophoresed at 24 volts and 300 mill amperes for 30 minutes. Slides were removed from the buffer and placed on a drain tray. After 5 minutes of washing with a Neutralization Buffer, the slides were stained with 80µL 1X Ethdium bromide 5minutes. They were immersed in cooled water. A coverslip was carefully mounted and counted slides instantly [34].

3. RESULTS AND DISCUSSION:

3.1. Results

3.1.1. Chemical part

The newly synthesized compound was subjected to thin-layer chromatography (TLC) and spectral studies ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy, and FTIR. The physical properties are described in Table1.

Table1. Physical Properties

Molecular Formula	Molecular Weight	Percentage yield	Melting point^{0}C
$C_{19}H_{17}CLN_2O_2$	340.80	85.2	240-242

IR Study

The IR results (400-4000 cm^{-1} range) of New Compound showed a strong absorption band of the new functional group (CH=N) at 1590 cm1 [35]. The sharp absorption band at 1651cm-1 was (C=O) presented in the new Schiff base compound [36]. The (C=C) group was identified by the frequency at 1516 cm-1 [37]. Absorption bands at 1222cm-1 were ascribed to the (C_N) [38]. Finally, the absorption at 1421cm^{-1} was employed by the (CH3) group, and 1098cm-1 belonged to the (C-O) group [39]. These top Absorption Bands support a Chemical structure of the novel Schiff base molecule Figure 2.

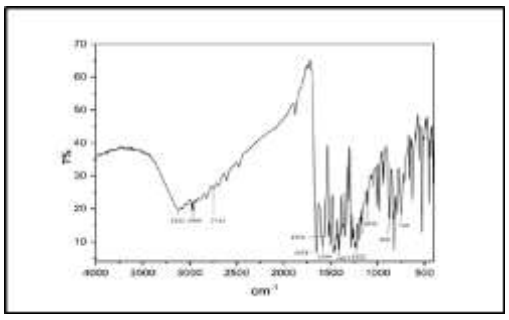

Figure 2. FTIR spectrum of 2-(5–Chloro- 3, 3 – Dimethylindolin–2-Ylidene)-3-((4-hydroxyphenyl)imino)propanal.

NMR results

^{1}H and ^{13}C NMR spectra were verified by using an Agilent Technologies (inova) spectrometer at 499.19MHz for 1H NMR and at 125.54 MHz for 13CNMR in ppm using TMS As a standard, (DMSO) as a solvent.

The HNMR spectrum Figure 3 revealed the two trans protons of (HCNH) appearing as doublets at δ=13.96ppm NH and δ= 8.52ppm CH [40]. Two signals were detected in the region at δ= 9.60 and 9.56 ppm, which attributed to free OH proton and to bonded OH [41]. 9.38 to (s, H, HCO), δ=(7.57–6.87) ppm (7H,ArH)

corresponding to seven protons (4H from p-hydroxyphenyl+3H from 5chloro-indoline ring[42], which confirm the 1:1 condensation of aromatic aldehyde with amine and 1.59 (6H, s, CH3); six protons atoms of two methyl groups [43].

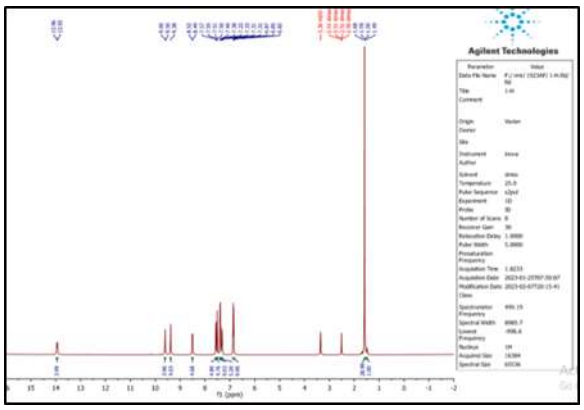

Figure 3. 1HNMR of 2-(5-Chloro-3, 3-Dimethylindolin-2-Ylidene)-3-((4-hydroxyphenyl)imino)propanal.

^{13}C NMR was reliable with ^{1}HNMR Figure 4, a signal at188.19ppm apportioned to the (C=O) group [44]. 184.08ppm to (Ar-N-C) group, an signal of (Ar-NH-C=C) group detected at 155.97ppm [45]. Two Signals at (107.62 and54.66) ppm assigned to(O=C-C and CH3-C-CH_3)groups respectively[46], 22.16ppm fits to ($2CH_3$). The other peaks in the range between 150.22-116.63 ppm were assigned to the carbon atoms of aromatic rings [47].

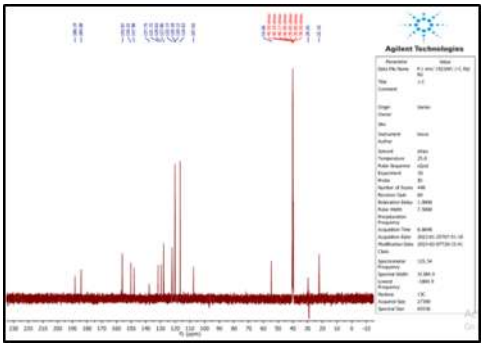

Figure 4. C13NMR of 2-(5-Chloro-3, 3-Dimethylindolin-2-Ylidene)-3-((4-hydroxyphenyl)imino)propanal..

3.1.2. Biological part

Cytotoxic activity

In vitro cytotoxicity of New Indole derivative against MCF7 breast cancer cells was time-dependent at 10, 20, 40, and 60µg/ml. The cytotoxic activity of this new compound as shown in Figure 5 displayed that (60µg/ml) demonstrates stronger cell-toxic activity against the MCF7 cell line with 62% at 48hrs. and 53% at 24hrs. Also, (40µg/ml) displayed inhibition rates of 59% and 47% after (48 and 24) hrs., respectively, when this new compound was tested against REF normal cell line, the inhibition rate of the concentrations 10 and 20 µg/ml gave less than 21% for 24 and 48 hrs. That made them ideal Concentrations for this compound for its safety toward REF cell growth. However, the concentration of 40 µg/ml showed 21% and 31% viability after exposure of 24 and 48 hrs., respectively. However, the inhibition rate for the higher concentration (60µg/ml) is 26% and 36% for 24 and 48 hrs., respectively. Therefore, the ideal concentration for these cell lines could be 60µg/ml because it inhibits the growth of the MCF7 cancer cell line, and it will be safe for REF normal cell viability.

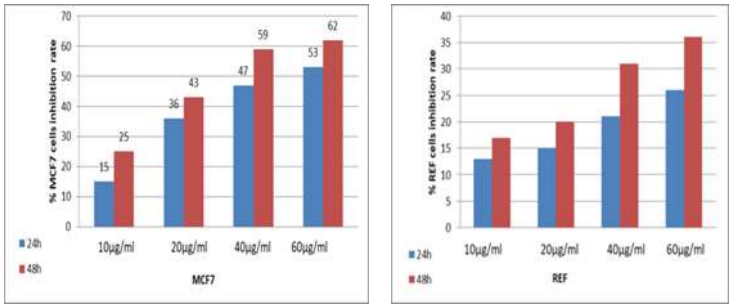

Figure 5. Cytotoxic activity of 2-(5-Chloro-3, 3-Dimethylindolin-2-Ylidene)-3-((4-hydroxyphenyl)imino)propanal against MCF7 cancer cell line and REF normal cell line.

Apoptosis activity

New Schiff Base was shown to be safe and did not show any DNA damage on (REF) normal cell line with 60 µg/ml /ml concentration for 24 and 48 hrs. Figure 6 compares the control results (treated with free serum media) without significant DNA damage. But when tested, the same concentration on the (MCF7) breast cancer cell line showed a significant DNA damage indicated by the long comet tail related to the control untreated cell Figure 7.

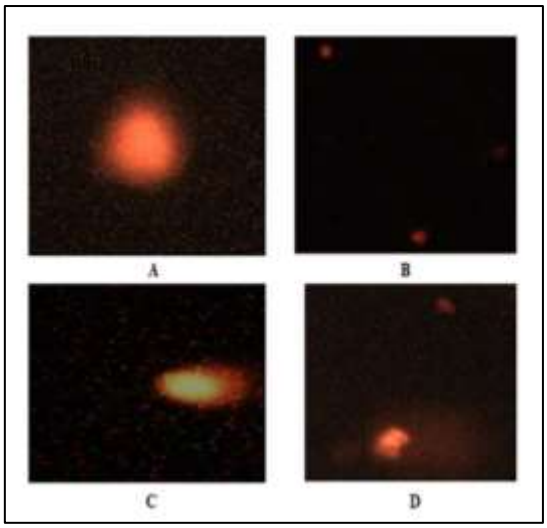

Figure 6. comet images for REF cell line. (A) control. (B) treatment with 60 µg/ml /ml concentration for 24 hrs. without significant DNA damage. (C) treatment with 60 µg/ml /ml concentration for 48 hrs. indicated by the mild comet tail. (D) Treatment with 60 µg/ml /ml concentration for 72 hrs, with moderate DNA damage.

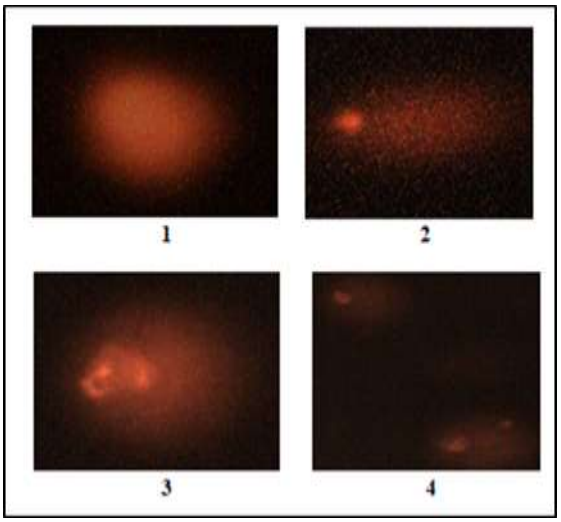

Figure 7. Comet images for MCF7 cell line. (1) control. (2) 60µg/ml concentration at 24hrs. With DNA damage indicated by the severe comet tail. 48 hrs. Showed a moderate comet tail. (3) 72 hrs. With DNA moderate damage.

3.2. Discussion

3.2.1. Chemical part

The new compound 2-(5-Chloro-3, 3-Dimethylindolin-2-Ylidene)-3-((4-hydroxyphenyl)imino)propanal. was stable, non-hygroscopic, had a high melting point, was soluble in organic solvents at room temperature, and was very soluble in DMSO.

IR spectra show a significant band at 1590cm-1, which is qualified to stretch vibration of (HC=N), indicating its specific chemical structure. The Carbonyl group had a pronounced absorption band at 1651cm^{-1}, while CH aromatic and CH aldehyde had bands at 2960υ and 2745υ, respectively. The new Schiff base structure was fitted these primary absorption bands.

A single signal at(δ= 13.96)ppm in HNMR spectrum was belonged to (N-H) indole Ring and δ = 8.52 ppm for the HC=N proton, Signal in the region at δ=(9.60, 9.56) ppm was detected to the free OH proton and bonded OH. The aromatic protons appear in the expected region (7.57–6.87) ppm corresponding to seven protons, which confirm the 1:1 condensation of aromatic aldehyde with amine and 1.59 to six protons atoms of two methyl groups.

In ^{13}C NMR data, the carbonyl group was at 188.19 ppm, the aromatic (N-C) group at 184.08ppm, the NH-C=C group at 155.97ppm, and (O = C - C and CH_3 -C- CH_3) groups at 107.62 and 54.66 ppm. Aromatic ring carbon atoms accounted for the other peaks between 150.22 and 116.63 ppm. All NMR peaks matched the newly compound's structure.

3.2.2. Biological part

Cytotoxic activity

The cytotoxic activity of concentration 60 µg/ml after 48 hours of exposure time appears to be higher cytotoxicity against the MCF7 cell line with 62%. Also, the concentration (40 µg/ml) displayed an inhibition rate of 59% after 48 hrs. of exposure time.

The concentrations (10 and 20) µg/ml for this new compound gave inhibition rates of 13% and 15% for 24 and 48 hrs., respectively, toward the REF normal cell line. That made them ideal concentrations for its safety toward REF cell growth. However, the cytotoxic activity of higher concentration (60µg/ml) toward REF showed 26% and 36% for 24 and 48 hrs. Therefore, the ideal concentration for these cell lines could be 60µg/ml because it inhibits the growth of the MCF7 cancer cell line, and it will be safe for REF normal cell viability.

Apoptosis activity

Genotoxicity with a concentration of 60 mg/ml was experienced against MCF7 breast cancer cell line and REF normal cell line. That is not toxic toward REF compared to MCF7 cancer cells.

The study found that 60 µg/ml of the novel indole Schiff base induced more DNA damage and genomic toxicity in MCF7 cells after 24 hours of treatment. The strong comet tail contrasts with the REF cell line without DNA damage. According to comet photos, the results above showed DNA damage, but after 48 hours of exposure, the mild comet tail in REF was linked to a moderate comet tail in MCF7 cells. The REF and MCF7 cell lines showed substantial DNA damage from the comet tail after 72 hours. The study found that the novel indole Schiff base molecule is more efficient in causing genotoxicity in breast cancer cell lines with extensive comet tails, while remaining safe on normal cell lines without causing significant DNA damage after 24hrs. of exposure at60μg/ml.

4. Conclusion

New compound 2-(5-Chloro-3, 3-Dimethylindolin-2-ylidene)-3-((4-hydroxyphenyl)imino) propanal. Was synthesized by reacting malonaldehyde with 4-aminophenol in refluxing methanol. The product's IR and NMR spectrum data match the newly synthesized chemical compound. The in vitro cytotoxicity against (MCF7) breast cancer cells showed that it inhibits cancer cells while protecting normal cell growth. The newly synthesized chemical caused moderate and severe comet tail DNA damage in MCF breast cancer cells. It has moderate DNA damage after 72 hours compared to REF normal cells, greater attention should be absorbed toward future investigations of these novel indole derivatives in cancer treatment.

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