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Synthesis, Characterization of 2-(5-Chloro 3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde and Testing its Cytotoxic Activity Against Cancer Cell Line

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Abstract:

Anew compound of indoleSchiff base derivatives have been synthesized by reaction of 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-malonaldehyde with 4-methoxy aniline. The chemical structure of the synthesized compound was characterized by TLC, FT-IR, 1H, 13C NMR and APT 13C NMR. The in vitro anticancer activity of the newly synthesized compound tested against AMJ13 breast cancer cell line. The revealed data showed that compound have promising anticancer activity. AMJ13 cell line was time dependent in both 40 and 60 µg/ml and the ideal inhibition rate to AMJ13 cells growth is 66 and 68 % after 72hs. of exposure. However, the lower concentration 20 µg/ml also displayed cytotoxicity against the tested cell line with 50 and 68 % inhibition rate determined after 48 and 72hs. of exposure. The Concentrations 10 and 20 µg/ml gave less than 10% inhibition rate when tested against REF cell line viability for 24, 48 and 72hs. Schiff bases compoundssuggested to have a potential effect by inhibitingcancer cell line, and further studies needed to determine the mechanism of their antitumor activity.

Keywords: *Schiff bases, NMR, AMJ13 cancer cell line, Cytotoxic Activity.*

Introduction:

Schiff bases were essential compounds in the biochemistry fields due to their biological activities [1]. Schiff bases containing an azomethine group (-C=N-) as a useful group have attention for a long time due to their medicinal and pharmaceutical activities [2]. Schiff bases were first informed by Hugo Schiff in 1864, they are formed by the condensation reaction of aldehydes or ketones with primary amines in the acid as a catalyst [3].They have many applications in different fields for examples: antitumor, antibacterial and antifungal activity [4].

Schiff bases get from different heterocyclic compounds mainly those including of indole molecule because they have a broad applications for example, anti-oxidant, anticancer [5], antifungal, anti-inflammatory and antiviral properties [6].

Cancer is a disease that makes cells growth in the body out of control like breast, lung, colon and prostate cancer are the most common types of cancer [7].

Breast cancer (BC) is one of the most common diagnosed types of cancer in women around the world an inducing to cancer death with almost 1.67 million new cases of cancer and more than 500,000BC deaths predicted to have appeared in 2012[8].

This research aimed to synthesis a new indole derivative as drawing in Figure1, and tested their cytotoxic activity against – AMJ13 breast cancer cell line [9].

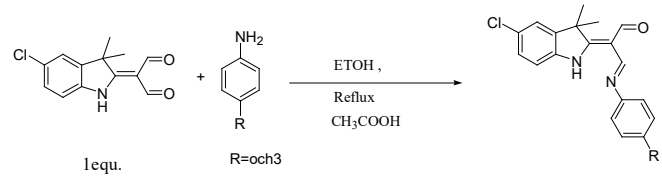

Figure 1: *the synthetic pathway to a new Schiff base*

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Materials and methods:

Chemistry part

Melting points were measured by using open capillary melting point device and the purification were taken by using Thin layer Chromatography was performed by using Silica gel sheets and the spots were observed using florescence analysis cabinet model CM-10. IR spectra were recorded on Perkin-Elmer spectrum in Diyala University,1H,13C NMR and APT13C NMR spectra were recorded in DMSO on a Bruker 400 MHz spectrometer, at 400MHz for1H NMR and at 100 MHz for13C NMR and APT spectra in Jordan, University of science and technology, College of science, Irbid city .

Chemicals and solvent

All the chemicals and solvents used in this research were obtained from different companies; they were used as received without further purificationSuch4-Methoxy aniline was gotten from Hopkin and Williams, Glacial acetic acid was gotten from BDH, all organic solvent obtained fromScharlau,4-chlorophenylhydrazinhydrchloride, Methyl isobutyl ketone, Dimethylformamide and Dimethylsulfoxide were obtained from Aldrich, Phosphoryl chloride and Sodium hydroxide were obtained from Thomas Baker.2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-malonaldehydewas synthesized with reform of a procedure described by[10].As shown in figure (2).

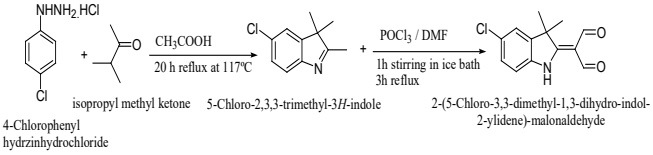

Figure 2: *the synthetic pathway of 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-malonaldehyde*

Synthetic methods:

Synthesis of 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde (compound 3) as demonstrated in figure (3)

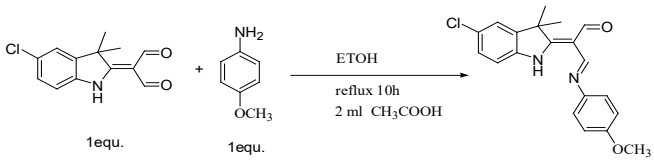

Figure (3): *The synthetic pathway of2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde*

A solution of (0.5g 2mmol) of 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-malonaldehyde was dissolved in 25ml ethanol and (0.25g, 2mmol) of 4-methoxy aniline was dissolved in (10ml) ethanol then (2ml) of glacial acetic acid was added to the solution. The mixture was refluxed in water a bath at 78ºC for10h.Solvent was reduced to one quarter, yellow precipitate was formed, filtered off, washed with ethanol and dried in oven at 78ºC. The purity of compound was determined by using TLC (4:2) hexane: ethyl acetate with pre-coated silica gel, which gave one spot. Yield (0,61g 85%), m.p.145-146 0C. IR data in (cm-1):3063 (CH aromatic) 2976 (CH aliphatic), 2734 (CH aldehyde), 1672 (CH=O), 1625 (CHN), 1603 (C=C), 1347 (CH3), 1263 (C-N), 1186 (C-O), 816 (C-Cl) and 783 (C-H bending).1HNMR (400MHz, DMSO, δ in ppm): δ=13.96(s,1H,NH), 9.37(s,1H,HCO), 8.54(s,1H,HCN), 7.58-7.02(7H,Ar-H), 3.78 (s, 3H, OCH3),and 1.56(6H,s, 2x CH3); 13CNMR (100MHz, DMSO, δ in ppm):δ = 187.79 (C=O), 183.47 (N-C=C), 156.37 (CH=N),157.11, 149.58, 147.45, 132.72, 129.22, 127.28, 121.61, 119.66, 119.53 and 114.87 (Ar-CH), 107.40 (O=C-C=C), 55.36(OCH3), 54.15 (CH3CCH3) and 21.64 (2x CH3).APT 13CNMR shown signals for CH and CH3 appeared at negative side (below base line of the spectrum) 187.61, 156.94,127.11, 121.43, 119.48, 119.35, 114.70, 55.19 and 21.46 whereas quaternary carbons, CH2 carbons and carbons diluted DMSO solvent were observed at positive side (above base line of the spectrum) 183.29, 156.94, 149.40, 147.27, 132.55, 129.04, 107.22 and 53.98.

Biological part

Cell lines have been used in this study are two types: (AMJ13): Breast cancer cell line and (REF): fibroblastic and epithelial cells with normal chromosomal pictures as normal murine cell line were used. Both of them were locally established in Iraqi center for cancer and medical genetics research / Mustansiriyah University as a gift from experimental therapy department and they are maintained for use.

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Solubility of compounds tested for in vitro cytotoxicity

Solubility of synthesized compound was approved according to protocol labeled mentioned by [11]. The new compound was dissolved in Dimethyl sulfoxide (DMSO) and diluted with medium RPMI-1640 to the chosen concentrations (10 µg/ml, 20µg/ml, 40µg/ml and 60µg/ml).

Both cell lines (AMJ13 and REF) were cultured in RPMI-1640 media which contains 10% fetal bovine serum, glutamine (2 mmol/L), streptomycin (100 U/ml) and penicillin (100 U/ml), then incubated in 5% CO2 at 37ºC for 24 hour. The falcon contains attached monolayer cells was de-attached with 1 ml of trypsin/versine to provide suspension of cells, then 10 ml added of prepared media to above solution. About 200µl of the cells were culture on clean sterile 96- well micro titer plate then let the cells for 24 hrs. to make monolayer to be ready to be treated with the our new compound.

Exposure day: empty the media from the cells and added 200µl from the dilutions new compound. Each concentration was triplicate and returns the microtiter plates to the incubator. Leave wells contains only cells without treatment contains serum free media representing control cells. Three different exposure times of the cells were included in this research, 24, 48 and 72 hour. The protocol of handing and treating the cells was prepared as described by Butler, 2004 [12].

Cell Viability Assay

The cytotoxicity was determined after each exposure time using crystal violate stain. Decant the contents of micro titer plate, add 200 µl was added of the crystal violate stain to each wells of the treated cells for 20 min. in the incubator at 37ºC. The crystal violate stain will stain the nuclei of the viable cell and the color will be visible to the eye. Then the plates were read by ELISA reader at 495nm. And then the inhibition percentage was calculated using the following equation as recommended by [13].

%Growth Inhibition = (C–T)/C ×100 %

Were, C represent absorbance of control and T absorbance of sample.

Statistical Analysis

In this study, we used student t-test to determine the differences between the concentrations in each cell line and to determine the differences between two cells in each exposure time. Graph Pad Prism V6 was used to determine this statistical test. Excel 2010 sheet was used to draw the curves.

Results:

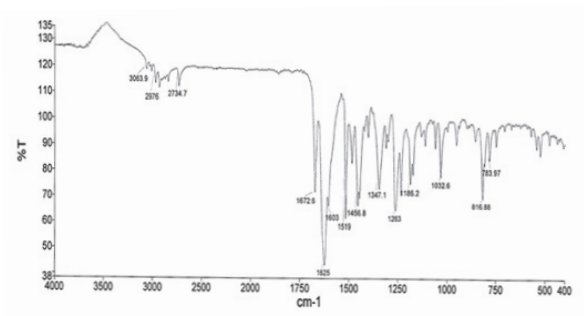
The new synthesized compoundwas subjected to TLC; spectral studies like HNMR, 13CNMR, APT13CNMR and FTIR, and their results are discussed below. The physical properties such as the percentage yield and melting point of the compounds is characterized in Table No.1

Table 1: *Physical properties of the synthesized compound*

Molecular formula	Molecular weight	Percentage Yield	Melting Point ºC
$C_{20}H_{19}Cl$-N_2O_2	354.83	85%	145-146^{0}C

IR Study

The IR results of the new synthesized compound was absorption bands in the 4,000 - 400 cm-1 range, especially the new functional group (azomethine group CH=N) at1625 cm-1for new synthesized compound [14]. Also strong absorption band at 1672cm-1 were gone to (C=O) of the carbonyl group [15]. As well as stretching frequency at 1603cm-1 for new compound was denoted to (C=C) group [16]. at the same time the synthesized compound was appeared an absorption bands at 1263cm-1 which attributed to (C-N) group [17]. Finally the absorption band at 1347cm-1was appointed to (CH3) group and the absorption band at 1186cm-1 was belonged to (C-O) group [18]. All these main absorption bands are approved the chemical structures of the new synthesized compound as shown in figure (4).



***figure*(4).** *IR Spectrum of the 2-(5-Chloro 3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde*

NMR Study

1H-NMR, 13C-NMR and APT 13C-NMR spectra were reported in DMSO (dimethyl sulfoxide) with chemical shifts in ppm and using TMS (tetramethylsilane) as a standard.

1H-NMR

The 1H-NMR results for this new compound figure (4) shown single signal at 13.96ppm was appointed to proton of (NH) of indole ring [19].A singlet signal at 9.39ppm was referred to proton atom of carbonyl group (C=O) [20]. As well as, single signal at 8.54ppm was attributed to proton of Schiff base group (CH=N) [21]. Signals were appeared in the region between (7.58-7.02) ppm were assigned to protons of aromatic ring for this new compound [22]. Finally peak at 1.56ppm was belonged to six protons of two methyl groups and 3.78was denoted to OCH3 group [23].

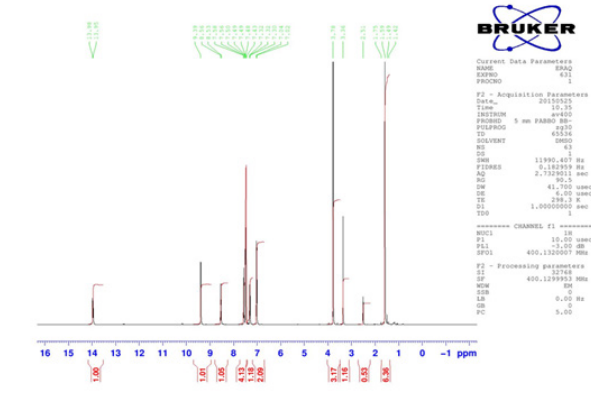


Figure (4): *1H NMR Spectrum of the 2-(5-Chloro 3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde*

13C NMR study

The 13C NMR results supported 1H NMR results for the new compound as shown on figure (5). A signal at 187.79ppmand at 156.37ppm which belonged to the carbon atom of the carbonyl group C=O and the carbon atom of the azomethine group (CH=N) respectively [24].The signals were appear in range between (149.58-114.87ppm) assigned to the carbon atoms of aromatic ring [25]. While the signal 107.40ppm was referred to carbon atom of (O=C-C=C)group [26], as well as a signal of carbon atom of (CH3CCH3) group was observed at 54.15. In addition, signal two groups of methyl were observed at 21.64ppm. Finally the signals of OCH3 were appeared at55.36 ppm [27].

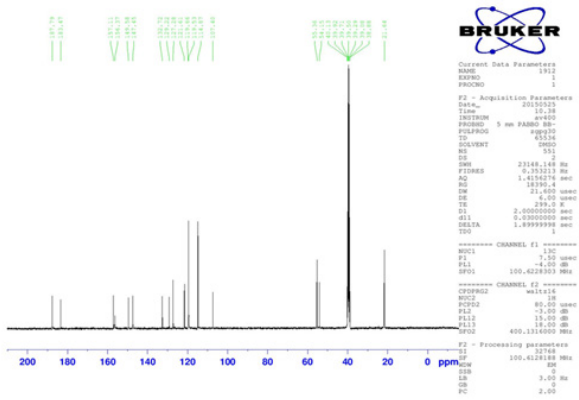


Figure (5): *13C NMR Spectrum of 2-(5-Chloro 3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde*

APT 13CNMR

APT 13C NMR results were further used to characterize the new compound. For example APT 13C NMR results of this compound figure (6) shown signals for quaternary carbons and solvent which appeared at positive side (above of the spectrum). While CH and CH3observed at negative side (below of the spectrum).1H, 13C NMR and APT spectrum results for the new compound match well with the expected signals and are regular with the formation of these compounds.

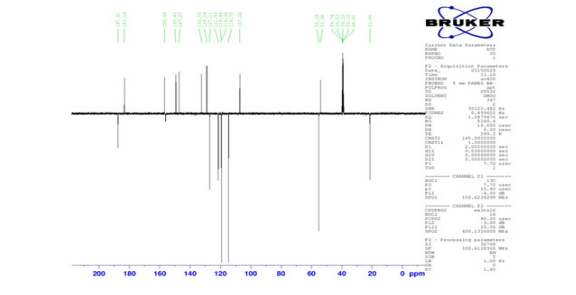


Figure (6): *APT NMR Spectrum of the 2-(5-Chloro 3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde*

In Vitro cytotoxicity effects of the new compound

Cytotoxic activity against AMJ13 cell line

The new synthesized compound was tested in vitro to determine their cytotoxicity toward AMJ 13breast human cancer cell line. Our results showed that the cytotoxic activity of this new compound figure (7) displayed cytotoxicity towards AMJ13 cell line and the effect was time dependent in both 40 and 60µg/ml and the highest inhibition of growth to AMJ13 cells was 66 and 68 % after 72hs. However, the lowest concentration (20 µg/ml) also displayed cytotoxicity against the tested cell line with 50 and 68 % inhibition rate determined after 48 and 72hour.The Concentrations 10 and 20 µg/ml gave less than 10% inhibition rate when tested against REF cell line viability for 24, 48 and 72hs. These concentrations are shown to be ideal Concentrations prepared from this new compound for their safety toward REF cell growth. Also, the concentration of 40 µg/ml reduced less than 10% of viability after exposure of 24 and 48hour, however, after 72hr this inhibition rate increased to about 60%. The higher concentration (60µg/ml)showed slight toxicity towards REF cell line. The inhibitory effect was time and concentration dependent (25, 45 and 65% inhibition rate for 24, 48 and 72h respectively). The ideal concentration for both cell lines could be 20µg/ml because it inhibits AMJ13 cancer cell line growth and is safe for REF normal cell viability after 27hrs.

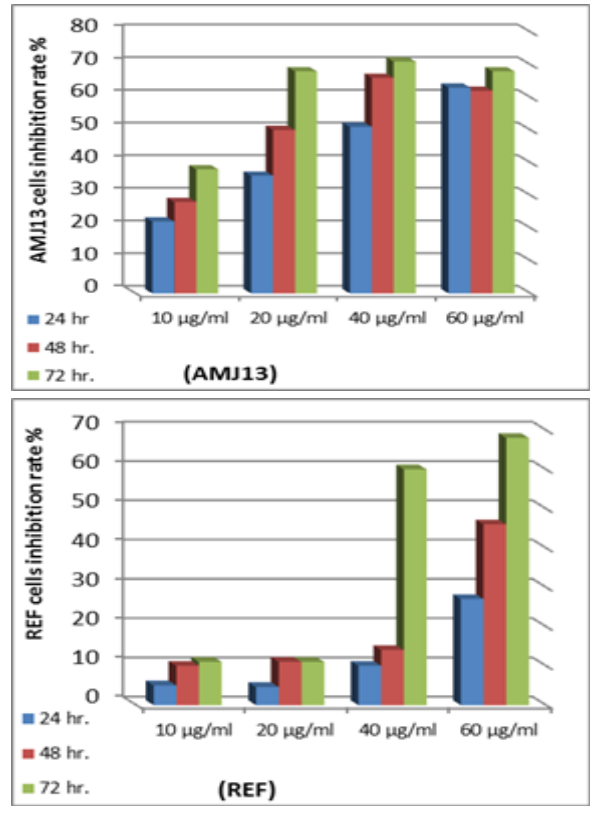


Figure (7): *Cancer Cell Lines AMJ13 and REF were treated with compound concentrations (10, 20, 40 and 60) µg/ml for Three Different Exposure Times 24, 48 and 72 h.*

Discussion:

The new compound 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehyde has been synthesized by reaction of 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-malonaldehyde with 4-methoxy aniline and subjected to TLC, spectral studies like HNMR, 13CNMR,APT 13CNMRand FTIR.

The in vitro anticancer activity of the newsynthesized compound tested against AMJ13breast cancer cell line. The revealed data showed that compound have promising anticancer activity. AMJ13 cell line was time dependent in both concentrations 40 and 60 µg/ml and the ideal inhibition rate to AMJ13 cells growth is 66 and 68 % after 72hs. of exposure. However, the lower concentration 20 µg/ml also displayed cytotoxicity against the tested cell line with 50 and 68 % inhibition rate determined after 48 and 72hs. of exposure. The Concentrations 10 and 20 µg/ml gave less than 10% inhibition rate when tested against REF cell line (normal cell line) viability for 24, 48 and 72hs.[28] The in vitro cytotoxicity of Unsymmetrical tetradentate Schiff base Fe(III) and Cu(II) complexes was tested for KB and Hep-G2 human cancer cell lines. The results showed that almost unsymmetrical tetradentate Schiff base complexes have high cytotoxicity. urea Schiff base complexes were tested against three cancer cell lines PC3, SKOV3, and HeLa displaying an cytotoxic activity with IC50 values of 0.71 ± 0.06, 0.12 ± 0.06, and 0.79 ± 0.23 µg/mL respectively[29]. these recent studies concluded and highlighted the importance of Schiff base in future cancer therapy.

Conclusions

The new Schiff base indole derivative 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-3-(4-methoxy-phenylimino)-propionaldehydehas been synthesized by reaction of 2-(5-Chloro-3,3-dimethyl-1,3-dihydro-indol-2-ylidene)-malonaldehyde with4-methoxy aniline, The chemical structure of the synthesized compound have been characterized and approved by TLC, FT-IR, 1H NMR, 13C NMR and APT13C-NMR techniques. The in vitro cytotoxicity of the compound prepared against breast cancer cell line AMJ13 revealed that this new compound has the ability to inhibit AMJ13 cells and in the same time safe to normal cell growth REF cell line. Let us to put a big highlight for further research on these compounds in cancer therapy models.

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