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Synthesis, characterisation, antioxidant and anticancer evaluation of novel schiff's bases of 2-quinolones

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ABSTRACT

A series of novel Schiff's bases of 2-Quinolones have been synthesized using Coumarin 4 aldehydes and different substituted amines as starting materials under conventional heating method and were obtained in good yields. The synthesized compounds were screened for their anti-oxidant activity by DPPH· method and were also tested for their antiproliferative activity against Lung cancer cell lines by MTT assay method. The details of the present work is presented here.

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Introduction

The quinoline skeleton is present in numerous natural products, especially in alkaloids. Many quinolines display interesting pharmacological activities and have found applications as pharmaceuticals, e.g., antimalarial drugs, such as quinine or chloroquin.

Quinoline ring is commonly obtained by *ortho*-condensation of benzene ring with pyridine

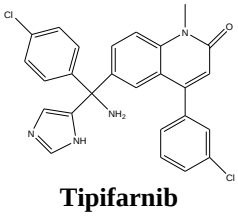
A number of biological activities have been associated with quinoline-containing compounds such as anti-inflammatory[2], antiallergic[3], antimalarial[4], antibacterial[5], antiproliferative[6], anticancer[7] and antiparasitic[8] activities.

In the last few years, extensive work has been done on quinolinones, with the aim to investigate and compare their anticancer activity.

The 4(1H)quinolone structure plays an extremely important role in the area of new investigational drug discovery programme. These compounds have been used as precursors for anticancer agents[9], anti-malarial agents[10] and reversible (H^+/ K^+) ATPase inhibitors[11].

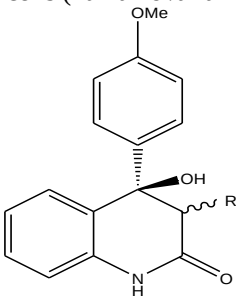
Quinolin-2-ones or 1-azacoumarins (carbostyrils) , is a part of quinoline alkaloids are known for their diverse biological activity and recently, a 6-functionalized 1-aza coumarins are undergoing human clinical trials as an orally active anti-tumor drug in view of its farnesyl protein-inhibiting activity in the Nano molar range.[13]

Tipifarnib, a new inhibitor of Farnesyltransferase, and potential novel anticancer drug candidate targeting Farnesyltransferase having structural features associated with 4-substituted carbostyrils.[14]



Tipifarnib

The diastereoisomeric quinolinones A and B were isolated from *P. janczewskii* derived from surface water (GermanBight, Helgoland Island). Both compounds were cytotoxic to a range of human tumour cell lines, with B strongly cytotoxic to SKOV-3 cells (human ovarian carcinoma).[15]



A R = α OH

B R = β OH

In view of our earlier observation on the diverse structural features and versatile biological properties associated with 4-substituted carbostyrils, it was thought of interest to synthesize 4-substituted 1-aza coumarins (carbostyrils).

In the present study, an attempt was made to synthesize other correlated structures nearing the existing quinolones present in the marketed drugs, to achieve improved biological activities of the parent compounds and a novel series of schiff's bases derived from 4-CHO Quinoline moiety is reported here.

Experimental

General Methods

Melting points were recorded on Toshniwal Melting point apparatus and were found to be uncorrected. IR spectra (KBr disks) were recorded on Shimadzu FTIR instrument. NMR spectra were recorded on Bruker DPX300 instrument in CDcl3 with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values are mentioned in δ (ppm) and coupling constants are given in Hz. Mass spectra were recorded on GCMS (Shimadzu).

Progress of the reactions was monitored by TLC on 2 × 5 cm pre-coated silica gel 60F254 plates of thickness of 0.25 mm

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(Merck). The chromatograms were visualized under UV 254-366 nm and iodine.

Chemical synthesis

2.2.1.1 General procedure for the synthesis of Aceto acetanilide.

9.313 ml (0.1 mole) of aniline and 13.014 ml of ethyl acetoacetate were taken together in a 250 ml round bottom flask. The reaction mixture was then refluxed at 160˚C for 36 hrs. on heating mantle continuously. At the end of the reaction period, 250 ml of hot water was added to the flask and the contents were heated to boiling. The mixture was then filtered; the filtrate was chilled in refrigerator till the white crystals appeared. The crystals were then retrieved, dried in air, recrystallized from toluene and was characterized.

General procedure for the synthesis of 4-Methylquinoline-2(1H)-one

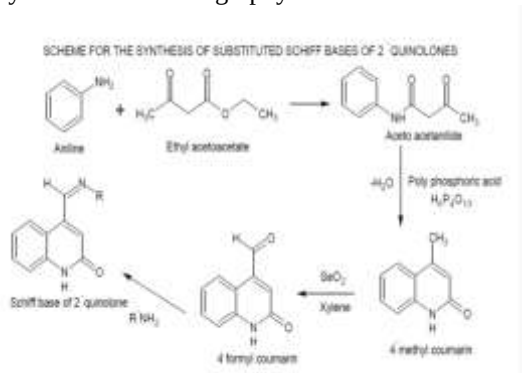
In a 100 ml conical flask, 10 gm. of polyphosphoric acid was taken and heated up to 100˚C on the magnetic heater with stirrer. 750 mg of acetoacetanilide was then added in the flask and stirred continuously for 3 hrs. During the reaction period, temperature was maintained between 95-100˚C. At the end of the reaction period, reaction mixture was poured into ice cold water; stirred the solution till the lumps dissolved completely. This solution was then neutralized with 4N Sodium hydroxide solution. The solution was kept in refrigerator for cooling till the compound settled. The precipitate was then retrieved, dried in air and was characterized.

Synthesis of 2-Oxo-1, 2-dihydro-quinoline-4-carbaldehyde

30 ml of xylene was taken in 100 ml round bottom flask and was heated on a mantle. A mixture of 600 mg of 4-methylqunoline-2(1H)-one, 3 gm. of selenium dioxide and molecular sieves (5A, 8-12 mesh) were added into hot xylene and was refluxed until the starting material disappeared. Reaction mixture was then poured into 50 ml beaker and after cooling; the solid was filtered off and dried.

Synthesis of substituted Schiff's bases of 2-Quinolones

To a solution of 2-Oxo-1,2-dihydro-quinoline-4-carbaldehyde in tetrahydrofuran (1-7 ml), aromatic amine was added at 0-5˚C and continuously stirred. The reaction mixture was then stirred under cold condition and reaction was monitored by TLC. Finally, the reaction mixture was poured into ice cold water. The product obtained was filtered, washed with distilled water until it was free from tetrahydrofuran and purified by column chromatography.



The analytical data of few of the synthesized Schiff bases is given as under

2-Oxo-1,2-dihydro-quinoline-4-carbaldehyde (JFB)

MASS (m/z) 173 [M]+, 145 [M-CO]+, 117 [M-CO,NH,CH2]2, 90 [M-CO,NH,CH2,CHO]+, 62 [M-CO,NH,CH2,CHO,C2H2]+

IR KBr (cm-1) NH (str) 2˚ amide 3315.74, C=O (str) 1662.69, C=O (str) aromatic aldehyde 1703.20, C=N (str) 1435.09, C=C (str) aromatic 1548.89, CH (str) aromatic methyl 2999.41.

4-(3'-chloro, 4'-fluoro benzilidine) quinoline-2(1H)-one (JMB-3)

MASS (m/z) 299 [M]+, 300 (M+ + 1), 301 (M+ + 2), 265 [M-Cl]+, 283 [M-F]+, 236 [M-Cl-CO]+ Base peak = m/z 91

IR KBr (cm-1) NH (str) 2˚ amide 3313.82, C=O (str) 1668.48, C=N (str) 1433.16, C=C (str) aromatic 1496.81, CH (str) aromatic methyl 2997.48, C-Cl 756, C-F 1261.49.

4-(4'-fluoro benzilidine) quinoline-2(1H)-one (JMB-4)

MASS (m/z) 265 [M]+, 266 (M++1), 245 [M-F]+ , Base peak = m/z 149

IR (cm-1) NH (str) 2˚ amide 3425.58, C=O (str) 1674.21, C=N (str) 1435.04, C=C (str) aromatic 1504.48, CH (str) aromatic methyl 2999.31, C-F 1232.51.

4-(3'-bromo benzilidine) quinoline-2(1H)-one (JMB-7)

MASS (m/z) 327 [M]+, 328 (M+ + 1), Base peak = m/z 149

IR KBr (cm-1) NH (str) 2˚ amide 3408.22, C=O (str) 1668.43, C=N (str) 1473.62, C=C (str) aromatic 1591.27, CH (str) aromatic methyl 2997.38.

4-(4'-methoxy benzilidine) quinoline-2(1H)-one (JMB-5)

1H NMR δ 3.811 (s, 3H, CH3), δ 11.943 (s, 1H, NH), δ 7.053-7.030 (m, 3H, Aromatic H), δ 7.404-7.381 (dd, J = 8.4 Hz and J = 0.8 Hz, Aromatic H), δ 7.485-7.462 (q, 2 Aromatic H), δ 7.588-7.546 (m, 1 Aromatic H), δ 8.8895 (d, J = 7.6 Hz, 1H), δ 8.989 (s, 1H, H)

IR KBr (cm-1) NH (str) 2˚ amide 3406.29, C=O (str) 1660.71, C-O (str) ether 1251.80, C=N (str) 1433.11, C=C (str) aromatic 1506.41, CH (str) aromatic methyl 2993.52, CH (str) alkane 2841.15

Biology

Antioxidant activity

Free radicals are produced as a part of normal cellular metabolism as well as result of abnormal reactions stimulated by some disease process or xenobiotic. A radical is an atom or molecule that contains one or more unpaired electrons and may be charged or uncharged. Since, oxygen is so prevalent in biological systems, oxygen radicals are most common. This quantitative consideration, as well as the ready reactivity of carbon-centred radicals with oxygen, has resulted in the term, radicals and oxidants often being used interchangeably even though, they are not equivalent.

A free radical is the one, that has moved out of the immediate location of its generation and is no longer controlled by that environment. Radicals that are retained within their sites of generation have been called ´caged radicals`. The distinction between free radicals and caged radicals is important since, radicals produced by some reactions are controlled and the loss of this control has significant toxicological implication including the effects of antioxidants or other radical traps on these processes.

Characteristic features of a free radical

- High reactivity with short life span
- Self-perpetuating (autocatalytic) and diverse chemical reactivity
- Low chemical specificity
- Generated both *in vitro* and *in vivo*.

The Interaction of ROS with DNA

The interaction of reactive oxygen species (ROS) with deoxyribonucleic acid (DNA) may involve direct modification of DNA (i.e. the oxidation of DNA bases or sugars, or strand breaks), or they may be mediated through changes in transcription factors or enzymes involved in regulating gene expression. It has been suggested that ROS modulate the efficiency of the overall process of signal transduction at many

sites. ROS-induced alterations in gene regulation are either beneficial or detrimental to cell.

Measurement of Antioxidant Activity

A wide range of antioxidants both synthetic and natural, have been proposed in the treatment of human diseases. Hence, considerable attention has been devoted for the development of techniques in order to measure the antioxidant activity. A number of methods are available for screening of antioxidants including *in vitro* and *in vivo* methods.

(1) Measuring the ability to donate an electron or hydrogen atom to a specific reactive oxygen species or to any electron acceptor.

(2) Testing the ability to remove any source of oxidative initiation e.g. inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation.

The *in vitro* methods include conjugated diene assay, DPPH· method, inhibition of super oxide radical formation, hydroxyl radical scavenging activity, nitric oxide radical inhibition activity, ABTS method etc. The *in vivo* models include microsomal lipid peroxidation and erythrocyte ghost system.

***In vitro* Antioxidant Screening by DPPH· Method**

Principle

DPPH· (1, 1-diphenyl-2-picryl hydrazyl) is a stable free radical, that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the methanolic solution of DPPH· shows a strong absorption band at 517nm. DPPH· radical reacts with suitable reducing agents, resulting in the pairing of electron and subsequent loss of colour. The colour decreases stochiometrically with the number of electrons taken up and the decrease in the absorbance can be directly measured and compared with that of the standard ascorbic acid and the blank. The samples may not be necessarily freely available to react with DPPH·, hence they react at different rates and the reaction will often not go to completion in a reasonable assay time. Therefore, the sample size that can lower the initial absorbance of DPPH· solution by 50% has been chosen as the endpoint for measuring the antioxidant activity. This method is widely reported for screening of antioxidant activity.

Procedure

DPPH· Solution

The DPPH· stock solution was prepared by dissolving 3.9 mg of the DPPH· in 50 ml of DMSO.

Standard Solution

Ascorbic acid was used as a standard free radical scavenger. This was prepared by dissolving 1mg of ascorbic acid in 1 ml of DMSO to get a 1000 µg/ml stock solution. Serial dilutions were then made to get concentrations of 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 32.25µg/ml, 15.63µg/ml and 7.8µg/ml.

- **Test Solution**

The solutions of the test compounds were prepared in DMSO, by dissolving 10 mg of the test compound in 1ml DMSO and 0.2 ml of this solution was diluted up to 2 ml to get 1000 µg/ml stock solution. Serial dilutions were then made to get concentrations of 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml, 15.62µg/ml and 7.81µg/ml.

- **Method**

The assay was carried out in a 96 well microtitre plate. To 100 µl of DPPH· solution, 100 µl of each of the test sample or the standard solution were added separately in wells of the microtitre plate in triplicate. Control was prepared by adding 100 µl DMSO in 100 µl DPPH· solution. The plates were then incubated at 37°C for 20 min. and the absorbance of each solution was measured at 540 nm using ELISA reader against the corresponding blank and the remaining DPPH· was calculated. IC_{50} is the concentration of the sample required to scavenge 50 % of DPPH· free radicals.

Percentage inhibition was measured by:

$$\% \text{ inhibition} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

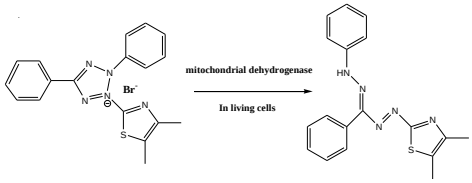
Anticancer activity

In vitro cytotoxicity on A549 (lung cancer cell line) by MTT assay

MTT [3-(4, 5-dimethylthiazol-2-ysl)-2, 5-diphenyltetrazolium bromide] assay

Principle

MTT is taken up by the viable cells and reduced to formazan by the "mitochondrial dehydrogenase" system that belongs to the mitochondrial respiratory chain functioning in metabolically active cells. Formazan formed, is a purple coloured water-insoluble product that is largely impermeable to cell membranes, thus resulting in its accumulation within the healthy cells which is solubilised by adding Dimethyl sulphoxide (DMSO). The optical density (OD) of purple coloured solution developed can be read using a conventional ELISA plate reader at 540nm (maximum absorbance). The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which, in turn, may be interpreted as a measure of viability and/or cell number.



MTT Formazan

Materials

1)Cell lines:Human Lung Cancer Cell Line, A549 procured from NCCS Pune, India.

2)DMEM (Dulbecco's Minimum Essential Medium) media supplemented with 10% fetal bovine serum (FBS) and MTT reagent from Himedia and Aldrich respectively.

3)Tissue culture flasks, 96 well microculture plates, Membrane Filters, eppendorfs, Pipette tips

4)Gentamycin sulphate, Methotrexate,

5)Tryphan blue dye

6)Absolute alcohol

7)Millipore water

Maintenance of cell lines

A-549 procured from NCCS Pune were grown in 25 cm^2 tissue culture flasks containing MEM and DMEM media supplemented with 10% FBS, 1% L- glutamine and 50µg/ml Gentamycin sulphate at 37ºC in CO_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in 25cm^2tissue culture flasks.

Sub culturing process of cell lines

- The culture media from the flasks containing monolayer culture was aspirated and washed with sterile phosphate buffered saline (PBS).
- To the flasks, 2ml of 0.1% trypsin-EDTA solution was added and after few seconds it was aspirated. The flask was then kept in incubator 2-3 min for detachment.
- The flasks were removed from the incubator and gently tapped to detach all the adhering cells. The cell detachment was confirmed by observing under an inverted microscope (Nikon Eclipse TE 2000-5, Japan).

- Once the cells were completely detached from the flasks, 2-3 ml of respective media containing 10% FBS was added and mixed well.
- Cell viability was checked with a small sample of the suspension by tryphan blue dye exclusion test.
- From the stock cell suspension, 1 x 10^4viable cells/ml suspended in media were seeded in 25cm^2tissue culture flask containing about 4ml of fresh media and incubated until the flasks attained 60-70% confluence.

Preservation of the tumour cells

Tumour cells from the first and second passage of transplantation were stored in liquid nitrogen in cryovials containing respective medium supplemented with 10% FCS and 10% DMSO as preservative at a concentration of 1x 10^6 cells /ml. This constituted the tumour bank. After every 10 passages, that tumour cell line was discarded and new passage was started using the original tumour cells from the tumour bank.

Trypsinization

To obtain a single cell suspension from a monolayer culture, cells were dislodged from the culture flasks by trypsinization.

- From a 60-70% confluent flask, the culture media was aspirated out using a micropipette.
- Cells were washed with 3 ml of PBS to remove trace amount of media.
- To each culture flask 2ml of trypsin-EDTA was added and after few seconds it was aspirated and the flask was kept in the incubator for 3-4 min for cell detachment.
- Culture flasks were observed under an inverted microscope (Nikon Eclipse, Japan) to ensure that cells were completely dislodged.
- Trypsin activity was stopped by adding 2-3ml media containing 10% FBS.

MTT assay method

Exponentially growing cell lines were harvested from 25cm^2 Tissue culture flasks and a stock cell suspension (1X10^5cell/ml) was prepared with respective media.

- A 96-well flat bottom tissue culture plate was seeded with 1x10^4cells in 0.1 ml of MEM and DMEM medium supplemented with 10% FBS and allowed to attach for 24hrs.
- Test compounds were prepared just prior to the experiment in 0.4% DMSO and serially diluted with medium to get the working stock of 200 µM/ml, 100 µM/ml, 50 µM/ml solution, 25 µM/ml.
- After 24 h of incubation, cells were treated with 100µl of test compounds and the plates were again incubated cells for 48 hrs.
- The cells in the control group received only the medium containing the 0.1% DMSO (vehicle).
- Each treatment was performed in triplicates. After the treatment, drug containing media was removed by inverting the plate onto a tissue paper.
- To each well of the 96 well plate, 100µl of MTT reagent (Stock: 1mg/ml in PBS) was added and incubated for 4hrs at 37ºC.
- After 4hrs of incubation the plate was inverted on tissue paper to remove the MTT reagent. To solubilize formazan crystals in the wells, 100µl of 100% DMSO was added to each well.
- The optical density (O.D) was measured by an Enzyme Linked Immunosorbent Assay (ELISA) plate reader at 540 nm. Percentage cytotoxicity of each extract was calculated by using this formula:

$$\frac{(\text{Control-Blank}) - (\text{Test-Blank})}{(\text{Control-Blank})} \times 100$$

- Percentage cytotoxicity was plotted against drug concentration and $IC_{50}$was calculated from the graph.

Results and discussions

Synthesis

All the starting materials used in the synthesis were checked for their authenticity using TLC, melting point and UV absorption. The solvents used for the synthesis were from LR-grade and AR-grade. They were further distilled and dried, by following the standard procedure. In order to prevent the entry of moisture, all the synthesized products were saved in a desiccator.

The synthesis of all the test compounds was achieved in 4 steps. All the test compounds were rechecked for their purity by melting point determination and thin layer chromatography.

The first step involved the synthesis of acetoacetanilide, using aniline and ethyl acetoacetate as starting materials. Aniline was treated with β-ketoester (ethyl acetoacetate) at high temperature to give acetoacetanilide. The formation of this product was supported by IR and the presence or formation of anilinide was qualitatively identified by performing characteristic Tafel's Test which showed positive result and confirmed the presence of anilide group.

The second step involved the cyclization of acetoacetanilide in presence of polyphosphoric acid to give 4-methyl-2-quinolone. The formation of this compound was supported by melting point and IR spectra.

The third step involved oxidation of 4-methyl-2-quinolone in presence of selenium dioxide to give the parent compound, 2-Oxo-1,2-dihydro-quinoline-4-carbaldehyde (JFB). It was then purified by recrystallization from methanol. Further, It structure was established by IR showing sharp peak of –CHO functional group at 1703cm-1 and mass spectral studies clearly showing the molecular ion peak at ….

In the final step, 15 Schiff's bases , compound JMB-1 to JMB-15 of the parent compound (JFB) were synthesized by the reaction between synthesized aldehyde and substituted aromatic amines. The purity of test compounds was checked by melting point determination and thin layer chromatography and structures was established by IR showing the absence of aldehyde peak, 1H-NMR and mass spectral studies showing the molecular ion peak.

Antioxidant activity

Antioxidant activity is a prerequisite for performing many related biological activities; including anticancer, antiallergic, anti-inflammatory and anti -diabetic activity .

The antioxidant activity of the synthesized test compounds JMB1-JMB15 containing quinoline-2-one ring system was carried out by using DPPH radical scavenging method. Ascorbic acid was used as the standard. The following table indicates the IC_{50} values of the synthesized test compounds JMB-1 to JMB-15.

Dpph Radical Scavenging

Among 15 synthesized test compounds, five compounds such as JMB-5, JMB-6, JMB-8, JMB-10 and JMB-11 containing 4-methoxy, 2-methoxy, 4-bromo, 3-chloro and 4-chloro respectively were found to have moderate antioxidant activity. Test compounds JMB-5,6,8,10 and 11 were found to have IC_{50} value at 322 µg/ml, 471.35 µg/ml, 517.57 µg/ml, 551.75 µg/ml

and 329 µg/ml respectively, when compared to that of the standard ascorbic acid having IC_{50} value at 4.3525 µg/ml, as shown in the Table 7.

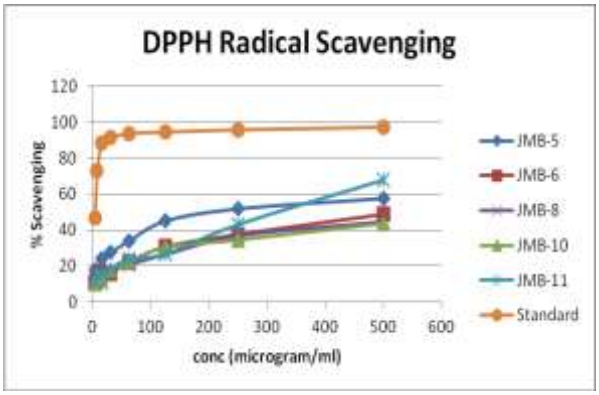


Figure 1 Showing % scavenging activity of synthesized test compounds

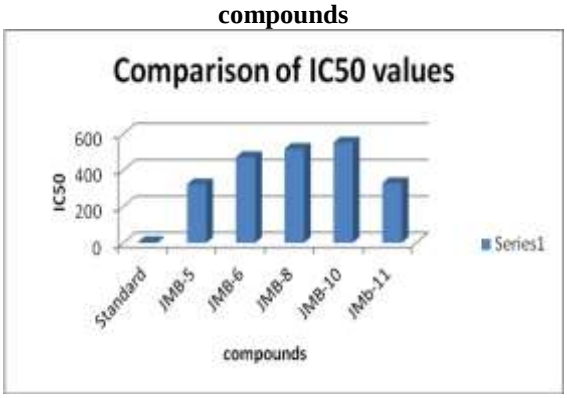


Figure 2 Showing IC_{50} values of synthesized test compounds

Thus, out of 15 synthesized compounds tested for their antioxidant activity, only two compounds such as JMB-5 and JMB-11 containing 4-methoxy and 4-chloro substitution were found to have the antioxidant activity comparable to that of standard ascorbic acid.

Anticancer activity

All the synthesized test compounds were screened for their *in vitro* anti-proliferative activity against A-549, human lung cancer cell line by MTT method. The activities of all the test compounds were compared to the standard drug Methotrexate, showing its cytotoxicity at a concentration of 1µM. Cytotoxicity was checked at 24 hours duration.

Table 8. Showing the % cytotoxicity against A549 human lung cancer cell line

Sr.No.	Compound code	Concentration (µM/ml)				IC_{50} (µM/ml)
		12.5	25	50	100	
1	JMB-3	12.89	15.40	25.50	39.61	132.375
2	JMB-7	-	9.52	37.15	68.89	70.33
3	JMB-8	-	-	32.18	42.71	135.0

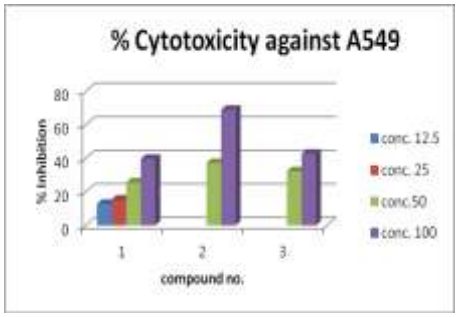


Figure 3: Showing the % Cytotoxicity of the synthesized test compounds

In our present study, the effect of 3-substituted quinolones was studied. Also, an attempt was made to explore the effect of Schiff base substitutions using different aromatic amines at third position of 2-quinolone.

Among the 15 compounds tested, none of the compounds were found to have significant cytotoxic inhibition except test compound JMB-3, JMB-7 and JMB-8. However, JMB-3 containing 3-chloro, 4-fluoro; JMB-7 containing 3-bromo and JMB-8 containing 4-bromo substituted aniline were found to have IC_{50} value at 132.375 µM, 70.33 µM and 135.0 µM respectively.

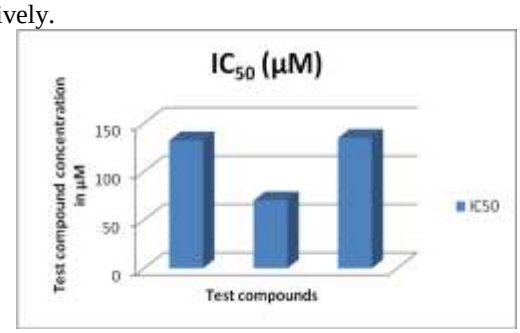


Figure 4: Showing the comparison of IC_{50} values of the synthesized test compounds

The present study showed that test compounds bearing the bromo aniline substitution at fourth position of 2-quinolone moiety were active. Thus, JMB-7 was found to be more potent test compound than the rest of the other compounds.

Conclusions

- All the final test compounds obtained are novel and the procedures were standardized to improve the yield and all the physical constant were fixed for them.
- The present study has prompted a better insight in developing more specific 4-substituted 2-quinolones, as potential anticancer agent.
- The present study revealed that, Schiff's bases of 2-quinolone ring system can be used for improving the chemical status of the existing structures which have shown some anticancer activity.
- This approach has also paved the way for generating more useful 2-quinolones as anticancer agents in future studies.

Table 7: Showing the DPPH˙ scavenging activity

Sr. No.	Conc.µg/ ml	% Scavenging					
		Standard	JMB-5	JMB-6	JMB-8	JMB-10	JMB-11
1	3.9	46.99	17.8896	10.6159	11.5379	10.2574	10.9745
2	7.81	73.08	16.5065	12.9722	11.0257	13.4332	14.3552
3	15.62	88.19	23.8827	16.5065	10.6159	13.1771	15.4309
4	31.25	91.30	27.5195	16.0455	17.6335	17.9920	18.1457
5	62.5	93.78	33.7687	22.1411	21.1166	22.4484	23.9339
6	125	94.61	44.9865	30.5929	26.4438	30.2855	26.5462
7	250	95.85	51.7992	37.508	36.8933	34.4858	43.0400
8	500	97.3	57.5874	48.7770	44.8328	43.5010	67.9856
9	IC_{50}	4.3525	322	471.35	517.57	551.75	329

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