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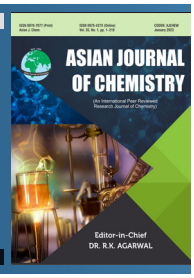

Synthesis and Evaluation of Anticancer Potential of Novel Benzopyran Derivatives Using MTT and *in vitro* Scratch Assays

Y. MANOGARAN[1], S. KARUPIAH[1], V. BALAKRISHNAN[2], N.K. FULORIA[1,*] and S. FULORIA[1,*]

[1]Faculty of Pharmacy, AIMST University, Bedong 08100, Kedah, Malaysia
[2]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia

*Corresponding authors: E-mail: nfuloria@aimst.edu.my; shivkanya_fuloria@aimst.edu.my

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Evidence over benzopyrans and azomethines high anticancer potential, intended present study to carry out the synthesis, characterization and anticancer activity of some new benzopyrans derivatives (NBD). Study involved synthesis of N′-(substituted benzylidene)-2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (**2a-d**) by hydrazination of substituted benzopyran ester (**1**). The structures of all the synthesized benzopyran derivatives (NBD **2a-d**) were elucidated based on the IR, ^{1}H NMR, ^{13}C NMR and mass spectral data. The NBD **2a-d** were further investigated for their cell viability (toxicity analysis) against HEK-293 and anticancer potential against MCF-7 cancer cell lines using MTT assay and *in vitro* scratch assay. Present study revealed that NBD **2a-d** structures were in agreement with their IR, NMR and mass spectrometric characterization data. The cell viability study, MTT and *in vitro* scratch assay of NBD **2a-d**, revealed compound **2d** to exhibit highest anticancer activity and safety when compared with standard (irinotecan). Present study concludes NBD **2d** to possess high anticancer potential and proven to be safer when compared with irinotecan, however further studies are needed to establish its clinical significance.

Keywords: Benzopyran, Imino analogues, Anticancer activity, Cytotoxicity.

INTRODUCTION

In recent decades, benzopyrans have attracted significant attention due to their remarkable chemical and biological characteristics [1]. Benzopyrans are the oxygenated fused heterocyclic nucleus containing compounds that are structurally elucidated as 1-benzopyran-2(2*H*)-one) and are highly persistent in natural and bioactive moieties [2,3]. The benzopyran synthesis was first time began in the mid of 19th century with discovery of Perkin's condensation reaction between acetic anhydride and salicylaldehyde [4]. Although there exist several methods to synthesize benzopyrans, however Perkin, Pechmann, Knoevenagel and Wittig condensation reactions are the classical ways to synthesized benzopyrans [5]. Attributed to their basic property, in living organisms, benzopyrans are known to exert the non-covalent interactions (such as hydrogen bonding, van der Waals interaction, electrostatic interactions, metal coordination, *etc.*) with several receptors, thereby are known to exert numerous therapeutic activities [3,6]. Evidence suggests breast cancer as the utmost common cause of mortality in female cancer patients [7]. Study estimated 297790 new breast cancer cases in year 2023 that are further estimated to reach over 3.0 million new cases and 1.0 million deaths in 2040 [8,9]. Although, diagnosis of most breast cancer cases occurs in early stage, but in 20-30% of breast cancer cases the patients suffer from distant relapse of cancer cells that spreads to distant body parts, such as brain, bones, liver, *etc.* [10]. Development of breast cancer is due to risk factors such as ageing, family history, imbalance of hormones, reproductive factors and lifestyle [11-13].

Though many therapies are indicated in treatment of breast cancer, such as hormonal and chemotherapy but associated side effects and resistance to these therapies is major problem for the health professionals. Hence, this creates a need to search for more selective and efficacious agent for the treatment of breast cancer. For medicinal chemists the benzopyran nucleus is of high importance from therapeutic point of view. Most importantly, benzopyrans are known to possess significant antitumor activity *via* inhibition of targeting of PI3K/Akt/mTOR

signaling pathways, carbonic anhydrase, induction of cell apoptosis protein activation, regulation of reactive oxygen species, inhibition of polymerization of microtubules, inhibition of angiogenesis and inhibition of tumor multidrug resistance, *etc.* [14]. Studies also revealed that incorporation of imino group enhances the anticancer potential of different benzopyran compounds [15,16]. Hence based on the problem of breast cancer, associated resistance, side effects of current anticancer drugs and high potential of benzopyrans and imines, motivated the workers of present study to propose the synthesis, characterization and anticancer evaluation of some novel benzopyran derivatives (NBD).

EXPERIMENTAL

All the chemicals for novel benzopyran derivatives (NBD) synthesis were procured from Sigma-Aldrich (USA), HmbG® Chemicals (Germany), Merck KGaA (Germany), Qrec Chemicals (Malaysia) and Friendemann Schmidt Chemical (USA). Synthesized NBDs were characterized based on their NMR spectra generated by ASCEND™ spectrometer at 700 MHz, FTIR spectra recorded on Jasco FTIR-6700 (4000 to 400 cm^{-1}) and generation of mass spectra recorded on Direct Infusion IonTrap Mass spectrometer (USA). Purity of NBDs was assessed using SMP11 Analogue apparatus. Reactions were monitored with TLC over aluminum sheet coated with silica gel 60 F_{254} (Merck Millipore) with CH_3OH:$CHCl_3$ (9:1) as eluant mixture using SPRECTROLINE® CM-26 UV cabinet [17,18].

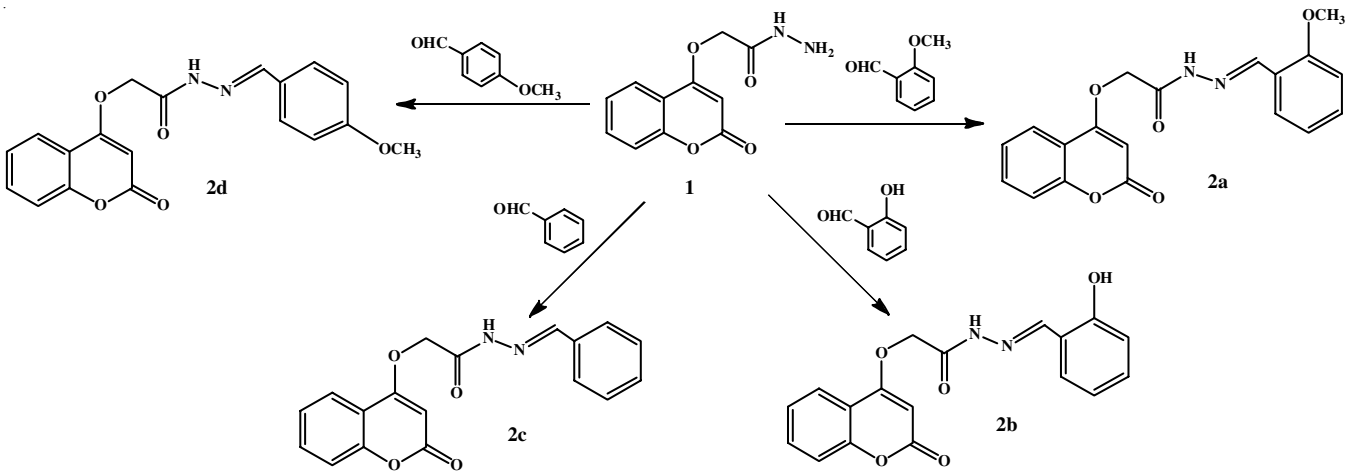
Synthesis of N′-(substituted benzylidene)-2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (2a-d): Synthesis of NBD **2a-d** was performed as per the standard procedure with minor modifications [19,20]. Briefly, a mixture containing equal molar concentration of 2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (**1**) (previously synthesized from hydrazination of ethyl ester of benzotetronic acid) and 4-methoxy benzaldehyde in dried ethanol was subjected to reflux for 8 h at 55 ºC. On completion of reflux, the crude mixture was dried and finally recrystallized with methanol and activated charcoal to yield pure compound **2a**. During experiment, anhydrous reaction conditions were maintained. Following similar protocol, other compounds **2b-d** were also synthesized and purified. The synthetic scheme for synthesis of compound **2a-d** is given in **Scheme-I**. All the new synthesized compounds **2a-d** were subjected to spectrometric characterization.

***N′*-(2-Methoxybenzylidene)-2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (2a):** Yellow crystalline, yield: 80%, m.p.: 90 ºC, R_f: 0.49; IR (KBr, ν_{max}, cm^{-1}): 2929 (C-H), 1609 (C=O), 1592 (C=N), 1452 (C=C); ^{1}H NMR (DMSO-d_6) δ ppm: 3.64 (s, 3H, O-CH_3), 4.82 (s, 1H, OCH_2), 5.09 (s, 1H, CH of pyran ring), 6.35-7.88 (m, 8H, Ar-H), 9.33 (s, 1H, NH-N) and 9.87 (s, 1H, N=CH); ^{13}C NMR (DMSO-d_6) δ ppm: 54.92 (CH_3), 78.16 (CH_2), 81.02 (=CH of pyran ring), 105, 108, 113, 114, 115, 117, 118, 122, 128, 129, 130, 131 (Ar-C), 160.18 (C=O of pyran ring), 151 (N-N=C), 164.23 (=C of pyran ring), 191.32 (O=C-NH); mass (*m/z*): 352 (M^+ peak).

N′-(2-Hydroxybenzylidene)-2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (2b): Yellow crystalline, yield: 76%, m.p.: 120 ºC, R_f: 0.49; IR (KBr, ν_{max}, cm^{-1}): 3425 (O-H), 2929 (C-H), 1609 (C=O), 1593 (C=N), 1452 (C=C); ^{1}H NMR (DMSO-d_6) δ ppm: 4.82 (s, 1H, OCH_2), 5.09 (s, 1H, CH of pyran ring), 5.94 (s, 1H, OH), 6.35-7.84 (m, 9H, Ar-H), 9.33 (s, 1H, NH-N) and 10.26 (s, 1H, N=CH); ^{13}C NMR (DMSO-d_6) δ ppm: 78.01 (CH_2), 80.85 (=CH of pyran ring), 105, 107, 108, 114, 115, 116, 118, 122, 126, 129, 130, 132 (Ar-C), 151 (N-N=C), 160.06 (C=O of pyran ring), 164 (=C of pyran ring), 191.29 (O=C-NH); mass (*m/z*): 338 (M^+ peak).

***N′*-Benzylidene-2-(2-oxo-2*H*-benzopyran-4-yloxy)-acetohydrazide (2c):** Brown crystalline, yield: 82%, m.p.: 70 ºC, R_f: 0.40; IR (KBr, ν_{max}, cm^{-1}): 2920 (C-H), 1609 (C=O), 1592 (C=N), 1454 (C=C); ^{1}H NMR (DMSO-d_6) δ ppm: 4.82 (s, 1H, OCH_2), 5.09 (s, 1H, CH of pyran ring), 6.42-7.92 (m, 9H, Ar-H), 8.72 (s, 1H, NH-N) and 9.86 (s, 1H, N=CH); ^{13}C NMR (DMSO-d_6) δ ppm: 78.01 (CH_2), 80.85 (=CH of pyran ring), 105, 107, 108, 114, 115, 116, 118, 122, 126, 129, 130, 132 (Ar-C), 151 (N-N=C), 160.06 (C=O of pyran ring), 161.49 (=C of pyran ring), 191.04 (O=C-NH); mass (*m/z*): 322 (M^+ peak).

***N′*-(4-Methoxybenzylidene)-2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (2d):** Brown crystalline, yield: 82%, m.p.: 115 ºC, R_f: 0.43; IR (KBr, ν_{max}, cm^{-1}): 2973 (C-H), 1609 (C=O), 1598 (C=N), 1491 (C=C); ^{1}H NMR (DMSO-d_6) δ: 3.65



Scheme-I: Synthesis of NBD **2a-d**

(s, 3H, O-CH_3), 4.82 (s, 1H, OCH_2), 5.09 (s, 1H, CH of pyran ring), 6.35-7.88 (m, 8H, Ar-H), 9.33 (s, 1H, NH-N) and 9.87 (s, 1H, N=CH); ^{13}C NMR (DMSO-d_6) δ ppm: 54.96 (CH_3), 78.17 (CH_2), 80.05 (=CH of pyran ring), 105, 108, 113, 114, 115, 117, 118, 122, 127, 129, 130, 134 (Ar-C), 160.18 (C=O of pyran ring), 151 (N-N=C), 164 (=C of pyran ring), 191.32 (O=C-NH); mass (*m/z*): 352 (M^+ peak).

***In vitro* anticancer activity:** The synthesized NBD **2a-d** were tested for the anticancer potential using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT) assay method with some modification [21,22]. Briefly, MCF-7 (ATCC, USA) cells were allowed for propagation using Dulbecco's modified eagle medium (DMEM) that was supplemented by 5% fetal bovine serum (heat inactivated) and subjected to 5% CO_2 incubator maintained at 95% relative humidity and 37 ºC temperature. For the experiment, MCF-7 cells were allowed to grow on in 96-well plate of 5000 cells per well capacity and followed by cell attachment by incubating for 12 h. To carry out experiment, NBD **2a-d** and standards were diluted using DMEM and administered into each well of plate to achieve eight different concentrations (twofold) ranging from 3.91 to µg/mL. Next, plates were exposed for 24 h to 5% CO_2 incubator maintained at 95% relative humidity and 37 ºC temperature. In experiment further MTT solution (10 µL) was added in each well and again plates were further incubated in dark for 4 h at 37 ºC. Next, the wells content of plate was pipetted out, followed by DMSO (100 µL) addition to each well to dissolve crystal formazan. Absorbance measurement was done at 570 nm (using Tecan Microplate Reader, Mannedorf, Switzerland) with reference of at 630 nm. Finally, based on the expression of eqn. 1, the cell cytotoxicity percentage (%) was calculated:

$$\text{Cytoxicity (\%)} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100 \quad (1)$$

Following similar protocol, the HEK-293 cells were also seeded in 96-well plate (with per well capacity of 35000 cells) and treated with NBD **2a-d** and finally measured for its absorbance to determine its cell viability using following formula given in eqn. 2:

$$\text{Cell viability (\%)} = 100 - \%\text{Cytotocity} \quad (2)$$

***In vitro* scratch assay (IVSA):** The synthesized NBD **2a-d** were evaluated for their wound healing ability or expansion capability on HEK 293 and MCF-7 cells, by measuring the migration rate of a cell population on scratched surfaces using *in vitro* scratch assay (IVSA) [23]. The wound healing activity of NBD **2a-d** was performed as per the standard protocol given in the literature [24]. Briefly, seeding of 200000 cells per mL was done overnight in cell culture plate. Later cells were subjected to phosphate buffered saline (PBS) washing and on the culture plate scratch was commenced using sterile 200 µL tip. The PBS washing was done to remove the resultant detached cells along with other cells debris. Next, the cellular treatment with NBDs **2a-d** and standard drug (irinotecan) was done and plates were subjected to incubation for 24 h. The cells that were untreated, were fixed as negative control. Finally, the cell migration or alteration in morphology was recorded in form of images through digital camera attached microscope (inverted), fitted with. All the experimental protocols were conducted three times (n = 3). Scratch width at different time (0, 3, 8 and 24 h) was analyzed using Image J software.

RESULTS AND DISCUSSION

Evidences over problem of breast cancer, associated resistance, side effects of current anticancer drugs and high potential of benzopyrans and imines were the motivation to carry out synthesis, characterization and anticancer evaluation of some novel benzopyran derivatives (NBD). Based on the literary facts [15-18], for present study the **Scheme-I** was designed, which offered all the NBDs (**2a-d**) in good yield.

Treatment of 2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide (**1**), with different aromatic aldehydes offered *N*′-(4-methoxybenzylidene)-2-(2-oxo-2*H*-benzopyran-4-yloxy)-acetohydrazide (**2a-d**) following Schiff's reaction. Importantly, NBDs synthesis was performed in anhydrous conditions. The synthesized NBDs were purified by recrystallizing with methanol and purity of NBDs was determined by their melting point (sharp) and TLC pattern (single spot) [18]. The structures of synthesized NBDs were characterized using FTIR, NMR and mass spectrometric data. Appearance of FTIR signal at 1592-1598 cm^{-1} related to C=N stretching, ^{1}H NMR signal at δ 9.86-10.26 ppm ascribed to N=CH protons, ^{13}C NMR signal at 151 corresponding to N-N=C carbon, the molecular ion peaks at δ 352, 338, 322 and 352 ppm in mass spectra of compounds **2a-d**, respectively, confirmed the chemical structure of NBDs **2a-d**. The characterized data of NBDs of current study also complied with the results of other studies on azomethines [23].

The cytotoxicity and cell viability studies of NBDs **2a-d** were evaluated by MTT assay against MCF-7 and HEK-293 cells using 96-well plate, respectively [17]. The percent cell cytotoxicity and cell viability were estimated using formulae given in equations **1** and **2**. The cytotoxicity experiment was performed as per the established protocol [21]. Results of cytotoxicity experiment suggest that NBDs **2a-d** are much effective and safer when compared with standard (Irinotecan), this is because when NBDs **2c** and **2d** were administered to MCF-7 at 500 µg/mL concentration, they offered 81.20 ± 5.49 % and 84.39 ± 1.21% inhibition respectively, that was higher than standard. The cell viability study results suggest that NBDs also offers a better safety profile when compared with standard, this is because there is no toxicity was exhibited by NBDs on HEK cells. Hence, this fact establishes the safety of newly synthesized compounds. The data of cytotoxicity and cell viability studies is presented in Figs. 1 and 2. The results of current studies were also in validated with other investigations results [21,22].

In a wound healing process, the cell migration is considered as an important stage as it determines whether applied drug will enhance the complete closure of scratch or wound [25,26]. So, effect of NBDs on proliferation of HEK 293 and MCF-7 cells was done using *in vitro* wound scratch assay. The *in vitro* wound scratch assay was done by seeding MCF-7 and HEK-293 cells in cell culture plates as per the established methodology [23]. The MCF-7 and HEK-293 cells were treated with

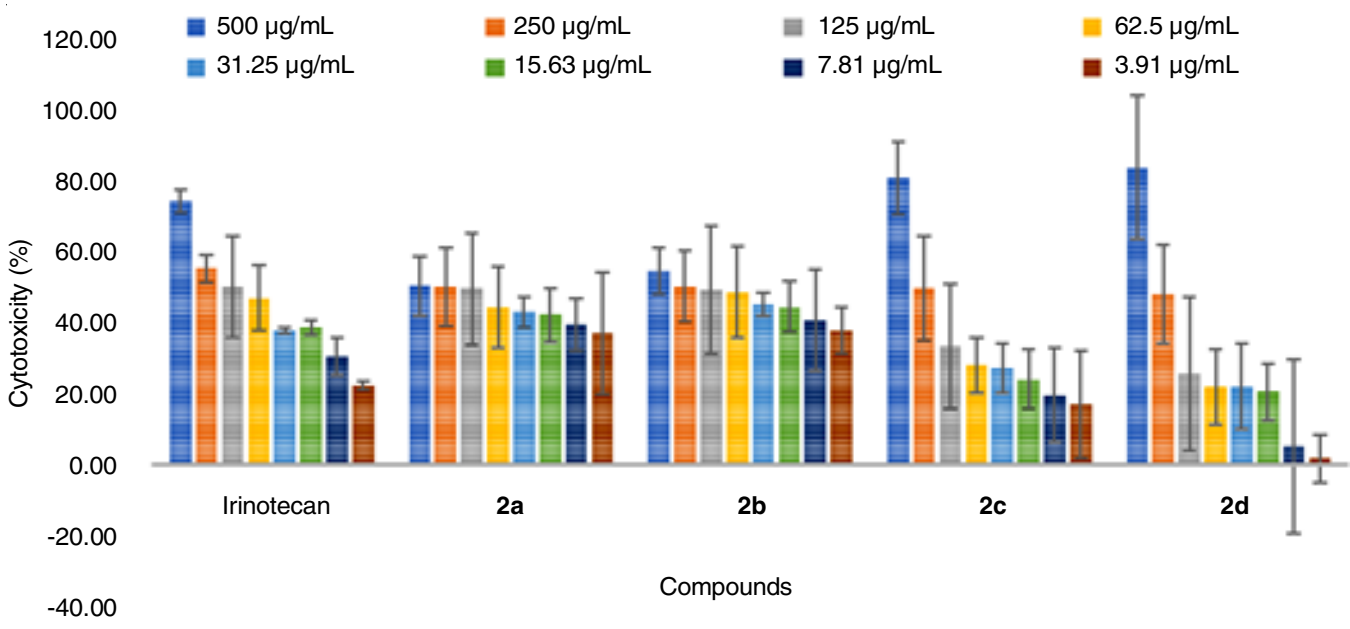


Fig. 1. Cytotoxicity of NBD **2a-d** against MCF-7 cells

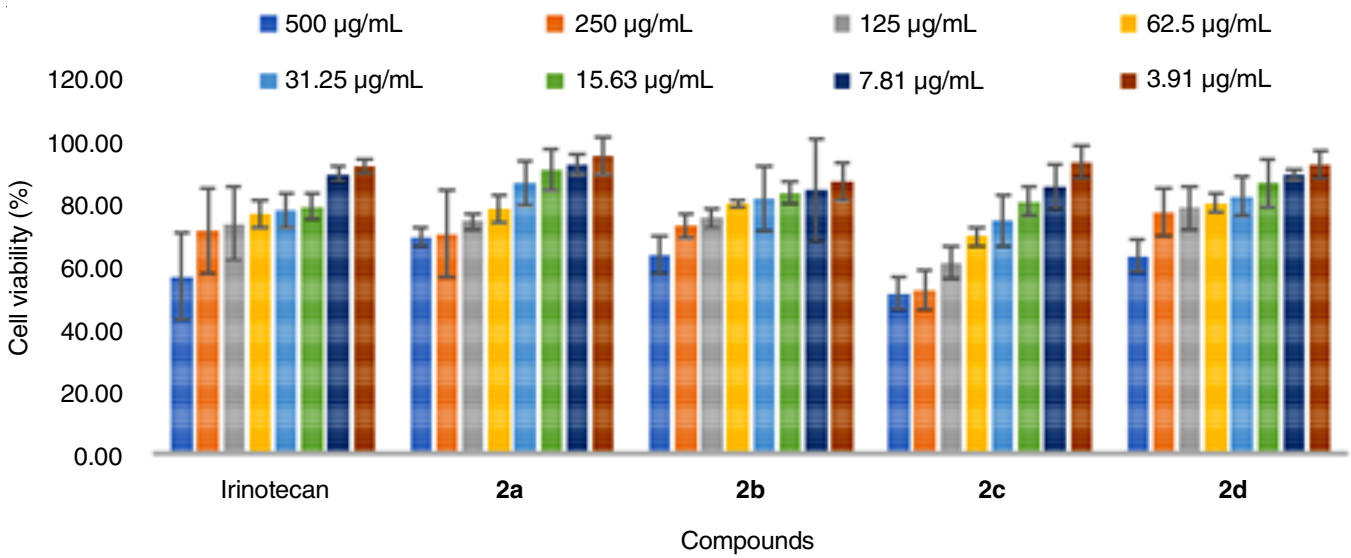


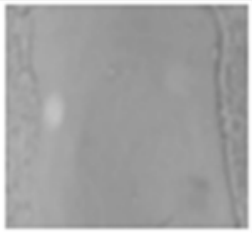
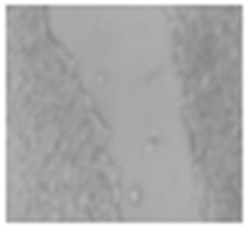
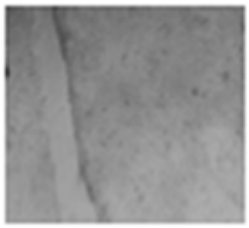
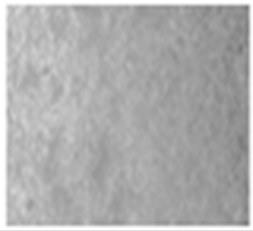
Fig. 2. Cell viability of HEK-293 cells against NBD **2a-d**

NBDs **2a-d** and images of scratched area were taken at regular intervals. The resultant data of *in vitro* wound scratch assay is given in Figs. 3 and 4. Based on the results of the *in vitro* scratch assay, it is revealed that scars on the NBD-treated plates of HEK-293 cells exhibited cell migration and closure of scratch after 24 h (Fig. 2); whereas NBD treated plates MCF-7 cells exhibited no migration of cells or no closure of scratch after 24 h. This indicates that NBDs **2a-d** synthesized in present study offers high safety against normal cells (HEK-293) on one hand and do not allow proliferation of cancer cells (MCF-7) as exhibited no cells migration. Hence, present study confirms NBDs **2a-d** as promising anticancer with high safety against normal cells.

Relating the results of cytotoxicity, cell viability & *in vitro* scratch studies and chemical structure of NBDs **2a-d**; it can be established that incorporation of electron withdrawing moieties for example -OCH_3 group at *para* position of benzylidene ring in the chemical structure of NBDs enhances their cytotoxicity and cell viability/safety. However, further preclinical and clinical investigations are needed to establish the clinical significance of NBD's.

Conclusion

Current study establishes the successful synthesis of new benzopyran derivatives (NBDs) from 2-(2-oxo-2*H*-benzopyran-4-yloxy)acetohydrazide *via* Schiff's base reaction. Chemical structures of NBDs were elucidated based on the IR, NMR and mass spectrometric data. This study also establishes the safety and anticancer potential of NBDs **4c** and **4d** against MCF-7, thereby supports their potential application in the treatment of breast cancer. Current study establishes that incorporation of electron withdrawing group (*p*-methoxy) in the NBDs offers

Hours	0	3	8	24
Culture (Negative control)				
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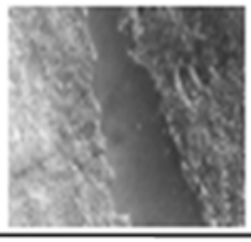
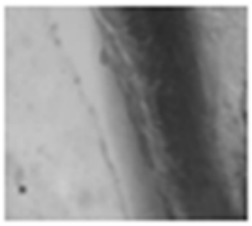
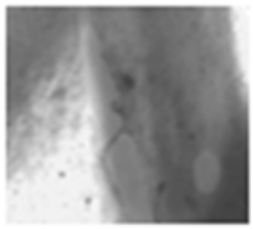
Irinotecan (Positive control)				
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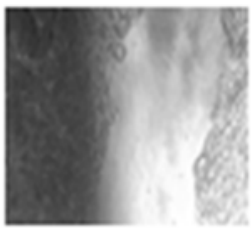
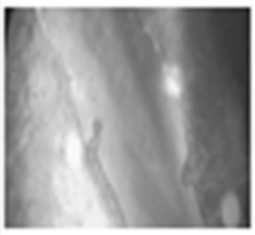
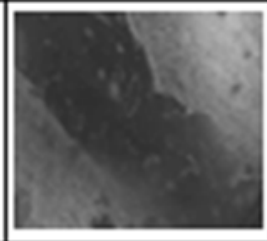
2a				
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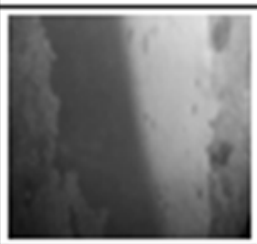
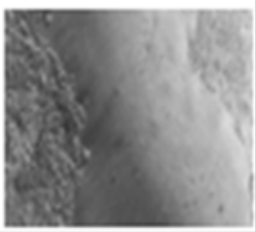
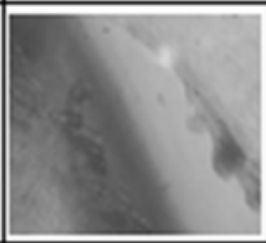
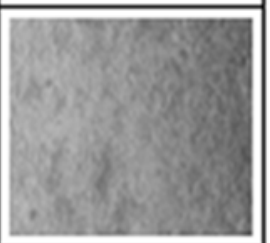
2b				
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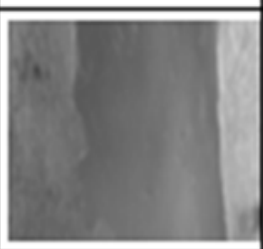
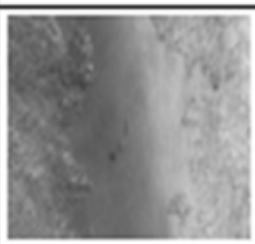
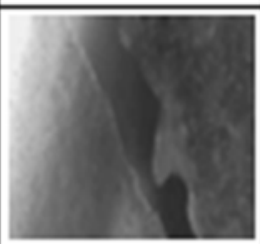
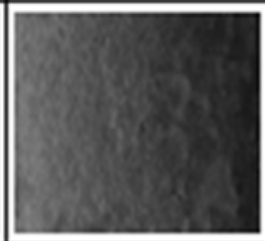
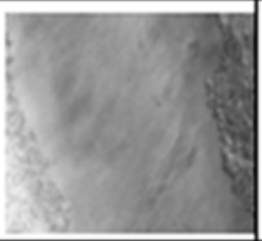
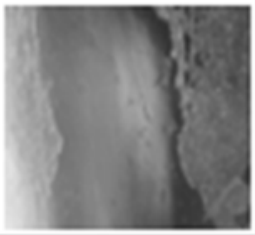
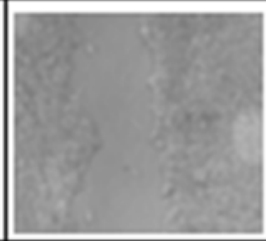
2c				
Distance (mm)	0.25	0.22	0.07	0
2d				
Distance (mm)	0.30	0.26	0.15	0

Fig. 3. Cell viability of HEK-293 cells against NBD **2a-d** (*in vitro* scratch assay)

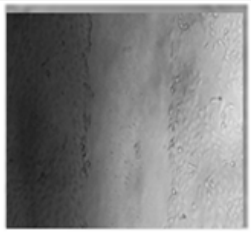
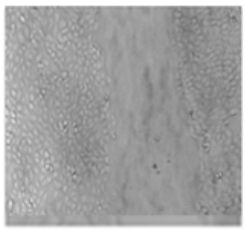
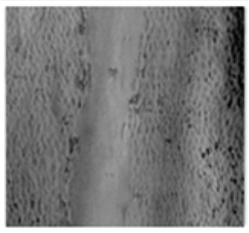
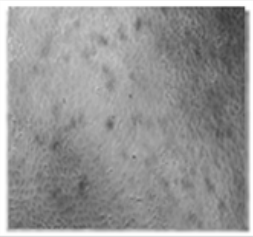
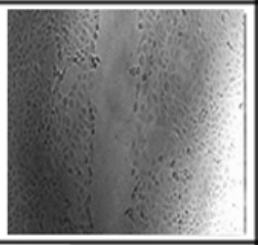
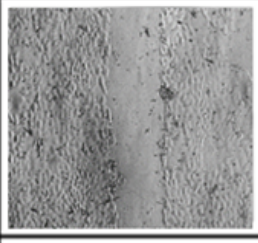
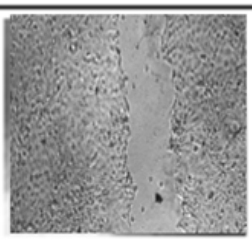
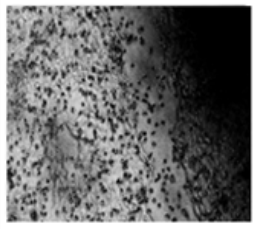
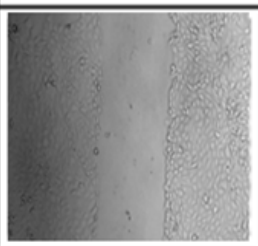
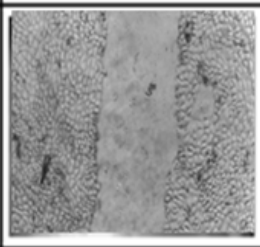
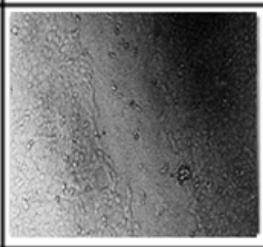
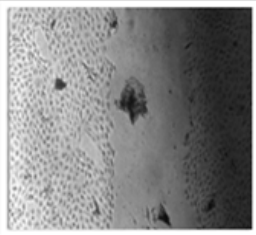
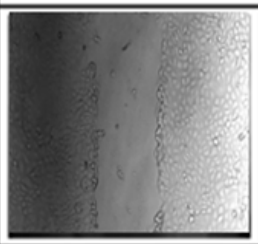
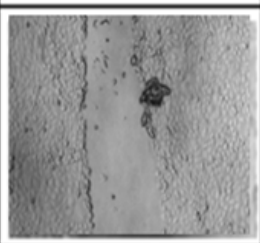
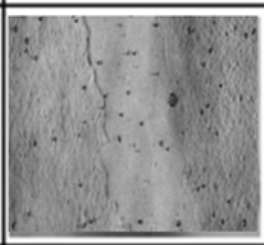
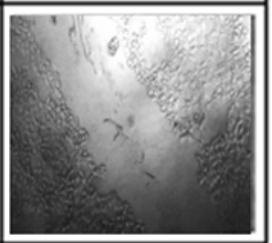
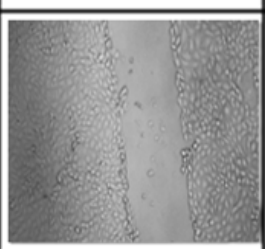
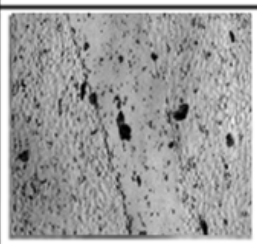
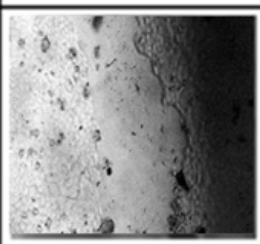
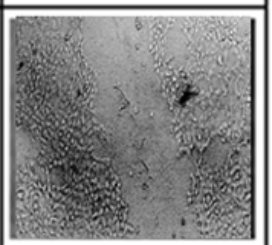
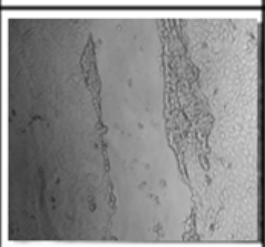
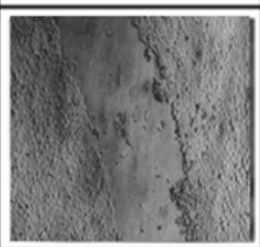
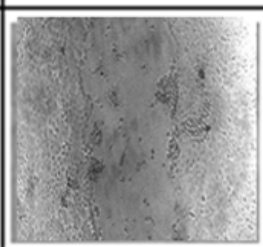
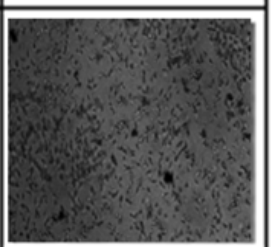
Hours	0	3	8	24
Culture (Negative control)				
Distance (mm)	0.15	0.12	0.08	0
Irinotecan (Positive control)				
Distance (mm)	0.10	0.10	0.10	0.10
2a				
Distance (mm)	0.15	0.15	0.15	0.15
2b				
Distance (mm)	0.10	0.10	0.10	0.10
2c				
Distance (mm)	0.12	0.15	0.15	0.15
2d				
Distance (mm)	0.13	0.15	0.15	N/A

Fig. 4. Cytotoxicity of NBD **2a-d** against MCF-7 cells (*in vitro* scratch assay)

maximum safety and anticancer activity. However, further clinical studies are required to further establish its clinical significance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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