DOI: 10.7860/JCDR/2023/61392.17703

Original Article

Biochemistry Section

# Anticancer Activity of the Amide-Imidazole Compound on Cancer Cell Lines: An In-Vitro Study

PRAMIT KUMAR[1], AMRESH KUMAR[2], SUDE KUMAR SINGH[3], PREETI SHARMA[4]

BY-NC-ND


## ABSTRACT

**Introduction:** The leading cause of morbidity and mortality in the world is cancer. Promising anticancer compounds include small heterocyclic chemicals. In many malignancies, cancer cells' resistance to therapy leads the recurrence and mortality after treatment. Drug resistance that develops during therapy encourages researchers to create compounds that are more useful and less harmful. Derivatives of amido-imidazole conjugates induce apoptosis in breast cancer cell line.

**Aim:** To investigate the effect of anticancer activity of amide-imidazole on various signalling and apoptotic protein in cancer cell lines.

**Materials and Methods:** This in-vitro study was designed in the Department of Biochemistry, Santosh Medical College, Ghaziabad, Uttar Pradesh, and AIIMS Patna from February 2021 to January 2022. The normal as well as cancer cell lines were cultured and grown in the medium, and the antiproliferative activity of compounds was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while various signalling proteins that regulate the proliferation and migration of cancer cell were assessed using the western blotting method. Statistical analysis of antiproliferative activity was estimated using graphical methods.

**Results:** The results showed that the amide-imidazole compound had variable antiproliferative potency in a variety of cancer cell lines. When HT-29, MDA-MB 231 and MCF-7 cancer cell lines were treated with the amide-imidazole compound at different concentrations (5, 10, 15, and 20 μM). Cell proliferation was inhibited, which is measured by MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assays. The growth in different cancer cells is HT-29 (94.16, 85.19, 77.54, and 77.86), Malondialdehyde (MDA)-MB 231 (100, 91.10, 86.82, and 79.96), and MCF-7 (74.01, 65.26, 60.42, and 36.99) at different concentrations, respectively. The western blot results of the Michigan Cancer Foundation-7 (MCF-7) cancer cell line showed a decrease in the concentration of various signalling pathways such as AKT, Extracellular signal-regulated Kinase (ERK), and Signal Transducer and Activator of Transcription-3 (STAT 3) and an increase in the cleavage of Poly (ADP-ribose) Polymerase (PARP) and Caspase-8, while also decreasing the antiapoptotic protein B-cell Leukaemia (BCL)-2.

**Conclusion:** In present study, amide-imidazole derivatives triggered the apoptosis and lowered the antiapoptotic cell protein in breast cancer cell lines (MCF-7). Hence, breast cancer, can be treated with amide-imidazole derivatives.


**Keywords:** Apoptosis, Breast cancer, Chemotherapy, Drug resistance, Signalling pathway

## INTRODUCTION

Cancer ranks as the second most common cause of mortality [1]. The GLOBOCON estimates that in 2018, there were 9.6 million global fatalities and 18.1 million new cases diagnosed [2]. Globally, 19.3 million cases were recorded by Global Cancer Observatory (GLOBOCON) throughout 2020 of which major cases were breast cancers (11.7%) and lung cancer (11.4%) [3].

The physiological functions of cells, including proliferation, growth, differentiation, metabolism, motility, survival, and death, are regulated by cellular signalling networks. Cellular signalling cascade abnormalities result in aberrant cell behaviour. It is widely acknowledged that cancer is caused by disruption of normal cellular regulatory signalling pathways [4]. Genetic modifications in oncogene and tumour suppressor genes have an impact on a number of signalling pathways [5]. Genetic alterations in oncogene and tumour suppressor genes are responsible for the activation of many signalling pathways. The estimated Glomerular Filtration Rate (eGFR), small Guanosine Tri-Phosphatase (GTPase) (RAS), serine/threonine kinase (AKT), cytoplasmic tyrosine kinase (Src & Abl), lipid kinase (PI3K), and developmental signalling pathways such as Wnt, Hedgehog, Hippo, and Notch are all commonly affected [6]. The p53, a gene necessary for cell division and other cellular stress responses including Deoxyribonucleic Acid (DNA) damage and programmed cell death, is the gene that is most frequently altered in cancer [5].

Traditional cancer therapies, including surgery, radiation, and chemotherapy, have been used to treat cancer in the past. There are several contemporary cancer therapy methods accessible today. Modern cancer medicines include targeted therapy, hormone therapy, immunotherapy, combination therapy, nanomedicine, precision medicine, stem cell therapy, and gene therapy [7-9].

Chemotherapies effectively kill cancer cells, but since they are adaptable to therapy, cancer cells frequently make chemotherapeutics ineffective. In many malignancies, cancer cells' resistance to therapy leads to recurrence and mortality after treatment. Because resistance in cancer cells is complex, it's probable that it arises from both acquired and innate resistance throughout therapy [10].

The Food and Drug Administration (FDA) database states that 59% of small molecule medications with different structural diversity and substitution patterns are nitrogen-containing heterocycles [11]. N-heterocycles are abundant in nature and play a key role in many biological compounds and pharmaceuticals, including vitamins, nucleic acids, antibiotics, serotonin, atropine, the infamous morphine, codeine (which works best when combined with acetaminophen or an Non Steroidal Anti-Inflammatory Drug (NSAID) like aspirin or ibuprofen), papaverine, caffeine, and nicotine). Due to its capacity for interaction, the N-heterocycle can target specific biological molecules for the therapy of disease [12].

A variety of imidazole derivatives have been created by several researchers, and they may be effective against various cancer cell

lines via diverse mechanisms. Baviskar AT et al., studied a substance with five imidazole derivatives and came to the conclusion that it inhibits topoisomerase IIR (Resistant strain) [13]. Zhao F et al., created six highly potent yet minimally toxic imidazole derivative compounds, and they came to the conclusion that compound C2 exhibits strong anticancer activity against the MCF-7 cancer cell line by inducing apoptosis and suppressing cell proliferation [14]. Dao P et al., created 26 imidazole derivatives, and they demonstrated that compound C3 has a higher anticancer potential by increasing the expression of FAK (focal adhesion kinase) in several cancer cell lines, including colon (HCT-116), breast (MDA-MB)-231, and prostate (PC-3) [15]. In Ehrlich Ascites Tumor (EAT) -bearing mice, a newly synthesised imidazole derivative drug inhibits the angiogenesis property of cancer, while a different research team has demonstrated antiproliferative action [16]. Alkahtani HM et al., created a novel imidazole derivative chemical and showed antiproliferative efficacy against several cancer cell lines by inhibiting Cyclin-Dependent Kinase 6 (CDK 6) and inducing apoptosis [17].

All the above researchers worked on different types of imidazole compound. In the present study, the authors used newly synthesised compound which is amide-imidazole which is different from others. Hence, the present study was conducted to identify the molecular mechanism of the anticancer (amide-imidazol) compound. The objective was to investigate the antiproliferative activity and effect on various cell signalling (apoptosis, cell cycle arrest) proteins in cancer cell lines and their side effects on normal cell lines.

## MATERIALS AND METHODS

The in-vitro study was designed in the Department of Biochemistry, Santosh Medical College, Ghaziabad, Delhi (NCR) and all the experiments was performed in Department of Biochemistry, AIIMS Patna from February 2021 to January 2022. The study was commenced after Ethical Committee Approval (IEC no. is SU/2021/092(13)).

The study was performed on different cancer cell lines as well as normal cell lines. The normal cell lines H9C2 and HEK 293T, as well as human breast cancer (MDA-MB-231), human breast adenocarcinoma (MCF-7), and human caucasian colon adenocarcinoma (HT-29), were purchased from National Centre for Cell Science (NCCS), Pune. A 10% of Foetal Bovine Serum (FBS) (Gibco) 100 units/mL penicillin, and 100 mg/mL streptomycin was added to Dulbecco's Modified Eagle's Medium (DMEM) to support the growth of all cells, in a humid environment at 37°C with 5% $CO_2$. After 70%-75% confluency, the cells were trypsinised and stored at -80°C for long-term (6 months) cryopreservation [18].

### Study Procedure

- **MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} Assay [19]:** The frozen cells were defrosted, seeded, and trypsinised normally at 70% confluence after 3-4 passes. The 96-well plates (signal plate) were used to cultivate 5000 cells per well of trypsinised (HT 29, MDA MB231, MCF 7) and normal cell lines (H9C2 and HEK 293T). To synchronise the cells into a single phase after 24 hours of incubation, serum-free medium was added. Then, after 6-8 hours, all of the cells were treated with amide-imidazole compound (5.0, 10.0, 15.0, and 20.0 µM), and fresh medium (for control cells) was added in a triplet fashion. Following a 24-hour treatment period, the cells were immediately injected with MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} solution (0.5 mg/mL) in 1X Phosphate-Buffered Saline (PBS), which was then incubated for three hours at 37°C. The absorbance was determined at 570 nm using an automated spectrophotometric plate reader (Eon, Biotech). Cell viability was expressed as percentage and compared to cells that were not treated.
- **Western Blot [20]:** On a 60-mm plate, MCF-7 cells {as determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, the amide-imidazole compound inhibited MCF-7 cell proliferation more effectively than other cell lines, such as MDA-MB-231 and HT-29} were seeded and left to attach to the plate overnight. The cells were given different doses of the amide-imidazole compound the following day for 24 hours (control, 5.0, 10.0, and 20.0 M). Cells were washed with cold, sterile PBS after being incubated for 24 hours, and then they were homogenised in Radioimmuno-Precipitation Assay (RIPA) Lysis solution with a protease inhibitor cocktail (Roche Germany). The Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)-resolved protein was put on the nitrocellulose membrane in an equal proportion (Bio-Rad), 5% non fat dry milk in 50 mM Tris-HCl (pH 7.4) with 0.05% Tween 20 for one hour (TBST).

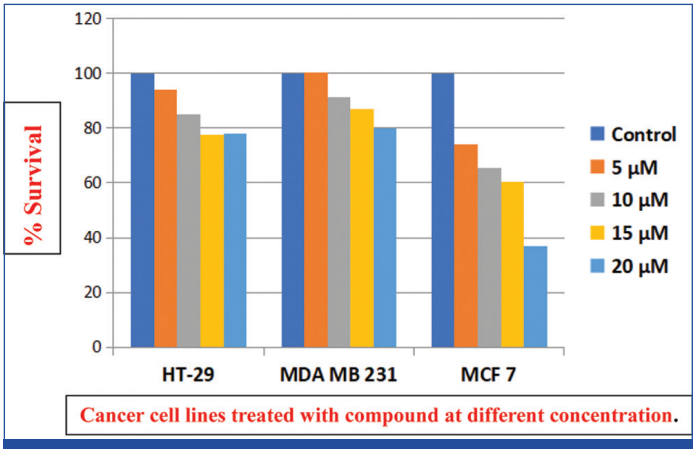
  The primary antibodies (P-STAT, STAT, P-AKT, AKT, P-ERK, Pro-PARP, Cleaved-PARP, Pro Caspase 9, Cleaved Caspase 3, BCL 2 and Actin) were incubated on the membrane for three hours at room temperature before the secondary antibody was added for an additional hour (Bio-Rad). The enhanced chemiluminescence substrate reaction ECL (Bio-Rad) was used to see protein bands, and the fluorescence hd2 (Protein Simple) gel documentation system will be used to see protein expression.

## STATISTICAL ANALYSIS

Statistical analysis of antiproliferative activity was estimated using graphical methods.

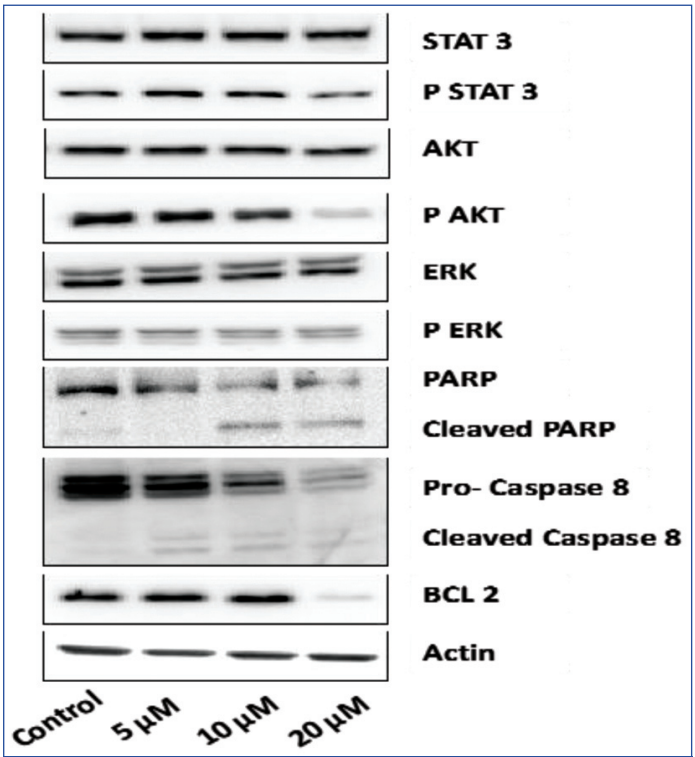
## RESULTS

**MTT cytotoxicity assay:** The results showed that the amide-imidazole compound had variable antiproliferative potency in a variety of cancer cell lines [Table/Fig-1]. When HT-29, MDA-MB-231, and MCF-7 cancer cell lines were treated with the amide-imidazole compound at different concentrations (5, 10, 15, and 20 µM) cell proliferation was inhibited, measured by MTT {3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide} assays. The growth in different cancer cells were: in HT-29 (94.16, 85.19, 77.54, and 77.86), MDA-MB-231 (100, 91.10, 86.82, and 79.96), and MCF-7 (74.01, 65.26, 60.42, and 36.99) at different concentrations, respectively. The compound amide-imidazole showed maximum activity against MCF-7 cell lines at a concentration of 20 µM. Compound amide-imidazole showed neither antiproliferative nor cytotoxic effects in the MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} experiment results against the normal cell lines Human Embryonic Kidney cell (HEK)-293 and H9C2 of rats. MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} experiments reveal that cell growth is inhibited in cancer cell lines but not in normal cell lines.




**[Table/Fig-1]:** MTT cytotoxicity assay.

Due to greater activity of amide-imidazole compound against MCF-7 cancer cell lines various signalling and apoptotic marker protein were further examined by using western blot techniques. At greater concentrations of compound amide-imidazole compound at 20 μM, antiapoptotic molecules like BCL-2 were found to be reduced, whilst apoptotic indicators like cleaved Poly (ADP-ribose) Polymerase (PARP) were found to be elevated. Procaspase levels of caspase-8 were reduced in cells treated with compound amide-imidazole compound at 20 μM despite an increase in the amount of cleaved caspase-8 conversion [Table/Fig-2].



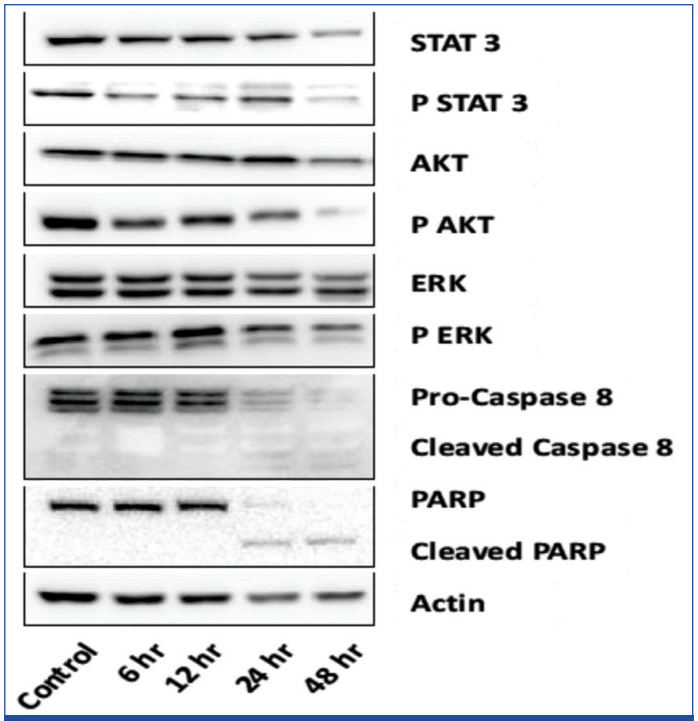

**[Table/Fig-2]:** Western Blots (signalling and apoptotic proteins) dose dependent.

The expression of various signalling molecules decreased after treatment, and this rise persisted until 48 hours, according to the kinetic analyses of different signalling molecules in MCF-7 cells treated with 20 μM of compound amide-imidazole compound at 0 hour, 6 hours, and 12 hours. amide-imidazole compound inhibits oncogenic signalling molecules like STAT-3, P-STAT3, AKT, P-AKT, ERK, and P-ERK while increasing the cleavage apoptotic markers PARP and caspase-8. Chemical amide-imidazole acts as a potent anticancer drug by inhibiting cell proliferation and controlling oncogenic signalling, both of which are necessary for cancer cell survival and development [Table/Fig-3].

## DISCUSSION

Chemotherapy against cancer is a crucial component that can be applied to treat almost any type of cancer. Traditional and tailored chemotherapy have both been used to treat cancer [21,22]. Traditional chemotherapies employ cytotoxic, non targeted chemicals. All anticancer drugs, both synthetic and biological, that target a specific molecular component of a cancer cell are referred to as targeted chemotherapy [23-25].

The present study was conducted to identify the molecular mechanism of the anticancer (amide-imidazol) compound for targeted therapy to inhibit the growth of cancer cell. In the present study, the authors use newly synthesised compound (amide-imidazole) which is different from others. Cancer cells have potency to generate chemo-resistance against most of the chemotherapeutic agents. So, researchers develop new compound to minimise/overcome the development of chemo-resistance as well as to reduce the cytotoxic effect on normal cells.




**[Table/Fig-3]:** Western Blots (signalling and apoptotic proteins) time dependent.

By changing the different groups of the core pharmacophore, it is possible to create and synthesise a variety of molecules with improved biological activity. For preclinical research, in-vitro testing of putative anticancer action is essential following design and synthesis. The multidirectional screening of prospective drugs enhances efficacy and defeats chemotherapeutic resistance built up by cancer cells. In order to examine their anticancer potential, molecular targets, and anticancer mechanism, the study screened in-vitro the probable anticancer effect of amide-imidazole compounds. Amido-imidazole compound had good anticancer activity against a variety of cancer cell lines, including phenotypically and genotypically distinct breast carcinoma cells (non metastatic cell MCF-7 and metastatic triple negative cells MDA-MB-231), colorectal carcinoma cells (HT-29). According to the preliminary antiproliferative assay results, amido-imidazole compound demonstrated a reduced cell survival rate against breast cancer cells MCF-7 when compared to other cancer cell lines.

The signalling pathways controlling cell survival, migration, and apoptosis were determined via western blot analysis. After 24 hours of treatment with varying dosages of 5 μM, 10 μM, and 20 μM, cell cycle-regulating molecules in MCF-7 cells were changed. The cell lines showed decreased activity in a number of cell survival and migratory markers at dosages of 20 μM. (P-STAT 3, P-AKT, and P-ERK). The authors further investigated the status of numerous apoptotic markers in MCF-7 cells after treatment with compound amide-imidazole compound. Antiapoptotic molecules like Bcl-2 were found to be decreased at higher concentrations of the compound amide-imidazole compound at 20 μM, whilst apoptotic indicators such as cleaved Poly (ADP-ribose) Polymerase (PARP) were found to be increased. Despite an increase in the amount of cleaved caspase-8 conversion in cells treated with compound amide-imidazole compound at 20 μM, procaspase levels of caspase-8 were decreased.

According to the kinetic studies of various signalling molecules in MCF-7 cells treated with 20 μM of compound imidazole at 0 hour, 6 hours, and 12 hours, the expression of various signalling molecules decreased after treatment, and this rise sustained until 48 hours [14-16]. The amide-imidazole compound increases the cleavage of apoptotic indicators PARP and caspase-8 while inhibiting oncogenic signalling components STAT-3, P-STAT3, AKT, P-AKT, ERK, and P-ERK. By inhibiting the creation of new cells and controlling

oncogenic signalling, two processes that are crucial for the survival and proliferation of cancer cells, the compound amide-imidazole acts as a potent anticancer therapy.

### Limitation(s)

This is an in-vitro study performed on different cancer cell lines. Hence, it could not study toxicity of the compound.

## CONCLUSION(S)

The current study conclude that compound amide-imidazole reduced the survival of breast cancer cells (MCF-7) by triggering apoptosis and cell cycle arrest, lowering the antiapoptotic protein BCL-2, and enhancing the cleavage of PARP and Caspase-8. In order to cure breast cancer, amide-imidazole derivatives can function as therapeutic molecules. The antiproliferative assays showed maximum growth inhibition against breast cancer cell lines (MCF-7) while no inhibition of growth on normal cell linens. The authors suggest further study using other cancer cell lines to understand the anticancer activity of compound and toxicity against normal cell lines.

## REFERENCES


[1] Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. J Thorac Dis. 2017;9(3):448-51. Doi: 10.21037/jtd.2017.02.75.
[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394-424. Doi: 10.3322/caac.21492.
[3] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209-49.
[4] Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nature Medicine. 2004;10(8):789-99.
[5] Sever R, Brugge JS. Signal transduction in cancer. Cold Spring Harbor Perspectives in Medicine. 2015;5(4):a006098.
[6] Sandri M, Hochhauser D, Ayton P, Camplejohn R, Whitehouse R, Turley H, et al. Differential expression of the topoisomerase IIα and β genes in human breast cancers. British Journal of Cancer. 1996;73(12):1518-24.
[7] Arruebo M, Vilaboa N, Gutierrez BS, Lambea J, Tres A, Valladares M, et al. Assessment of the Evolution of Cancer Treatment Therapies, Cancers. 2011;3:3279-330. Doi: 10.3390/cancers3033279.
[8] Al-Mahmood S, Sapiezynski J, Garbuzenko OB, Minko T. Metastatic and triple-negative breast cancer: Challenges and treatment options, Drug Delivery and Translational Research. 2018;8:1483-507. https://doi.org/10.1007/s13346-018-0551-3.
[9] Belete TM. The current status of gene therapy for the treatment of cancer. Biologics. 2021;15:67-77. Doi: 10.2147/BTT.S302095.
[10] Wang J, Xu B. Targeted therapeutic options and future perspectives for HER2-positive breast cancer. Signal Transduct Target Ther. 2019;4:34. https://doi.org/10.1038/s41392-019-0069-2.
[11] Vitaku E, Smith DT, Njardarson JT. Analysis of the structural diversity, substitution patterns, and frequency of nitrogen heterocycles among U.S. FDA approved Pharmaceuticals. J Med Chem. 2014;57(24):10257-74.
[12] Kerru N, Gummidi L, Maddila S, Gangu KK, Jonnalagadda SB. A review on recent advances in nitrogen-containing molecules and their biological applications. Molecules. 2020;25:1909. Doi: 10.3390/molecules25081909.
[13] Baviskar AT, Madaan C, Preet R, Mohapatra P, Jain V, Agarwal A, et al. N-fused imidazoles as novel anticancer agents that inhibit catalytic activity of topoisomerase iiα and induce apoptosis in G1/S phase. J Med Chem. 2011;54(14):5013-30. Doi: 10.1021/jm200235u.
[14] Zhao F, Lu W, Su F, Xu L, Dong Jiang D, Sun X, et al. Synthesis and potential antineoplastic activity of dehydroabietylamine imidazole derivatives. Med Chem Comm. 2018;9(12):2091-99. Doi: 10.1039/C8MD00487K.
[15] Dao P, Smith N, Tomkiewicz-Raulet C, Yen-Pon E, Camacho-Artacho M, Lietha D, et al. Design, synthesis, and evaluation of novel imidazo[1,2-a][1,3,5]triazines and their derivatives as focal adhesion kinase inhibitors with antitumor activity. J Med Chem. 2015;58(1):237-51. Doi: 10.1021/jm500784e.
[16] Roopashree R, Mohan CD, Swaroop TR, Jagadish S, Rangappa KS. Synthesis, characterization and in-vivo biological evaluation of novel benzimidazoles as potential anticancer agents. Asian J Pharm Clin Res. 2014;7(5):309-13.
[17] Alkahtani HM, Abbas AY, Wang S. Synthesis and biological evaluation of benzo[d] imidazole derivatives as potential anti-cancer agents. Bioorg Med Chem Lett. 2012;22(3):1317-21. Doi: 10.1016/j. bmcl.2011.12.088.
[18] Yadav UP, Ansari AJ, Arora S, Joshi G, Singh T, Kaur H, et al. Design, synthesis and anticancer activity of 2-arylimidazo [1, 2-a] pyridinyl-3-amines. Bioorganic Chemistry. 2022;118:105464. Doi: 10.1016/j.bioorg.2021.105464. Epub 2021 Nov 1.
[19] Weinberg R. The biology of cancer. New York, NY: Garland Science, Taylor & Francis Group. 2007.
[20] Kumar S, Prasad S, Sitasawad SL. Multiple antioxidants improve cardiac complications and inhibit cardiac cell death in streptozotocin-induced diabetic rats. PLoS One. 2013;8(7):e67009. https://doi.org/10.1371/journal.pone.0067009.
[21] Baldo BA, Pham NH. Adverse reactions to targeted and non targeted chemotherapeutic drugs with emphasis on hypersensitivity responses and the invasive metastatic switch. Cancer and Metastasis Reviews. 2013;32(3):723-61.
[22] Senapati S, Mahanta AK, Kumar S, Maiti P. Controlled drug delivery vehicles for cancer treatment and their performance. Signal Transduction and Targeted Therapy. 2018;3(1):01-19.
[23] Falzone L, Salomone S, Libra M. Evolution of cancer pharmacological treatments at the turn of the third millennium. Frontiers in Pharmacology, 2018;9:1300. Doi: 10.3389/fphar.2018.01300. eCollection 2018.
[24] Hartmann JT, Haap M, Kopp HG, Lipp HP. Tyrosine kinase inhibitors-a review on pharmacology, metabolism and side effects. Current Drug Metabolism. 2009;10(5):470-81.
[25] Tsimberidou AM. Targeted therapy in cancer. Cancer Chemotherapy and Pharmacology. 2015;76(6):1113-32.



**PARTICULARS OF CONTRIBUTORS:**
1. PhD Student, Department of Biochemistry, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, India.
2. Assistant Professor, Department of Biochemistry, Darbhanga Medical College, Darbhanga, Bihar, India.
3. Professor and Head, Department of Biochemistry, Darbhanga Medical College, Darbhanga, Bihar, India.
4. Professor, Department of Biochemistry, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, India.

**NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:**
Preeti Sharma,
Professor, Department of Biochemistry, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, India.
E-mail: pramit2510@gmail.com



**PLAGIARISM CHECKING METHODS:** [Jain H et al.]
- Plagiarism X-checker: Nov 30, 2022
- Manual Googling: Jan 06, 2023
- iThenticate Software: Feb 22, 2023 (5%)

**ETYMOLOGY:** Author Origin

**AUTHOR DECLARATION:**
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Nov 28, 2022**
Date of Peer Review: **Jan 12, 2023**
Date of Acceptance: **Feb 24, 2023**
Date of Publishing: **Apr 01, 2023**