NATURALISTA CAMPANO
ISSN: 1827-7160
Volume 28 Issue 1, 2024

Tailoring Methotrexate for Precision Cancer Therapy: Synthesis, Characterization, and Anti-Cancer Profiling of Novel Analogues

Rahul Subhash Buchade[1], Krishna Chandra Panda[2], Yasmin Khatoon[3], Touseef Begum[4*], Vineet Joshi[5], Yogesh Baban Zambare[6], R senthilraj[7], Jisha Joseph[8]

[1]Department of Pharmaceutical Chemistry, SCES's Indira College of Pharmacy "Niramay", S.No.89/2A, New Pune Mumbai Highway, Tathwade., Pune, Maharashtra, India 411057.
[2]Roland Institute of Pharmaceutical Sciences, Berhampur, Odisha, Pin-760010
[3]Institute of Pharmacy, Shri Ramswaroop Memorial University, Barabanki, Uttar Pradesh 225003.
[4*]Department of Pharmaceutical Sciences, Ibn Sina National College for Medical Studies, P. O. Box 31906, Jeddah Kingdom of Saudi Arabia 21418.
[5]College of Pharmacy, Shivalik Campus, Dehradun, Uttarakhand, 248197.
[6]Department of Pharmaceutical Chemistry, Krishanarao Bhegade Institute of Pharmaceutical Education and Research Talegaon Pune 410507.
[7]Faculty of pharmacy, Dr MGR Educational and Research institute Velappanchavadi Chennai 600077.
[8]School of Pharmacy, Vishwakarma University, Kondhwa Budruk, Pune 411048.

Corresponding Author: [4*]Touseef Begum
[4*]Department of Pharmaceutical Sciences, Ibn Sina National College for Medical Studies, P. O. Box 31906, Jeddah Kingdom of Saudi Arabia 21418.

Abstract: Background: One of the most widely used chemotherapeutics is methotrexate, a structural counterpart of folic acid, which is particularly useful in haematological malignancies, different solid tumours, and inflammatory illnesses.
Objective: Two methotrexate analogues have been synthesized in the current study and are being characterised and biologically assessed.
Methods: To obtain the desired methotrexate analogues, a thoroughly examined and studied scheme was employed. The compounds were synthesised by a two-step process followed by a substitution reaction at the pteridine nucleus. The structures of analogues of Methotrexate were elucidated with spectral techniques viz. infrared, nuclear magnetic resonance and mass spectrometry. The antitumor efficacy of the produced compounds was assessed in HCC 1937 cells.
Results: Using spectral methods, two successful analogues of methotrexate were synthesised and described. At various concentrations, the anticancer activity was assessed and contrasted with controls.
Conclusion: The results indicated that both the analogues of Methotrexate demonstrated significant cell growth inhibition activity.

Keyword: Methotrexate, Analogues of Methotrexate, Cancer, Anticancer activity, HCC 1937 cells

1. Introduction

Methotrexate (MTX) is a folate antagonist that was initially created to treat cancers (1). It has since been utilised as an immunosuppressive and/or anti-inflammatory medication to treat non-neoplastic disorders. MTX is being used to treat chronic inflammatory illnesses such as psoriasis, primary biliary cirrhosis, and intrinsic asthma, as well as refractory rheumatoid arthritis (RA) (2, 3). When taken alone or in combination with cyclosporin A and/or prednisone, MTX is also beneficial in preventing acute graft-vs-host disease (GVHD) (4-6). Additionally, MTX has been utilised as a supplement to treat moderate cardiac allograft rejection that is chronic (7). The majority of pharmacological research has focused on the use of MTX in cancer treatment, where dosages can be increased to 30 g/m2 by giving the antidote leucovorin afterwards (folinic acid, citrovorum factor) (4-6).

However, the recommended weekly dosage of MTX for autoimmune disorders and allografts is typically 7–15 mg, administered orally or by intramuscular injection. The dosages can be given as a single dose, divided into two or three, and spaced out by 12 hours. Since MTX is primarily thought of as an anti-inflammatory medication, it was unclear if the antiproliferative and cytotoxic properties of the drug that were shown in cancer cells also applied to low-dose therapies. Nonetheless, we have recently documented that even after brief exposure to MTX and subsequent activation in drug-free media, MTX specifically causes apoptosis in activated lymphocytes but not in resting lymphocytes. These findings provide the first proof of an immunosuppressive effect at modest doses given intermittently while using MTX (8-10). Currently, more is known about methotrexate's (MTX) metabolism, toxicity, pharmacokinetics, and clinical profile than any other medication used in cancer or rheumatology. Antifolate medications, primarily MTX, have been used to treat millions of patients with autoimmune and malignant disorders in the 56 years after Farber et al. initially reported clinical remissions in children with acute leukaemia following therapy with the folate antagonist aminopterin (1). As of right now, MTX is by far the most widely used disease-modifying antirheumatic medication, with at least 500,000 RA patients given to it globally (DMARD) (10, 11). In fact, more RA patients receive prescriptions for MTX than for all the biologic medications now in use put together. When taken with other DMARDs, it is the most often researched and given medication when a definite additive therapeutic benefit is shown. Despite our combined 20 years of expertise and success with MTX, there is a shockingly low degree of general knowledge on the numerous problems and complexity related to its use. Without solid scientific backing, myths are frequently spread regarding maximum weekly dosages, usage in the elderly, blood test monitoring, when to "give up" and add additional medications to an MTX regimen, and other topics. It's frequently thought that knowledge of the medication's numerous cellular impacts is unimportant when it comes to treating rheumatoid arthritis. These actions and ideologies make sense because, despite the overwhelming and frequently complex body of research supporting the use of agents like MTX, extremely busy clinicians with ten patients do not have the time or resources to read every nuanced publication outlining the optimal way to conceptualize clinical decisions regarding their use (11). This contribution does not aim to synthesize papers describing the clinical effects of MTX; rather, it reviews key and important principles about MTX metabolism that may be useful to the treatment of rheumatic illness. Throughout the conversation, it will become clear that the majority of our knowledge on MTX metabolism comes from cancer literature (11, 12). After familiarising themselves with the principles, clinicians ought to be more capable of prescribing the medication in a more logical and efficient way. Furthermore, new knowledge about the function of naturally occurring genetic variation in the cellular pathways involved in the metabolism of MTX shows promise for forecasting the medication's toxicity as well as its effectiveness. As of this writing, MTX is still a mainstay for the treatment of RA and other rheumatic diseases, both now and in the future, despite actual and perceived gaps in our knowledge of its effects (12-16).

For the treatment of rheumatoid arthritis and other rheumatic conditions, methotrexate is still a vital component. The antiproliferative effects of methotrexate against malignant diseases are known to be facilitated by folate antagonism. However, concurrent administration of folic or folinic acid does not reduce the anti-inflammatory potential of this agent, indicating the possibility of other mechanisms of action at work. Though methotrexate's anti-inflammatory effects cannot be fully explained by a single mechanism, both in vitro and in vivo studies have shown that cells produce adenosine (13, 17-19). Through the suppression of polyamines, methotrexate may potentially have anti-inflammatory effects. Understanding how methotrexate works against inflammatory disorders while also producing some of its well-known side effects has been made possible by the biological effects on inflammation linked to adenosine production. These actions add to the intricate and varied processes that render methotrexate effective in the management of inflammatory conditions (20-23).

One of the most often used chemotherapy drugs is methotrexate, a structural counterpart of folic acid that is particularly effective in treating inflammatory diseases, solid tumours, and haematological malignancies. Methotrexate suppresses the production of purine and pyrimidine precursors by interfering with the metabolism of folate, mostly through the inhibition of dihydrofolate reductase. Methotrexate appears to have cytostatic, cytotoxic, and differentiating effects due to the depletion of nucleic acid precursors (23-26). Another folate-dependent mechanism that methotrexate also affects is methylation of biomolecules. Moreover, methotrexate can alter cellular functions and metabolic pathways other from folate metabolism. It was anticipated and subsequently shown that methotrexate may block histone deacetylases due to its structural similarities with several of the functional groups of histone deacetylase inhibitors. According to recently released research, methotrexate may also have an impact on the antioxidant and glyoxalase systems. Even though methotrexate has been used for more than 60 years as an antagonist of folate metabolism in anticancer therapy, research into the drug's other molecular targets in cellular metabolism is currently ongoing (23, 27-31).

Considering all the facts and data, this present study aimed to scheme and synthesize two methotrexate analogues followed by characterization and evaluation of the compounds in vitro mechanistic cell line model for anticancer activity.

2. Materials and Methods

Chemical, Reagents, and Instruments

Pharmaceutical businesses provided gift samples of Methotrexate; the drug sample used in all of the experiments. All additional chemicals and reagents used in the synthesis and assessment were obtained only from authorized suppliers and were of both synthetic and analytical quality.

Physico-chemical properties and characterizations

Brown spots were noted in a firmly closed chamber after all chemical reactions were seen using TLC on silica gel G plates using benzene and ethyl acetate as the developing solvent system in an 8:2 ratio. The device used to measure the melting points of synthetic compounds is an open capillary approach melting point equipment. Using an FTIR-RXI spectrophotometer, the FTIR spectra of the chemicals/drugs in the KBr pellet was recorded (PERKIN ELMER). 1H-NMR using TMS as an internal standard (Chemical shift in δ ppm), spectral data of synthesised compounds were recorded in deuterium-substituted chloroform using a Bruker NMR spectrophotometer.

Synthetic Procedure for Methotrexate analogues

Pathway for synthesis of the Methotrexate analogues

For the synthesis of the analogues of Methotrexate, anthranilic acid and benzoyl chloride were used as starting material to synthesize the 2-phenyl-4-oxo-3,1 benzoxaine and then another step involving methotrexate will lead to the synthesis of the analogues. Similarly, the other analogue was also synthesized accordingly. The pathway and schemes for the synthesis of the analogues has been designed and presented as follows in figure 1 and figure 2 (32).

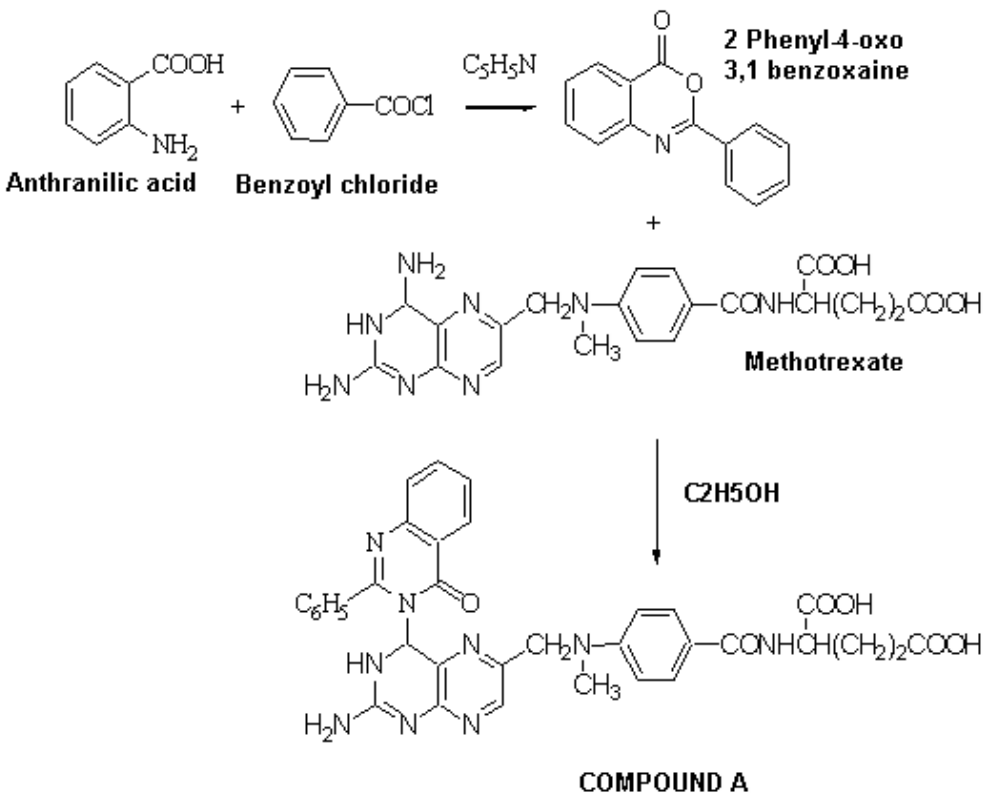


Figure 1. Pathway for synthesis of compound A (MA-1)

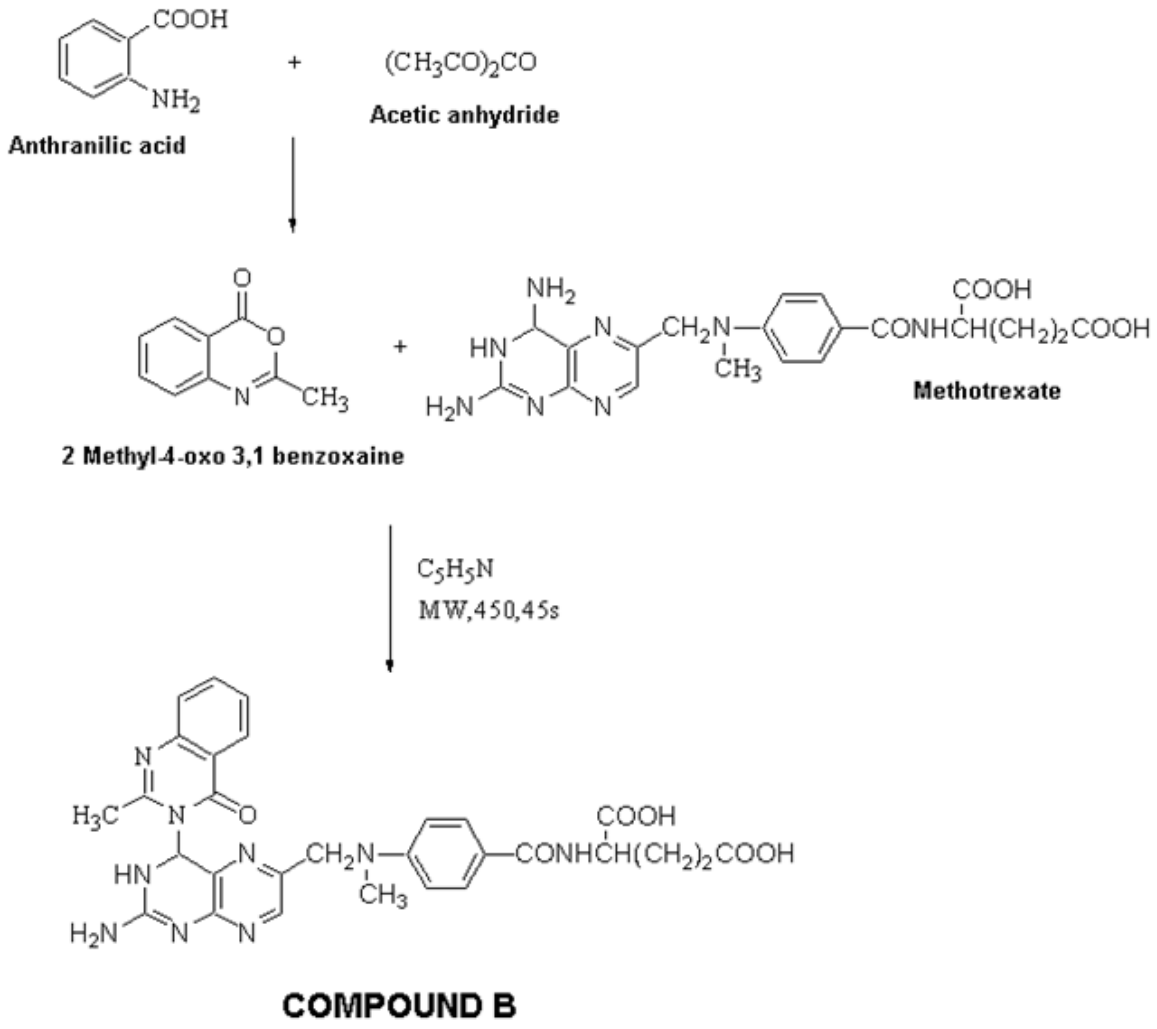


Figure 2. Pathway for synthesis of compound B (MA-2)

Synthesis of compound A
For the synthesis of compound, A code-named as MA-1 the following two step procedure was employed (32).

Synthesis of 2-phenyl-4-oxo-3,1 benzoxaine (Step 1)
Anthranilic acid (0.01 mol) was dissolved in dry pyridine (30 ml) by stirring slowly at room temperature. The solution was cooled to 0 ^{0}C and a solution of an aromatic acid chloride (0.02 mol) in dry pyridine (30 ml) was added to this solution slowly with constant stirring. When the addition was complete, the reaction mixture was further stirred for half an hour at room temperature and set aside for 1h. The pasty mass obtained was diluted with water (~50ml) and treated with aqueous sodium bicarbonate solution to remove the unreacted acid. When the effervescence ceased, it was filtered off and washed with water to remove the inorganic materials and the adhered pyridine. The crude benzoxaine thus obtained was dried and recrystallized from 95% ethanol.

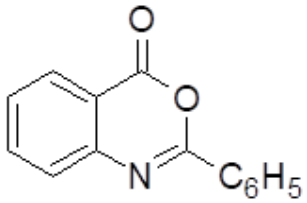


Figure 3. Structure of 2-phenyl-4-oxo-3,1 benzoxaine.

Synthesis of (S)-2-(4-(((2-amino,4-N-(2-phenyl-4-oxo-3,1 benzoxaine) diaminopteridin-6-yl) methyl) methylamino) benzamido) pentanedioic acid (Step 2)
Methotrexate (0.2 mole) and Phenyl-benzoaxone (0.2 mole) were separately dissolved in ethanol and the pH was adjusted to 4 and refluxed for 10 hrs at 50 ^{0}C. The product, which separated, was filtered off after cooling. The precipitate so obtained was filtered and washed with water and allowed to dry. It was finally purified by recrystallization from 95% ethanol. The melting points are reported.

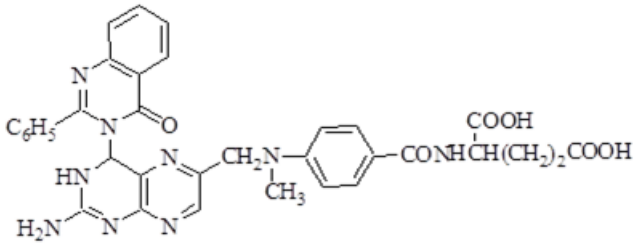


Figure 4. Structure of (S)-2-(4-(((2-amino,4-N-(2-phenyl-4-oxo-3,1 benzoxaine) diaminopteridin-6-yl) methyl) methylamino) benzamido) pentanedioic acid

Synthesis of compound B:
For the synthesis of compound B, code-named as MA-2 the following two step procedure was employed (32).

Synthesis of 2-Methyl-4-oxo-3,1 benzoxaine (Step 1)
Anthranilic acid (0.01 mol) and acetic anhydride (0.02 mol) was taken in 250 ml round bottom flask and reflux for 2h. An excess of acetic anhydride was distilled off under reduced pressure and the residue was dissolved in pet ether and kept aside overnight. The product thus separated was filtered by vacuum filtration and recrystalized from pet ether.

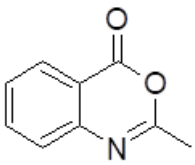


Figure 5. Structure of Methyl-4-oxo-3,1 benzoxaine

Synthesis of (S)-2-(4-(((2-amino,4-N-(2-methyl-4-oxo-3,1 benzoxaine) diaminopteridin-6-yl)methyl) methylamino) benzamido) pentanedioic acid (Step 2)
Methotrexate (0.2 mole) and Methyl-benzoaxone (0.2 mole) were separately dissolved in pyridine to get a clear solution. Both were mixed slowly with continuous stirring in ice-cold condition. The mixture is kept in micro-oven under 450 W for 45 sec. The product, which separated, was filtered off after cooling. The precipitate so obtained was filtered and washed with distilled water and allowed to dry. It was finally purified by recrystallization from a suitable solvent. The melting points are reported.

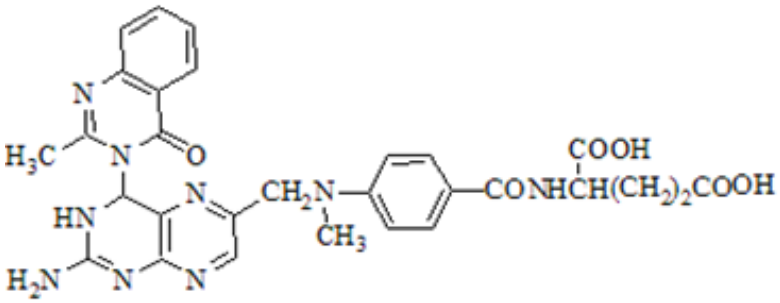


Figure 6. Structure of (S)-2-(4-(((2-amino,4-N-(2-methyl-4-oxo-3,1 benzoxaine) diaminopteridin-6-yl) methyl) methylamino) benzamido) pentanedioic acid

Evaluation of Pharmacological Activities
Anti-cancer activity against HCC1937 cell line
The suphorhodamine (SRB) test, as previously reported (33, 34), was used to evaluate the anti-cancer properties of the two drugs. The method is based on the SRB dye's stoichiometric binding to proteins, and it extrapolates the bound dye's absorbance value to show cell proliferation (35, 36). In RPMI 1640 media supplemented with 10% (V/V) foetal bovine serum (FBS) and 2 mM glutamine, HCC1937 cells from ATCC (CRL-2336) were seeded on triplicate in a 96-well plate at a cell density of 1 x 10^4 cells per millilitre. The cells were then left to adhere overnight at 37 ^{0}C in 9% CO_2. The compounds were added to cells at varying doses (0.1 to 100 µg/ml) and diluted in 1% (v/v) dimethyl sulfoxide (DMSO) before being cultured for 72 hours. As a negative control, wells with only DMSO and no chemicals in the cells were used. The cells were incubated at 4^{o}C for an hour after being fixed with 50% (W/V) trichloroacetic acid after 72 hours. Four times, the plates were gently submerged in water to wash them. and let to dry at room temperature on paper towels. 50 µl of 0.04 percent (W/V) suphorhodamine (SRB) was used to dye the plates. They were then incubated for an hour at room temperature, washed four times with 1 percent (V/V) acetic acid, and allowed to air dry. To dissolve the bound dye, 50 µl of 10 mM Trisbase solution (pH 10.5) was added to each well, and the mixture was shaken on an orbital shaker (Star lab smart equipment) for 10 minutes at room temperature. Using GraphPad Prism, non-linear regression was used to calculate the half-maximal inhibitory concentration (IC_{50}) after absorbance was measured at 511 nm in a microplate reader (BioTek®). % inhibition of cell growth was computed using the following formula:

% Cell growth = Absorbance of sample /absorbance negative control x 100%
% Inhibition = 100 - % cell growth

Statistical analysis
Mean ± SD (n = 6) was used to express the results. Using the GraphPad Prism software program, statistical analyses were carried out using one-way analysis of variance (ANOVA) and *post hoc* "Dunnett's Multiple Comparison Test." "P" values were regarded as statistically significant if they were less than 0. 05.

3. Results

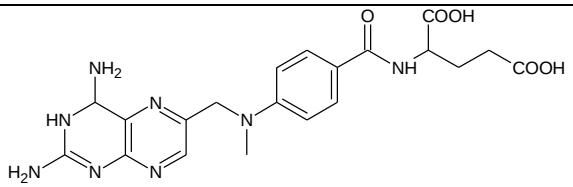
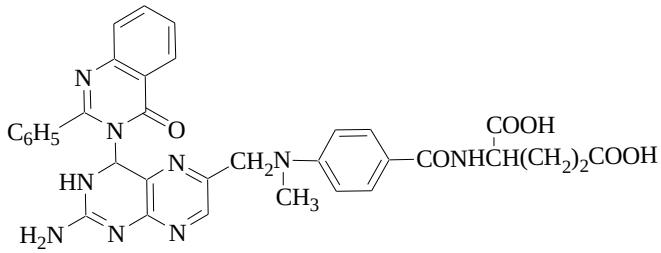
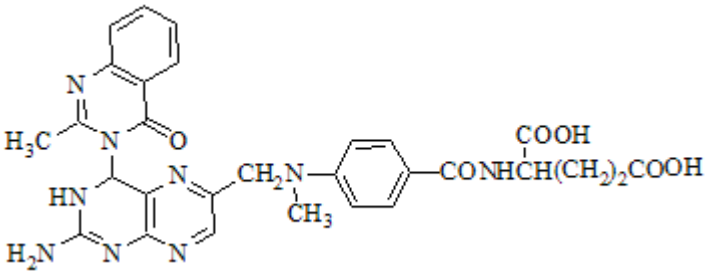
Synthesis and characterization of Methotrexate analogues
The Methotrexate analogues were synthesized as per the scheme and pathways presented earlier in this manuscript. Physical and Analytical Data including Melting point, percentage yield and Rf values of the synthesized Methotrexate analogues were presented in table 1. Benzene and Ethyl acetate in the ratio of 8:2 was used a mobile phase. The findings provide important details on the molecular makeup, molecular weight, melting temperatures, yields, and Rf values of methotrexate, compound A (MA-1), and compound B (MA-2). Methotrexate is a well-known chemotherapeutic medicine; its chemical formula is $C_{20}H_{24}N_8O_5$ and its molecular weight is 457.51. It does not have a defined melting point. MA-1 has a molecular weight of 658,78, a melting point range of 89-91°C, a yield of 71%, and an R_f value of 0.71. Its chemical formula is $C_{34}H_{31}N_9O_6$. These features point to its possible size and complexity, and the high yield suggests a successful synthesis. Analogously, MA-2, with a molecular weight of 596.73 and a formula of $C_{29}H_{29}N_9O_6$, has a greater melting point range of 149-164°C, a marginally reduced yield of 63 percent, and an R_f value of 0.52. A higher melting point suggests a more complex structure, and a lower yield might hint to problems or adverse reactions during synthesis. In conclusion, the information supplied enables a rapid comparison of these substances, MA-1 and MA-2, as near counterparts of methotrexate, ultimately signifying a successful synthesis of the analogues with advantageous characteristics. The results revealed that the produced compounds' molecular weight and melting point differed from that of methotrexate, suggesting that the molecule had not yet formed. Through TLC analysis, the compounds' purity was verified. The compounds that underwent infrared and mass spectroscopy confirmation are displayed in Table 2 and Figures 7 & 8.
Different functional groups with distinctive frequencies are shown in the FTIR spectrum characterization. At 3416 cm^{-1}, the molecule exhibits N-H stretching vibrations, which are suggestive of amine groups. Furthermore, the existence of carbonyl functional groups within the structure is suggested by the presence of C=O (carbonyl) vibrations at 1504 cm^{-1}. Understanding the compound's reactivity and possible interactions with other molecules depends on this information. MA-1 exhibits a structure that is comparable yet complicated, with many functional groups. Amine groups are indicated by the N-H stretching vibration at 3413.39 cm^{-1}, while carbonyl functionalities are indicated by the C=O vibrations at 1613.16 cm^{-1} and 1541.81 cm^{-1}. Additionally, at 902.523 cm^{-1}, the molecule exhibits aromatic C-H vibrations, suggesting the presence of an aromatic ring. Characterizing Compound A's chemical characteristics and possible uses is made easier with the help of this knowledge. MA-2 displays a distinct

range of vibrations, which include 1509.99 cm^{-1}, C=O vibrations at 1637.27 cm^{-1}, and N-H stretching at 3446.17 cm^{-1}. In comparison to methotrexate and compound A, the existence of C=O carboxylic and C=O aromatic conjugate vibrations implies a more complicated structure. The compound's structural variety is further enhanced by the C-H aromatic-1,4 disubstitution vibration at 1100.19 cm^{-1}. Mass Spectral analysis was done in APCI mode with Positive Polarity using a solvent system of acetonitrile and water and the analysis of fragmentation patterns suggested that M+ ion at m/z 658.78 (MA-1) characteristically with M^+ peak. The parent peak has been recorded at 531.15. Analysis of the spectra for MA-2 revealed that M^+ ion at m/z 596.73 (B) characteristically with M^+ peak. The parent peak has been recorded at 576.66.

Table 1. Physical and Analytical Data including Melting point, percentage yield and R_f values of the synthesized Methotrexate analogues.

Cmpd.	**Molecular Formula**	**Molecular Weight**	**Melting Point [^{0}C]**	**Yield [%]**	**R_f Value**
Methotrexate	$C_{20}H_{24}N_8O_5$	457.51	121-131		
A	$C_{34}H_{31}N_9O_6$	658.78	89-91	71	0.71
B	$C_{29}H_{29}N_9O_6$	596.73	149-164	63	0.52

Table 2. Spectral interpretation by FTIR and findings of Methotrexate and synthesized analogues

Compound	**Structure**	**Freq.**	**Functional Group**
Methotrexate		3415	N-H
		1502	C=O Carboxylic
		1382	C=O Aromatic Conjugate
		617	C-H Aromatic
Compound A		3412.28	N-H
		1612.09	C=O
		1543.97	N-H 1°
		1449.29	C=O Carboxylic
		905.19	C-H Aromatic
Compound B		3445.29	N-H
		1635.31	C=O
		1502.98	C=O Carboxylic
		1386.28	C=O Aromatic
		1109.03	conjugate C-H Aromatic-1,4 disubstitution

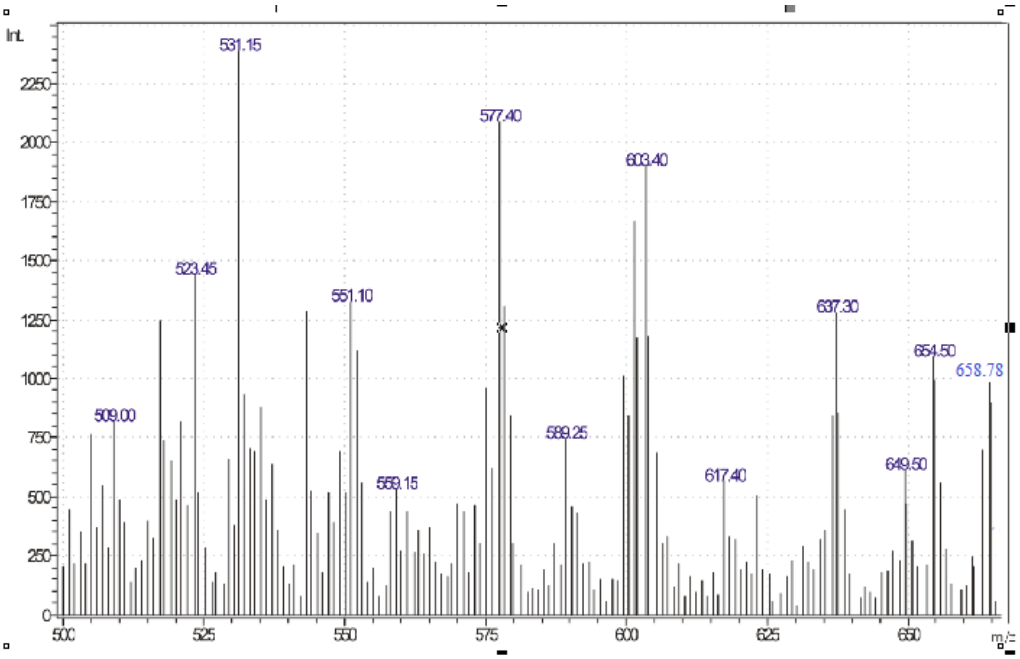


Figure 7. Mass Spectra of compound A (MA-1; $C_{34}H_{31}N_9O_6$; 658.78)

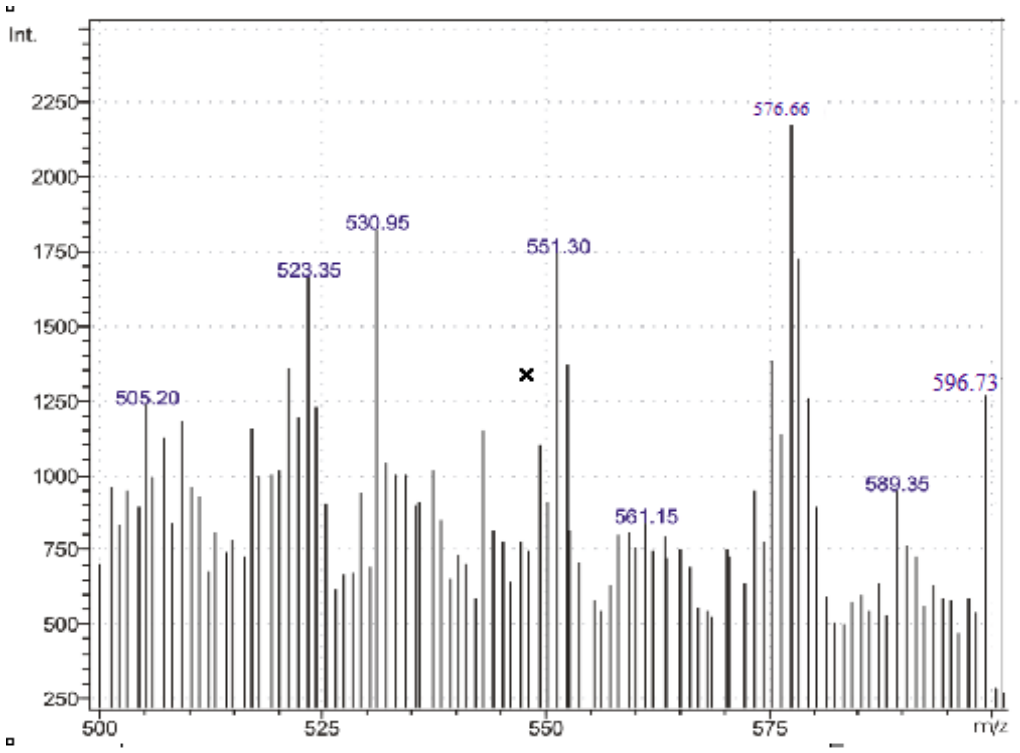
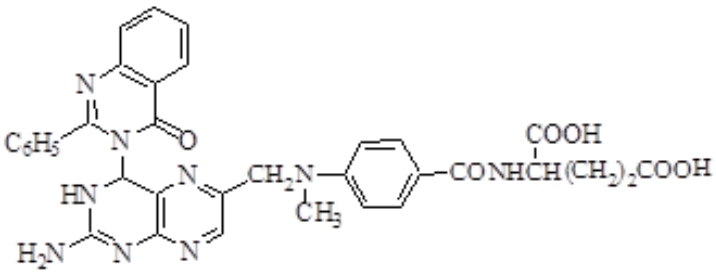
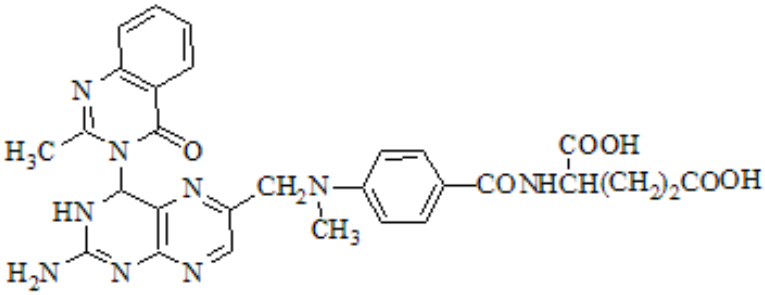


Figure 8. Mass Spectra of compound B (MA-2; $C_{29}H_{29}N_9O_6$; 596.73)



Compound A (MA-1)



Compound B (MA-2)

Figure 9. Structures of compound A and compound B (MA-1 and MA-2)

Biological and Pharmacological activity

Anti-cancer activity of plant extracts against HCC1937 cell line

Table 3 displays the mean absorbance of the compounds-incubated wells as well as the control at 511 nm. Wells treated with the chemicals showed lower absorbance values (poor cell survival) than the control at all tested doses. The means were determined to be substantially different with a P<0.0001, and the analysis of variance was performed using a one-way ANOVA with a P value of <0.05. The two substances were found to have a percentage suppression of cell growth based on their respective absorbance values at all doses (Table 3). The observed trend indicates that the chemicals at all tested doses inhibited the development of the HCC1937 breast cancer cell line more than the control did. For example, at 90 µg/ml, the percentages of cell growth inhibition caused by MA-1 and MA-2 were 82.94 and 80.29 percent, respectively. At lower chemical doses, the difference in the suppression of cell development was more noticeable. For instance, MA-1 significantly inhibited cell proliferation (45.0 percent) at 0.1µg/ml compared to MA-2 (20.59 percent). Both substances inhibited cell growth in a dose-dependent manner, with a reduction in inhibitory efficacy at lower doses. Using non-linear regression in GraphPad Prism 9, the two drugs' half-maximal inhibitory doses (IC50) were found (Figure 10). The two drugs were determined to have half-maximal inhibitory doses (IC50) of 2.316 µg/ml and 4.748 µg/ml, respectively.

Table 3. Mean absorbance at 511 nm of treated wells containing HCC 1937 cells.

Mean absorbance (*p* value < 0.001)							
Concentration µg/ml	**0.1**	**10**	**20**	**30**	**40**	**70**	**90**
MA-1	0.187	0.181	0.172	0.124	0.110	0.105	0.058
MA-2	0.270	0.227	0.220	0.209	0.191	0.153	0.067
Control	0.34	0.34	0.34	0.34	0.34	0.34	0.34

Table 4. Cell growth as % inhibition of HCC 1937 cells by MA-1 and MA-2

	% inhibition						
Concentration µg/ml	**0.1**	**10**	**20**	**30**	**40**	**70**	**90**
MA-1	45.00	46.76	49.41	63.53	67.65	69.12	82.94
MA-2	20.59	33.24	35.29	38.53	43.82	55.00	80.29

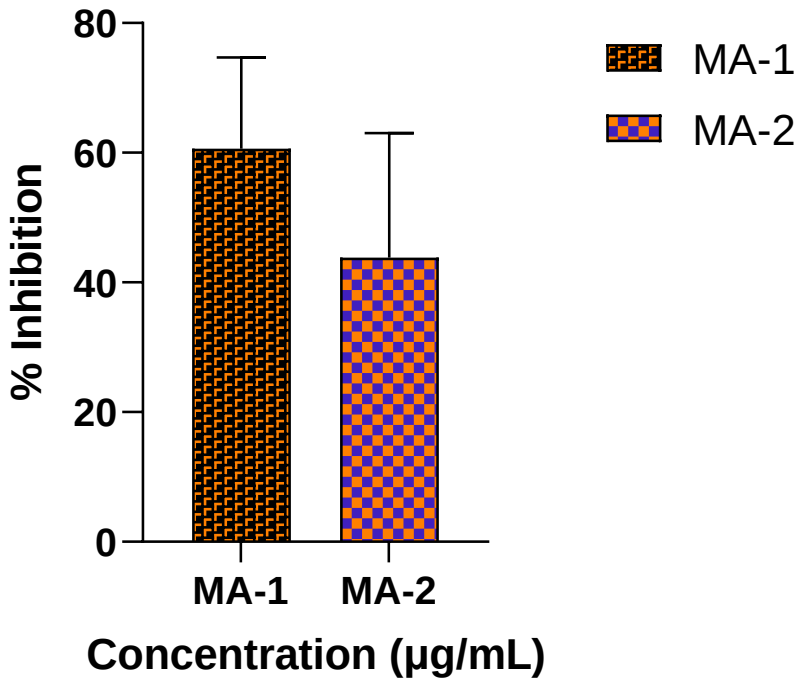


Figure 10. % inhibition of cell growth of HCC 1937 cells by MA-1 and MA-2

4. Discussion

The goal of the current work was to create better, physiologically active substitutes for methotrexate. The goal of the current study was to assess the anticancer potential of Compounds A and B, also known as MA-1 and MA-2, which are methotrexate analogues that substitute pteridine nucleus, in cell lines. Cell toxicity evaluation is a valid criterion for assessing an anticancer drug's efficacy. Physiochemical and spectral examinations were used to describe and confirm the novel entity of methotrexate analogues for the produced MA-1 and MA-2. In earlier research, we discovered that the test chemicals were safe when administered orally to experimental animals in our lab up to a level of 5 mg/kg body weight. The current study's findings demonstrated that MA-1 and MA-2 were successful in preventing the growth of cancer through in vitro screening. The findings supported the finding that MA-1 had stronger anticancer properties than MA-1. Conclusion: By replacing the methyl quinazoline nucleus in the methotrexate molecule at position 4, a strong analogue with notable anticancer activity may be easily produced. The synthetic methotrexate analogues' structural formulas were Compound A ($C_{34}H_{31}N_9O_6$) and Compound B. ($C_{29}H_{29}N_9O_6$). The synthesised chemicals were validated using spectroscopic and physiochemical analyses. In methotrexate, Compound A, codenamed MA-1, has a quinazoline nucleus that has been substituted with phenyl at the fourth position of the pteridine nucleus, whereas Compound B, designated MA-2, has a quinazoline nucleus that has been substituted with methyl at the same location. The test chemicals' in vitro anticancer activity results showed that MA-2 inhibited the HCC 1937 cells more potently than MA-1. The replacement of a methyl quinazoline nucleus at the fourth position of the pteridine nucleus in methotrexate may be the cause of this strong action. HCC 1937 cell line model was used to test in vitro anticancer activities (5, 13, 21, 23, 33).

5. Conclusions

The successful synthesis of two methotrexate analogues using a reliable and effective method was demonstrated in the current work. Additionally, it was discovered that the yields were both acceptable and noteworthy, demonstrating the effectiveness of this method for synthesising methotrexate analogues. Following the successful synthesis of two Methotrexate analogues, their structures were clarified and characterised. The two methotrexate analogues showed strong anticancer potential as well, according to the current study. Furthermore, in the HCC 1937 cell line model, two analogues of methotrexate showed notable anticancer effect in terms of a decrease in

the percentage of cell growth inhibition. This work opened the door to successfully synthesise Methotrexate analogues and then evaluate them physiologically to get novel biologically active medicinal moieties.

List Of Abbreviations

°C	Degree Centigrade
mol	moles
gm	Gram
h	Hours
mL	Milliliter
mol L-1	Mol per litre
m.p.	Melting point
b.p.	Boiling point
MTX	Methotrexate
$CHCl_3$	Chloroform
THF	Tetrahydrofuran
TEA	Triethylamine
TFA	Trifluoroacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
FT-IR	Fourier transform infrared spectroscopy
^{1}HNMR	Nuclear Magnetic resonance spectroscopy

Conflict of Interest: The authors affirm that the article does not include any conflicts of interest.

Funding: None

6. References

1. Farber S, Toch R, Sears EM, Pinkel D. Advances in chemotherapy of cancer in man. Advances in cancer research. 4: Elsevier; 1956. p. 1-71.
2. Weinblatt ME, Coblyn JS, Fox DA, Fraser PA, Holdsworth DE, Glass DN, et al. Efficacy of low-dose methotrexate in rheumatoid arthritis. New England Journal of Medicine. 1985;312(13):818-22.
3. Felson DT, Anderson JJ, Boers M, Bombardier C, Chernoff M, Fried B, et al. The American College of Rheumatology preliminary core set of disease activity measures for rheumatoid arthritis clinical trials. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 1993;36(6):729-40.
4. Colville-Nash PR, Scott DL. Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. Annals of the rheumatic diseases. 1992;51(7):919.
5. Helliwell PS, FitzGerald O, Fransen J, Gladman DD, Kreuger GG, Callis-Duffin K, et al. The development of candidate composite disease activity and responder indices for psoriatic arthritis (GRACE project). Annals of the Rheumatic Diseases. 2012:annrheumdis-2012.
6. Kremer JM. Toward a better understanding of methotrexate. Arthritis & Rheumatism. 2004;50(5):1370-82.
7. Olsen RW, Tobin AJ. Molecular biology of GABAA receptors. The FASEB Journal. 1990;4(5):1469-80.
8. Cronstein BN. The mechanism of action of methotrexate. Rheumatic disease clinics of North America. 1997;23(4):739-55.
9. Kremer JM, Alarcón GS, Lightfoot Jr RW, Willkens RF, Furst DE, Williams HJ, et al. Methotrexate for rheumatoid arthritis. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 1994;37(3):316-28.
10. Bedoui Y, Guillot X, Sélambarom J, Guiraud P, Giry C, Jaffar-Bandjee MC, et al. Methotrexate an old drug with new tricks. International journal of molecular sciences. 2019;20(20):5023.
11. Friedman B, Cronstein B. Methotrexate mechanism in treatment of rheumatoid arthritis. Joint bone spine. 2019;86(3):301-7.
12. Solomon DH, Glynn RJ, Karlson EW, Lu F, Corrigan C, Colls J, et al. Adverse effects of low-dose methotrexate: a randomized trial. Annals of internal medicine. 2020;172(6):369-80.
13. Hanoodi M, Mittal M. Methotrexate. StatPearls [Internet]: StatPearls Publishing; 2023.
14. Tugwell P, Bennett K, Gent M. Methotrexate in rheumatoid arthritis. Indications, contraindications, efficacy, and safety. Ann Intern Med. 1987;107(3):358-66.

15. Wang W, Zhou H, Liu L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. Eur J Med Chem. 2018;158:502-16.
16. Warren RB, Chalmers RJ, Griffiths CE, Menter A. Methotrexate for psoriasis in the era of biological therapy. Clin Exp Dermatol. 2008;33(5):551-4.
17. Schep GN. Methotrexate therapy. Can J Gastroenterol. 1998;12(1):26-7.
18. Skibińska L. [Pharmacokinetics of methotrexate]. Pol Tyg Lek. 1992;47(51-52):1163-6.
19. Torres RP, Santos FP, Branco JC. Methotrexate: Implications of pharmacogenetics in the treatment of patients with Rheumatoid Arthritis. ARP Rheumatol. 2022;1(3):225-9.
20. Woolf R, Smith C. Methotrexate. Handbook of Systemic Drug Treatment in Dermatology: CRC Press; 2023. p. 195-202.
21. Haberman RH, Herati R, Simon D, Samanovic M, Blank RB, Tuen M, et al. Methotrexate hampers immunogenicity to BNT162b2 mRNA COVID-19 vaccine in immune-mediated inflammatory disease. Annals of the rheumatic diseases. 2021;80(10):1339-44.
22. Koźmiński P, Halik PK, Chesori R, Gniazdowska E. Overview of dual-acting drug methotrexate in different neurological diseases, autoimmune pathologies and cancers. International journal of molecular
23. Juge P-A, Lee JS, Lau J, Kawano-Dourado L, Serrano JR, Sebastiani M, et al. Methotrexate and rheumatoid arthritis associated interstitial lung disease. European Respiratory Journal. 2021;57(2).
24. Grosflam J, Weinblatt ME. Methotrexate: mechanism of action, pharmacokinetics, clinical indications, and toxicity. Curr Opin Rheumatol. 1991;3(3):363-8.
25. Lester RS. Methotrexate. Clin Dermatol. 1989;7(3):128-35.
26. Puig L. Methotrexate: new therapeutic approaches. Actas Dermosifiliogr. 2014;105(6):583-9.
27. Westhovens R, Rigby WFC, van der Heijde D, Ching DWT, Stohl W, Kay J, et al. Filgotinib in combination with methotrexate or as monotherapy versus methotrexate monotherapy in patients with active rheumatoid arthritis and limited or no prior exposure to methotrexate: the phase 3, randomised controlled FINCH 3 trial. Annals of the rheumatic diseases. 2021;80 (6):727-38.
28. Bangert CA, Costner MI. Methotrexate in dermatology. Dermatol Ther. 2007;20(4):216-28.
29. Bannwarth B, Labat L, Moride Y, Schaeverbeke T. Methotrexate in rheumatoid arthritis. An update. Drugs. 1994;47(1):25-50.
30. Barnhart K, Coutifaris C, Esposito M. The pharmacology of methotrexate. Expert Opin Pharmacother. 2001;2(3):409-17.
31. Cronstein BN. The mechanism of action of methotrexate. Rheum Dis Clin North Am. 1997;23(4):739-55.
32. Tiwari AK, Singh VK, Bajpai A, Shukla G, Singh S, Mishra AK. Synthesis and biological properties of 4-(3H)-quinazolone derivatives. European Journal of Medicinal Chemistry. 2007;42(9):1234-8.
33. Knorr A, Mestieri LB, Pinheiro LS, Mendes RA, Gonzalez Hernandez PA, Barletta FB, et al. Cytotoxicity and Bioactivity of Calcium Silicate-based Cements in a Culture of Stem Cells from the Apical Papilla. Iran Endod J. 2021;16(4):225-31.
34. Vajrabhaya L, Sithisarn P, Wilairat P, Leelaphiwat S. Comparison between Sulphorhodamine-B dye staining and 51Cr-release method in cytotoxicity assay of endodontic sealers. J Endod. 1997;23(6):355-7.
35. Thaher BA, Al-Masri I, Wahedy K, Morjan R, Aliwaini S, Al Atter IM, et al. Synthesis and bioassay of 3-Aryl -1-(pyridin-4-yl)benzo[4,5]imidazo[1,2-d][1,2,4]- triazin-4(3H)-ones as anti-cancer agents. Naunyn Schmiedebergs Arch Pharmacol. 2023;396(8):1797-810.
36. Wang D, Nie Z, Jiang X, Ye J, Wei Z, Cheng D, et al. Pyrrolo [3,4-b]-quinolin-9-amine compound FZU-0038-056 suppresses triple-negative breast cancer partially through inhibiting the expression of Bcl-2. Aging (Albany NY). 2020;12(10):9621-32.