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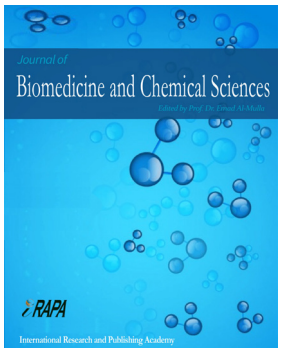

In Silico Study, Synthesis and Evaluation of Cytotoxic Activity of New Sulfonamide-Isatin Derivatives as Carbonic Anhydrase Enzyme Inhibitors

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ABSTRACT

Design, molecular docking, synthesis and evaluation of cytotoxic activity of new compounds I, II, III and IV that have isatin-sulfonamide derivatives. For chemical synthesis, chemical compounds such as sulfonamide, 4-aminoethyl benzoate, isatin and its derivatives were used. For the docking study, the MOE software program version 2015.10 was used. And MTT assay for the prediction of cytotoxic activity. The synthesised compounds demonstrated significant inhibition of carbonic anhydrase XII activity through molecular docking and significant inhibition of cancer cell viability. Compounds II and IV show higher S-scores than acetazolamide. Also, the MTT assay shows IC50 against MCF-7 cells (0.06 µM and 0.105 µM) of compounds II and IV, respectively, when compared with IC50 0.394 µM of acetazolamide. IC50 against Hct116 cells (0.063 µM and 0.114 µM) of compounds II and IV, respectively, when compared with IC50 0.901 µM of acetazolamide. The MTT assay explains that compounds II and IV have better cytotoxic activity compared with acetazolamide. New compounds that were produced showed signs of cytotoxicity and carbonic anhydrase inhibitory qualities.

INTRODUCTION

After cardiovascular disorders, cancer is the second most common cause of mortality in the US (Siegel, et al., 2019; Aziz Alibeg, et al., 2020). Sex and age are the most important in cancer susceptibility and treatment, and the male are more subjected to cancer than the female (Dorak & Karpuzoglu, 2012; Edgren, et al., 2012). Most cases of cancer death from colorectum and breast cancer in women and prostate, lung and colorectum cancer in men (Machlowska, et al., 2020). Antitumor medication resistance in cancer cells is the primary cause of the increased incidence of cancer treatment failure, creating significant difficulties for the healthcare system (Shaldam, et al., 2023). Overcoming this barrier still presents a big issue that calls for more research and creative fixes. The family of enzymes known as carbonic anhydrases (CAs) is one of the novel targets for tumor cells, with the goal of treating the tumor microenvironment to minimize chemotherapy resistance. CAs are widely distributed members of the metalloenzymes group, with eight gene groups spanning from α- to ι encoding their genes. They are present in various mammals (Naji, et al., 2023). All human CAs (hCAs) are members of the α-class; fifteen isozymes have been found so far; these vary in terms of molecular characteristics, oligomeric arrangement, cellular localization, organ and tissue distribution, expression levels, kinetic properties, and reactivity to various inhibitor classes (Noor, 2017).

Hypoxia increases the expression of carbonic anhydrase, which primarily shows up in peri necrotic

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tumor cell patterns. CA IX and CA XII have been linked to cancer development (Aljubouri & Naser, 2023). It has been suggested that CA XII regulates cell adhesion, proliferation, and malignant cell invasion. It is noteworthy that human epithelial cancers originating from tissues that typically do not express these isoforms—such as carcinomas of the cervix, lungs, kidneys, prostate, and breast—overexpress CA IX (Aggarwal, 2013). Sulfonamides, or R-SO2NH2, are a significant class of compounds with the ability to inhibit CA isoenzymes, which are implicated in various physiological and pathological processes (Sağlık, et al., 2019). One sulfonamide-based CA inhibitor that is used in clinical practice is acetazolamide (Tafreshi, et al., 2014). Several CAIs, such as acetazolamide, ethoxzolamide, and dichlorphenamide, have been used in clinical practice for many years as diuretics and systemically acting antiglaucoma medications. CAIs use their deprotonated sulfonamide nitrogen atom to coordinate the zinc ion at the catalytic site (Salerno, et al., 2021). The diverse chemical structures of heterocyclic compounds have long been a source of interest for drug discovery due to their wide range of pharmacological potential (Abbas, et al., 2023). One such biologically active heterocyclic molecule is isatin, also referred to as [indole quinone or indanedione, or 1H-indole-2,3-dione (Al-Khuzaie, et al., 2022).

EXPERIMENTAL

Pure raw materials and modern apparatus were used to prepare, confirm, and identify the synthesized samples. The Electro Thermal Technique (SMP30) type was used to detect the melting point. Thin Layer Chromatography 1020 GS- Silica gel 60 type was used to follow the reaction's progression. The Pharmacy Faculty at Kufa University used a Fourier transform infrared spectrophotometer-8400s, Shimadza (KBr), to record the spectra to identify the synthesized chemicals. Using TMS as an internal reference, nuclear magnetic resonance (^{1}H, ^{13}C-NMR) data were obtained at Teheran University- Iran using a Bruker-400 MHz apparatus. The Molecular Operating Environment (MOE) Program was utilized in an in-silico investigation to demonstrate the precise binding of the resultant compounds with the target receptors.

Chemical Section

Synthesis of Schiff Base Derivatives (Ia-Id)

In general, isatin and its derivatives (5-Methyl Isatin, 5-Methoxy Isatin, and 5-Fluoro Isatin) were condensed with 4-amino ethyl benzoate (in equal moles) using hot 100% ethanol as a solvent to create Schiff base derivatives (Alibeg & Mohammed, 2024; Altalhi, 2024). Drop by drop, three to four drops of glacial acetic acid were added to the mixture. The final mixture was refluxed for 48 hours. TLC analysis was used to follow the reaction steps (hexane: ethyl acetate, 3:1 v/v). After the finished product was poured over broken ice water, the precipitate was separated, filtered, and repeatedly cleaned with hot distilled water and ethanol. It was then dried and recrystallized from ethanol.

Table 1
Physical properties of Schiff bases derivatives (Ia-Id)

Symbol	Chemical formula	Color	Yield %	M.Wt (g/mol)	M.P	R_f
Ia	$C_{17}H_{14}N_{20}3$	Yellow powder	83	294.1	216-219	0.6
Ib	$C_{17}H_{13}FN_2O_3$	orange powder	29	312.30	170-172	0.5
Ic	$C_{18}H_{16}N_2O_4$	Light yellow powder	78	324.33	253-255	0.4
Id	$C_{18}H_{16}N_2O_3$	Dark Brown powder	88	308.33	95-97	0.66

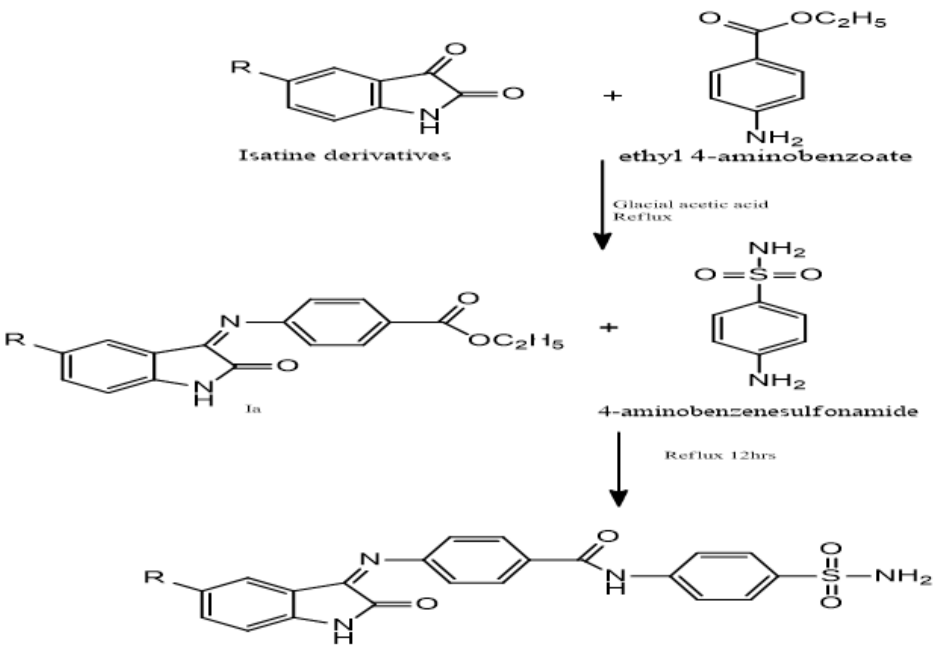
Synthesis of Final Products (I-IV)

In equal moles, the prepared ligands in the first step were combined with 4-aminobenzene sulfonamide using absolute ethanol: DMF (1:1 v\v) as solvent (Alibeg & Mohammed, 2024; Patil, et al., 2023). The reaction mixture was reflux for (12hrs). Thin Layer Chromatography (hexane: ethyl acetate (3:2 v/v)) gave its approval for the reaction to end. Crushed ice was added once the solution was reduced to half. The raw material was removed with a filter, cleaned in cold water, and then recrystallized from the water after a day.

Table 2
Physical properties of final products

Symbol	Chemical formula	Color	Yield %	M.Wt (g/mol)	M.P	R_f
I	$C_{21}H_{16}N_4O_4S$	Light yellow powder	96	420.09	205-207	0.4
II	$C_{21}H_{15}FN_4O_4S$	Yellow powder	08	438.08	198-200	0.4
III	$C_{22}H_{18}N_4O_5S$	Orange powder	59	450.47	227-230	0.42
IV	$C_{22}H_{18}N_4O_4S$	Brown-black powder	73	434.1	150-153	0.5

Chemical Synthesis



R=H compound I, R=F compound II, R=OCH3 compound III, R=CH3 compound IV

Scheme. 1. Synthesis of intermediate and final product.

Spectroscopic Analysis

Compound Ia ($C_{17}H_{14}N_2O_3$) FT-IR (cm^{-1}) 3269 stretching band of the (N-H) group of amides, 2924 stretching band of aromatic (C-H), 1737 stretching band of ester (C=O), 1647 stretching vibration of the imine or Schiff base group (C=N), 1610 stretching band of the amide (C=O), 1573 stretching band of aromatic ring (C=C), 1182 and 1114 stretching bands of ester (C-O) (Pavia, et al., 2014).

Compound Ib ($C_{17}H_{13}FN_2O_3$) FT-IR (cm^{-1}) 3417 stretching band of the (N-H) group of amides, 3068 stretching band of aromatic (C-H), 1720 stretching band of ester (C=O), 1662 stretching vibration of the imine or Schiff base group (C=N), 1610 stretching band of the amide (C=O), 1610 and 1487 stretching bands of aromatic ring (C=C), 1278 and 1201 stretching bands of ester (C-O).

Compound Ic ($C_{18}H_{16}N_2O_4$) FT-IR (cm^{-1}) 3271 stretching band of the (N-H) group of amides, 2980 stretching band of aromatic (C-H), 1710 stretching band of ester (C=O), 1676 stretching vibration of the imine or Schiff base group(C=N), 1604 stretching band of the amide (C=O), 1492 stretching band of aromatic ring (C=C). `

Compound Id ($C_{18}H_{16}N_2O_3$) FT-IR (cm^{-1}) 3280 stretching band of the (N-H) group of amides, 2983 stretching band of aromatic (C-H), 1708 stretching band of ester (C=O), 1602 stretching vibration of the imine or Schiff base group(C=N), 1523 stretching band of aromatic ring (C=C), 1276 and 1174 stretching bands of ester (C-O).

Compound I ($C_{21}H_{16}N_4O_4S$) FT-IR (cm^{-1}) 3468 and 3404 stretching bands of the (N-H) group of the primary amines, 3230 stretching band of the (N-H) group of amides, 2983 stretching band of aromatic (C-H), 1716 stretching band of the newly amide (C=O),1656 stretching vibration of the imine or Schiff base group (C=N), 1602 stretching band of the amide (C=O). ^{1}H NMR (ppm) The 1H of the amide appeared as a singlet at 7.5-8 ppm and the hydrogens of the three aromatic rings showed as multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. ^{13}C-NMR (ppm): 129 as singlet of carbonyl C=O and 118-163 as singlet of aromatic C.

Compound II ($C_{21}H_{15}FN_4O_4S$) FT-IR (cm^{-1}) 3421 and 3473 stretching bands of the (N-H) group of the primary amines, 2985 stretching band of aromatic (C-H), 1705 stretching band of the newly amide (C=O), 1660 stretching vibration of the imine or Schiff base group(C=N), 1620 stretching band of the amide (C=O), 1473 stretching band of aromatic ring (C=C). ^{1}H NMR (ppm) The 1H of the amide appeared as a singlet at 7.5-8 ppm and the hydrogens of the three aromatic rings showed as multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. ^{13}C-NMR (ppm): 129 as singlet of carbonyl C=O and 118-163 as singlet of aromatic C.

Compound III ($C_{22}H_{18}N_4O_5S$) FT-IR (cm^{-1}) 3468

and3435 stretching bands of the (N-H) group of the primary amines, 2927 stretching band of aromatic (C-H), 1668 stretching vibration of the imine or Schiff base group (C=N), 1602 stretching band of the amide (C=O). ^{1}H NMR (ppm) The 1H of the amide appeared as a singlet at 7.5-8 ppm and the hydrogens of the three aromatic rings showed as multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. Three hydrogens of methyl appeared as singlet at 2.5 ppm. ^{13}C- NMR (ppm): 129 as singlet of carbonyl C=O, 118-163 as singlet of aromatic C and 55 as singlet of C of OCH_3.

Compound IV ($C_{22}H_{18}N_4O_4S$) FT-IR (cm^{-1}) 3427 and 3468 stretching bands of the (N-H) group of the primary amines, 3244 stretching band of the (N-H) group of amides, 2983 stretching band of aromatic (C-H), 1718 stretching band of the newly amide (C=O), 1604 stretching vibration of the imine or Schiff base group (C=N). ^{1}H NMR (ppm) The 1H of the amide appeared as a singlet at 7.5-8 ppm and the hydrogens of the three aromatic rings showed as multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. Three hydrogens of methoxy appeared as singlet at 2.8 ppm. ^{13}C- NMR (ppm): 129 as singlet of carbonyl C=O, 118-163 as singlet of aromatic C and 21 as singlet of C of CH_3.

Docking Study

The research includes building the protein and ligand structures for a molecular docking analysis use the 2015.10 MOE (Molecular Operating Environment) software. Using ChemDraw Professional 12.0, the ligand structures were precisely drawn. After that, the ligands were protonated in the Molecular Operating Environment (MOE) in three dimensions. partial charges were added, and energy minimization was performed before the results were saved. The genetically modified carbonic anhydrase XII crystal structure (PDB: 1JCZ /chain A) was acquired from the PDB website and integrated into MOE for the receptor. To prepare the target protein, the remaining chains and small molecules were removed, leaving only the chain sequences essential to the protein's function. Additionally, water molecules were eliminated. The protein's atom potentials were modified and hydrogen bonds were added before the active site was found. Lastly, MOE was used to load the previously produced ligands from the stored data, and the docking procedure was carried out.

Study of Cytotoxic Cell Lines

The National Cell Bank of Iran (Pasteur Institute, Iran) provided the human normal cell line HUVEC, the human colorectal cancer cell line Hct116, and the human breast cancer cell line MDA-MB-231. Gibco's RPMI-1640 medium, supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) was used to cultivate the cells. Trypsin/EDTA (Gibco) and phosphate-buffered saline (PBS) solution were used to passage the cells, which were then maintained at 37 °C in humidified air with 5% CO_2. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide] (Sigma-Aldrich) assay was used to measure the proliferation and vitality of the cells. Trypsin was used to digest the cells, after which they were extracted and adjusted to a density of 1.4 × 104 cells/well. The cells were then planted into 96-well plates containing 200 µl of new media per well and allowed to grow for a full day. The cells were treated to 600–7.4 µg/ml of the compounds for a whole day at 37 °C and 5% CO_2 after forming a monolayer. Following a 24-hour treatment period, 200 µl/well of MTT solution (0.5 mg/ml in phosphate-buffered saline [PBS]) was added, and the supernatant was disposed away. After that, the plate was incubated for a further four hours at 37 °C, all the while maintaining the monolayer culture in the initial plate MTT solution: The collected cell supernatant was followed by the addition of 100 µl of dimethyl sulfoxide to each well. The cells were cultured at 37 °C on a shaker plate until the crystals completely dissolved. The vitality of the cells was measured by measuring absorbance at 570 nm using an ELISA reader (Model wave xs2, BioTek, USA). The concentration of the compounds that induced 50% of cell death (IC_{50}) was determined using the appropriate dose-response curves.

Result of Docking Study

Reference compound must be identified when working in a docking study like acetazolamide. Use reference acetazolamide to compare the binding properties of designed compounds to the active site of carbonic anhydrase XII enzyme with binding properties of acetazolamide to the active site of the same enzyme. Two factors depend on docking study results to assess the activity and selectivity of compounds: the S-score and RMSD (root mean square deviation). S-score refers to the binding affinity between the desired enzyme and newly designed compounds, with more binding properties when less S-score value. RMSD refers to the distance between atoms of posed of the active site of designed compounds and the posed ligand. In the preparatory stages, hydrogen atoms are added to the structure of the carbonic anhydrase XII enzymes to correspond to the natural PH. Table 3 shows the results of docking compounds that explain that newly

designed compounds have good binding affinities with carbonic anhydrase XII enzyme. The binding energy of synthesized compounds with the active site of selected enzyme ranges from -6.64 to -7.12 Kcal/mol compared with the -5.82 Kcal/ mol binding energy of acetazolamide. acetazolamide forms three bonds with amino acids of the active site of the enzyme while newly synthesized compounds show more than three bonds forming with the active site of the enzyme. So, when comparing the results of energy of binding of synthesized compounds with acetazolamide, shows more selectivity of synthesized compounds to carbonic anhydrase XII enzyme (1JCZ code in PDB).

Table 3

Binding properties of newly synthesized compounds with CA XII (PDB: 1JCZ/ chain A)

Compound	R group	Docking S- scores in ΔG (Kcal/mol)	RMSD	Number of binding sites	Molecules that involve in binding
ACTAZOLAMIDE	----	-5.82	2.094	3	Zn 3:901, Thr199, Lys67
I	H	-6.64	1.321	4	Zn 3:901, Thr199, His96, His94
II	F	-6.89	1.678	5	Zn 3:901, Thr199, His96, His94, Ser132
III	OCH_3	-7.12	1.921	6	Zn 3:901, Thr199, His119, His96, Ser132, Leu198
IV	CH_3	-6.75	1.211	7	Zn 3:901, Thr200, Thr199, His94, Lys67, Ser132, Leu198

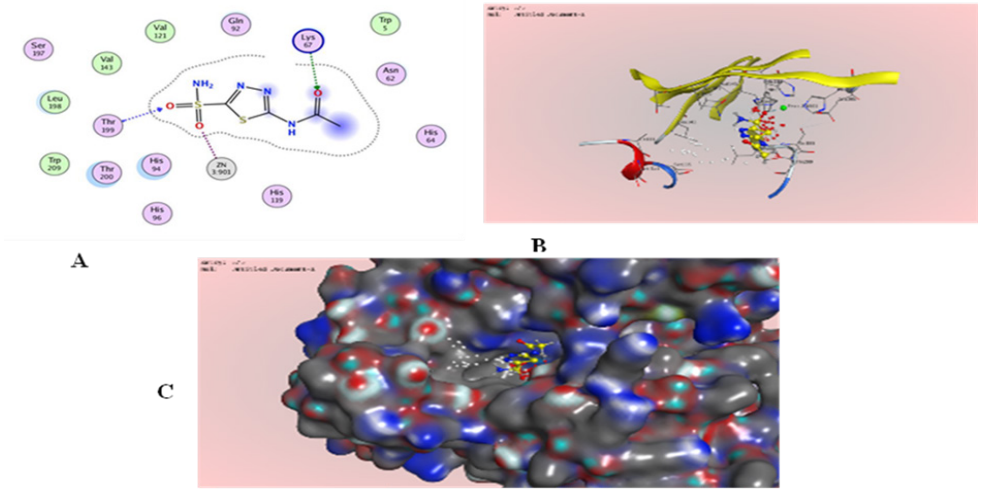


Fig. 1. Acetazolamide with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Acetazolamide with active site, (B) explain the 3D picture of binding Acetazolamide with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

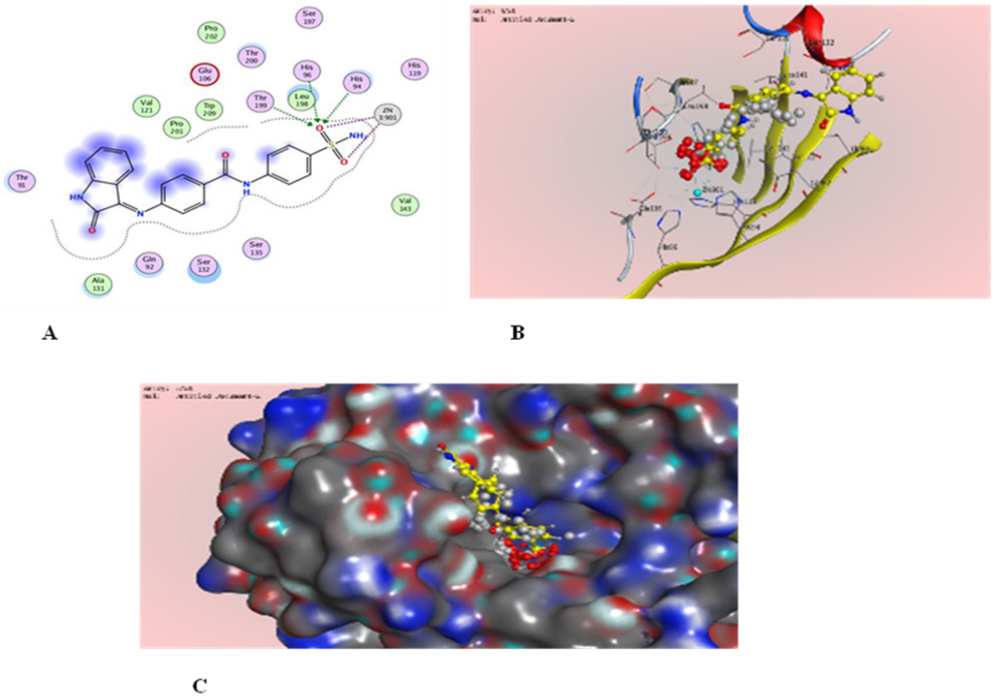


Fig. 2. Compound I with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound I with active site, (B) explain the 3D picture of binding Compound I with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

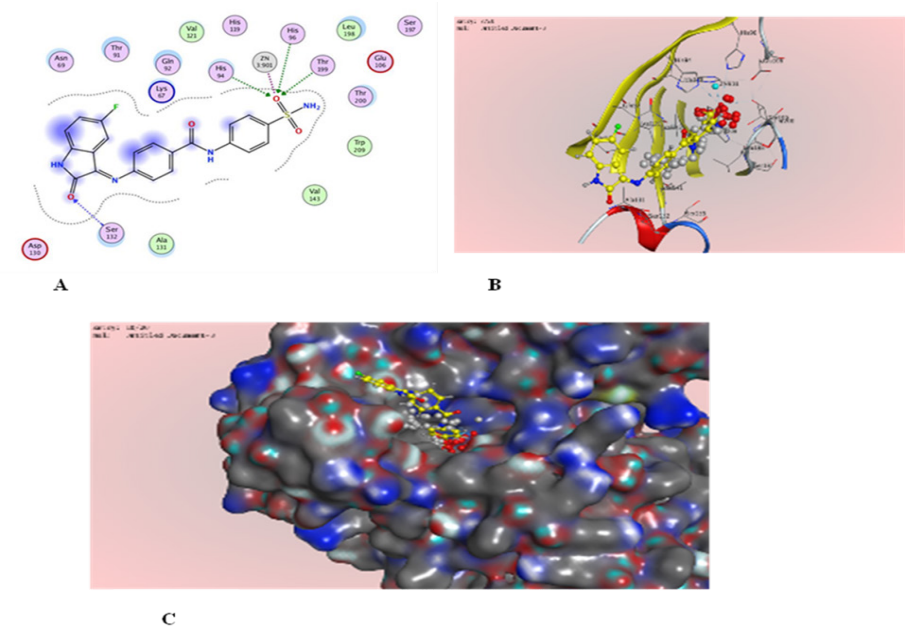


Fig. 3. Compound II with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound II with active site, (B) explain the 3D picture of binding Compound II with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

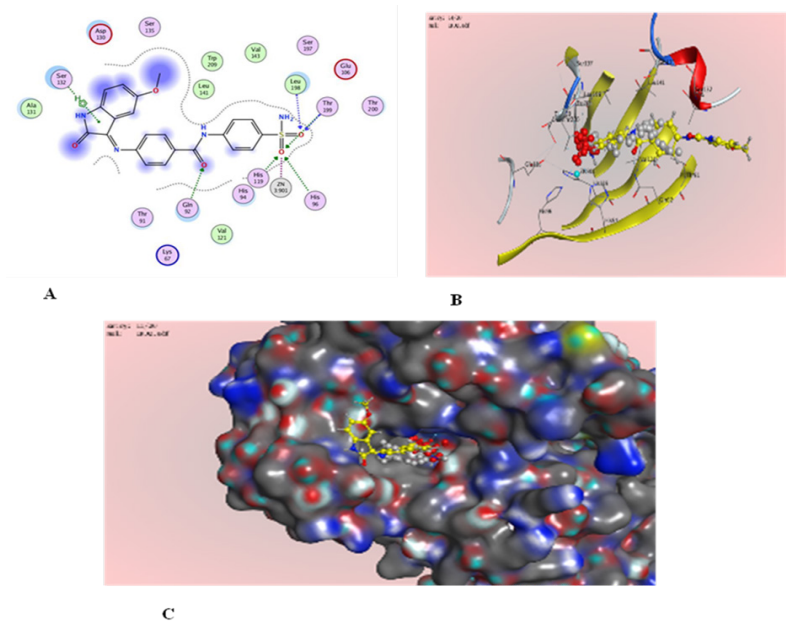


Fig. 4. Compound III with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound III with active site, (B) explain the 3D picture of binding Compound III with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

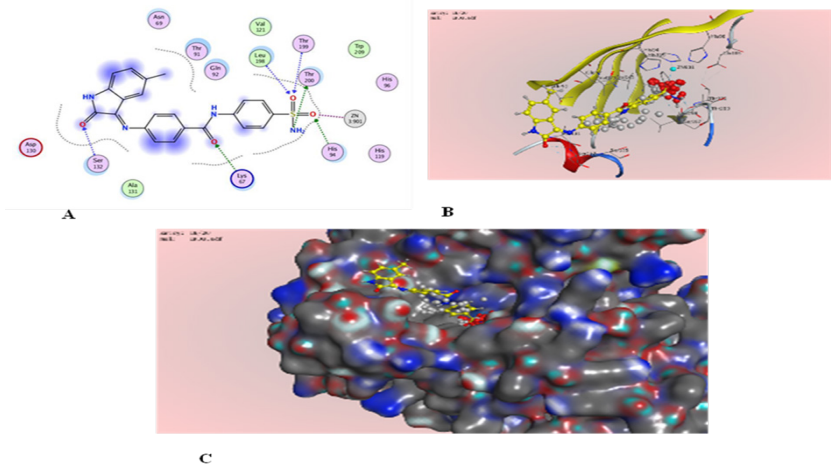


Fig. 5. Compound IV with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound IV with active site, (B) explain the 3D picture of binding Compound IV with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

Cytotoxic Evaluation

The discovery of new anticancer medications with fewer side effects and more selectivity against cancer cells is essential. Normal cells around cancerous cells in the human body (Tuğrak, et al., 2021). Therefore, substances meant to be used in clinical settings should be more lethal to tumor cells than normal ones. In a cell culture environment, the cytotoxicity effect of the substances (I-IV) was evaluated using cell line MTT tests. The effects of the chemicals were investigated using cell types: MCF7, which represents breast cancer cells, HCT-116, which represents colorectal cells, and MCF10, which represents normal cells. In vitro studies show superior cytotoxic activity of newly synthesized compounds by utilizing MTT assay. Study inhibition ability against cancerous cell proliferation to assess the activity of newly synthesized compounds. As the results shown in Table 4 explain good anti-cancer activity toward carbonic anhydrase XII enzyme.

Table 4
Anti-cancer activity toward CA XII enzyme

compound	MCF-7 IC_{50} µm	Hct116 IC_{50} µm	HCF10a IC_{50} µm
ACETAZOLAMIDE	0.394	0.901	1.313
Compound I	0.147	0.144	16.535
Compound II	0.060	0.063	0.578
Compound III	1.553	1.660	0.558
Compound IV	0.10.5	0.114	0.503

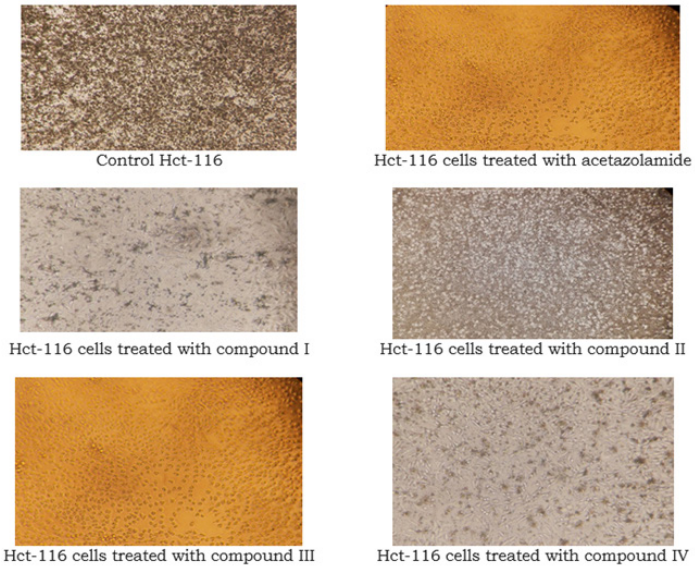


Fig. 6. Show the morphology of the Hct-116 control cells and the morphology of Hct-116 cells treated with acetazolamide and newly synthesized compounds I, II, III and IV at IC_{50}, note the number of living cells are less when treated with newly synthesized compounds.

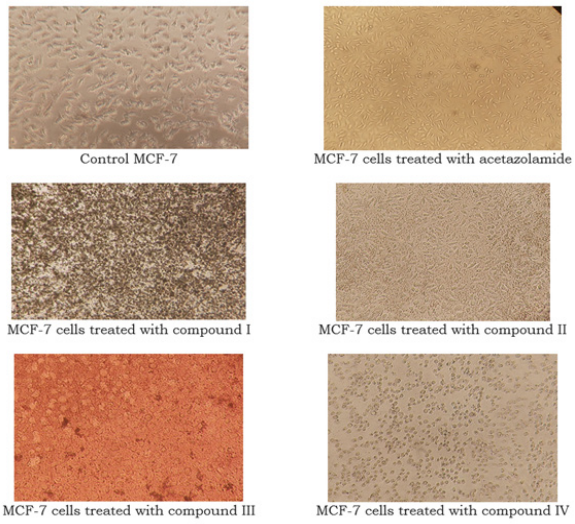


Fig. 7. Show the morphology of the MCF-7 control cells and the morphology of MCF-7 cells treated with acetazolamide and newly synthesized compounds I, II, III and IV at IC_{50}, note the number of living cells are less when treated with newly synthesized compounds.

CONCLUSION

In this study, shows that has effective anti-cancer compounds acting on the Carbonic Anhydrase XII enzyme (PDB code: 1JCZ). Three main steps act on it that is design, synthesis and evaluation of biological activity. Use ^{1}H NMR, ^{13}C NMR and FT-IR to identify and characterization of compounds. Anti-cancer efficiency tested toward MCF-7 and Hct116 cell lines by MTT assay. This research explains two of four compounds have better S.score and RMSD values than acetazolamide in MOE docking. Also, compounds II and IV have good cytotoxic activity IC_{50} (0.06 µM and 0.105 µM) respectively in MCF-7 when compared with IC_{50} of acetazolamide (0.394 µM) and compounds II and IV have better cytotoxic activity IC_{50} (0.063 µM and 0.114 µM), respectively, in Hct116 when compared with IC_{50} of acetazolamide 0.901 µM.

Competing Interest

The authors had no competing interests.

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