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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

Microwave-assisted synthesis of N-benzylidene-4-fluoroaniline and N-benzylidene-4-Nitroaniline and their inhibitory activities on hCA isoenzymes

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Abstract: In this study, N-benzylidene-4-fluoroaniline (3a), N-benzylidene-4-nitroaniline (3b) as a result of condensation of benzaldehyde (1) and 4-fluoroaniline (2a), 4-nitroaniline (2b) using a microwave method was synthesized. The structures of the synthesized compounds were characterized by Fouirer Transform İnfrared Spektrofotometre (FTIR spectroscopy), Nuclear Magnetic Resonance (NMR spectroscopy) and elemental analysis methods. The prepared compounds were tested for their inhibitory effects on carbonic anhydrase isoenzymes (hCA-I and hCA-II). The results showed that the synthesized compounds 3a and 3b had a strong inhibitory effect on hCA-I and hCA-II enzymes activity in vitro. By testing these compounds in other CA isoenzymes, more effective CA inhibitors can be formed. Thus, new therapeutic applications can be made for enzyme activators in the near future.

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1. INTRODUCTION

Schiff bases derived from the reaction of primary amines with carbonyl compounds are used in a wide range of different fields. [1]. Schiff bases are indicated by the general formula RCH = NR. R and R 'are alkyl or aryl substituents [2]. Schiff bases are widely used in various fields such as pharmaceutical preparation, agricultural and plastic industry, polymer production, electronics industry, cosmetics, dyestuff production due to their biological and structural properties [3]. Schiff bases have antifungal properties as fungicides, insecticides, chemotherap eutic, anticancer agents in antioxidants, antimutagenic and antiviral drugs [4-8]. Nitro and halo derivatives of Schiff bases are known to have antimicrobial and anti-tumoral activity.

For the first time in 1986 Gedye used microwave in organic synthesis. The synthesis of microwave methods in scientific studies has started to attract great interest in recent years [9]. The microwave is a low frequency electromagnetic energy state at the end of the electromagnetic spectrum between the radio and infrared waves in the frequency range 300-300.000 MHz [10]. Microwave chemistry continues its development with scientific studies [11-

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15]. Because of the direct heating of the substances in the microwave reactions, the products are obtained with pure and high yields in less time than the classical heating methods [16, 17].

Carbonic anhydrase enzymes (CA, EC 4.2.1.1) are metalloproteins containing zinc (Zn^{2+}) which catalyze the conversion of CO_2 to bicarbonate (HCO_3^-) and proton (H^+) release. In addition, the carbonic anhydrase enzyme plays a role in many important reactions such as transport of CO_2 in tissues, pH and CO_2 homeostasis, transport of ions, biosynthetic reactions, bone formation. CA enzymes have been found in many tissues such as salivary glands, muscles, brain, nerve myelin sheath, pancreas, prostate and endometrial tissues by histochemical methods [18].

$$CO_2 + H_2O \longrightarrow H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The carbonic anhydrase isoenzymes are encoded by five different family of genes that are evolutionally independent, including α-, β-, CA-, δ- and ε- CA. Six teen different CA isoenzymes and CA-linked proteins (CARPs) were identified in the mammals. hCA-I and hCA-II are two main cytosolic CA isoforms found in mammalian red blood cells [19, 20].

For this reason, in this study it is aimed to synthesize Schiff base derivative N-benzylidene-4-fluoroaniline (**3a**), N-benzylidene-4-nitroaniline (**3b**) by micro wave method and investigate the inhibition potentials on CA-I and hCA-II isoenzymes.

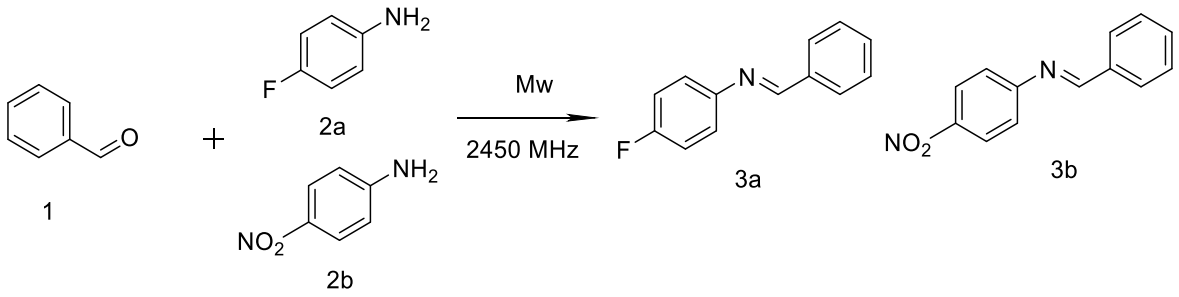
2. MATERIALS and METHODS

2.1. Chemicals

Benzaldehyde, 4-fluoroaniline are commercially available (Merck, Sigma-Aldrich) and were used without further purification. Sepharose-4B, sulphanylamide, L-tyrosine, Tris, Na_2SO_4, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from Merck. Reactions were monitored via thin-layer chromatography (TLC). ^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 (100) MHz Varian spectrometer using $CDCl_3$. Column chromatography was performed on silica gel 60 (70–230 mesh ASTM), and TLC was carried out on silica gel (254–366 mesh ASTM). Melting points were determined on a capillary melting apparatus (Buchi 530) and are uncorrected. Infrared (IR) spectra were obtained from solutions in 0.1-mm cells with a Perkin-Elmer spectrophotometer (Waltham, MA). Elemental analyzes were performed on a Leco CHNS-932 apparatus.

2.2. General Method for Synthesis of 3a, 3b

4-floroanilin (**2a**) (1mmol), 4-nitroanilin (**2b**) (1mmol) was added into the benzaldehyde (**1**) (1 mmol) mixture, and then the reaction mixture was exposed to microwave radiation at 2450 MHz. The progress of the reaction was monitored by thin layer chromatography (TLC) (Runner phase, *n*-hexane-ethylacetate (4: 1) was used). It was determined that the reactions were completed in 8 minutes for all aniline derivatives. The crude products **3a** ve **3b** were obtained in pure form (Scheme 1).



Scheme 1. The synthesis of the compounds **3a, 3b**

2.3. Purification of Carbonic Anhydrase Isozymes (hCA-I and hCA-II) from Human Erythrocytes by Affinity Chromatography

Fresh human blood was obtained from Atatürk University, Blood Center, kept at 4°C and used within 2-3 days at most. Blood samples were centrifuged for 15 minutes at 2500 rpm to separate the erythrocytes, and the remaining plasma and leucocytes layers were carefully removed and discarded. Subsequently, the remaining erythrocytes were washed twice with 0.9% NaCl solution and the upper portions were discarded. Then, the erythrocytes were hemolyzed with pure water at 0°C and mixed for half an hour, and the cell membranes were separated by centrifuging the hemolyzate at 20,000 rpm at 4°C for half an hour. The pH was adjusted to 8.7 with solid Tris. Thus, the hemolyzate came to be applied to the colonic [21].

The affinity was prepared on gel Sepharose-4B matrix. After activating Sepharose-4B with CNBr, L-tyrosine was covalently attached as the extension arm. Tyrosine was then coupled by diazotization of the sulfanilamide as the ligand. The hemolysate was applied to the prepared Sepharose-4B-L-tyrosine-sulfanylamide affinity column equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were stored at 4 ^oC [22].

2.4. Hydratase Activity Assay

Carbonic anhydrase activity was determined using the Wilbur-Anderson Method modified by Rickli et al. [23]. This method is based on the determination of the pH change from the H^+ ion resulting from the hydration of CO_2 to the bromine thymol blue indicator and measuring the permeation rate. The time (tc) interval was determined between addition of CO_2 solution and occurrence of a yellow-green color. The same interval was recorded without enzyme solution (to). The activity was calculated from the formula**.** One Wilbur – Anderson Unit = (to- tc) / tc

2.5. Inhibition Assays

In order to calculate the IC_{50} values of hCA I and hCA-II enzymes, inhibitor effects of Schiff bases derivatives were run at different concentrations, keeping the substrate concentration constant on hydratase activity. The activities of the enzymes in the uninhibited medium were used as 100% activity. Hydratase activities were measured in the presence of different concentrations of enzymes, and % activity values were calculated. IC_{50} values were calculated for each inhibitor using the scheme of % Activity- [inhibitor] [24].

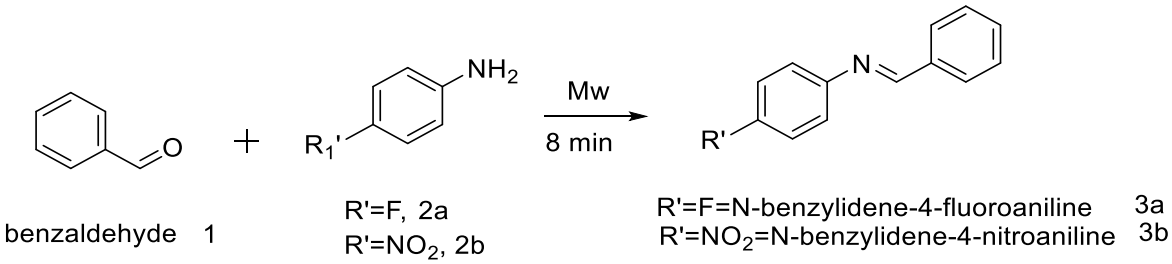
2.6. Protein Determination

The protein assay for all purification steps was performed according to the Bradford method [25]. This method is based on the principle that coomassie brilliant blue G-250 binds

to proteins in a phosphoric acid medium. The resulting complex shows maximum absorbance at 595 nm. The sensitivity of this method is between 1 - 100 micrograms.

3. RESULTS

N-benzylidene-4-fluoroaniline (3a), N-benzylidene-4-nitroroaniline (3b), were prepared from reaction of benzaldehyde (1), 4-floroaniline (2a), and 4-nitroaniline (2b) by microwave method (Scheme 2). It was determined that the reactions were completed in 8 minutes for two Schiff bases derivatives. Its physical properties, analytical and spectral data of the imine's compounds are summarized below.



Scheme 2. The synthesis route of the compound (**3a**,**3b**)

3.1. N-Benzylidene-4-fluoroaniline (3a)

Yield: 95 %, M.p. 104 °C, yellow solid.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 H (s, 1H), 7.90 (dd, *J* = 7.0, 2.6 Hz, 2H), 7.48 (dd, *J* = 5.2, 2.0 Hz, 3H), 7.21 (dd, *J* = 8.6, 5.0 Hz, 2H), 7.08 (t, *J* = 8.5 Hz, 2H) (Figure 1). ^{13}C NMR (400 MHz, $CDCl_3$) δ 160.4, 131.7, 129.0, 129.0, 122.6, 122.5, 116.2, 115.9, 29.9 (Figure 2). FTIR ($CDCl_3$, cm^{-1}): 3408 (aromatic CH), 3058 (aromatic CH), 2923, 2852 (CH=N), 1627 (aromattic C=C), 1505, 1261, 1222, 1094 (C-F), 801, 700 cm^{-1} (Figure 3). $C_{13}H_{10}FN$ Anal. calc. for: C,78.37; H, 5.06; N, 7.03 % Found: C, 78.71; H, 4.870; N, 7.093 %

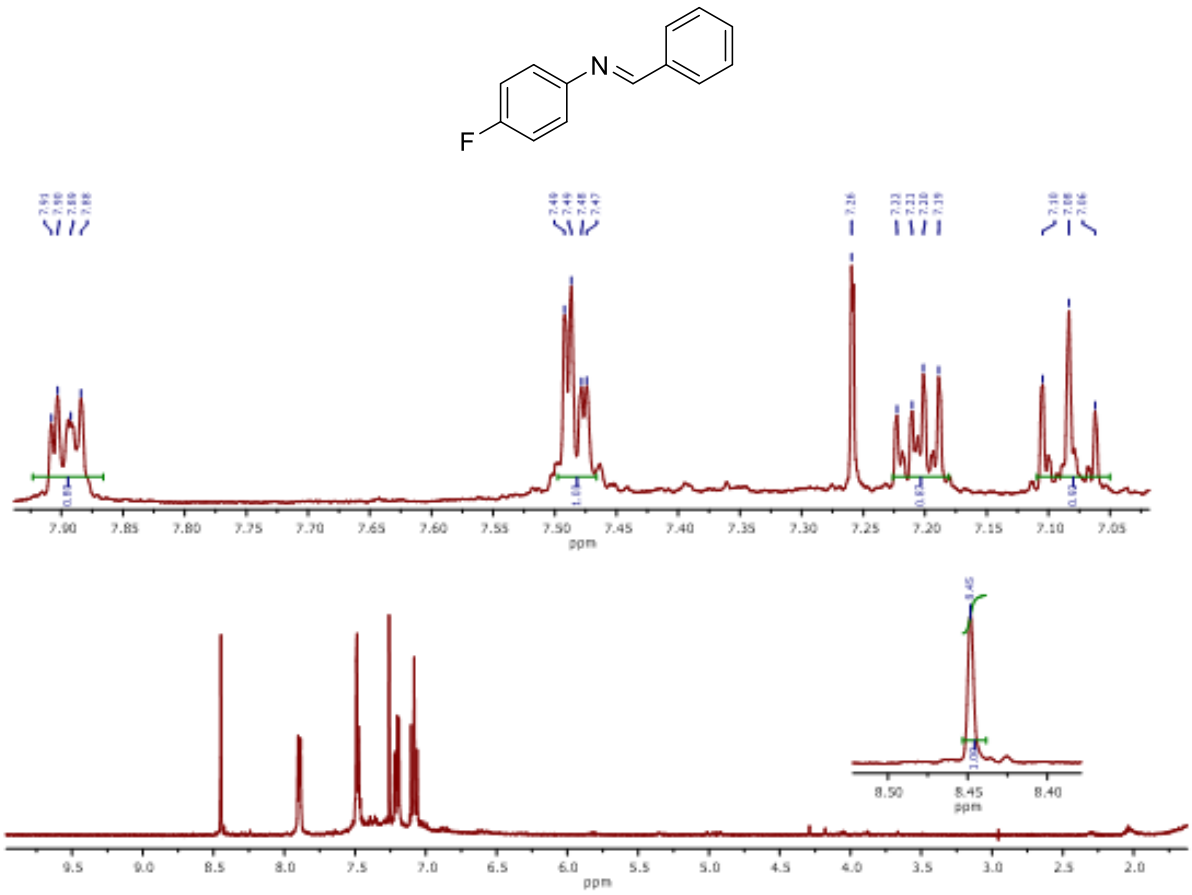


Figure 1. ^{1}H-NMR spectrum (400 MHz) of Compound **3a** ($CDCl_3$, δ ppm)

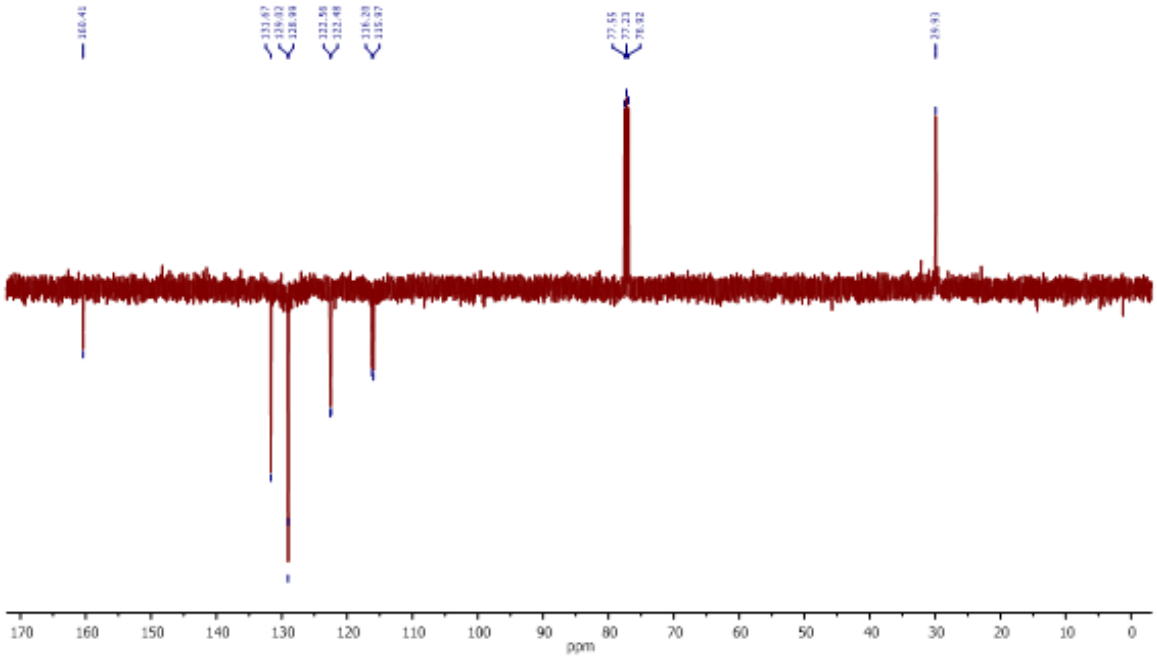


Figure 2. ^{13}C-NMR Spectrum (400 MHz) of Compound **3a** ($CDCl_3$, δ ppm)

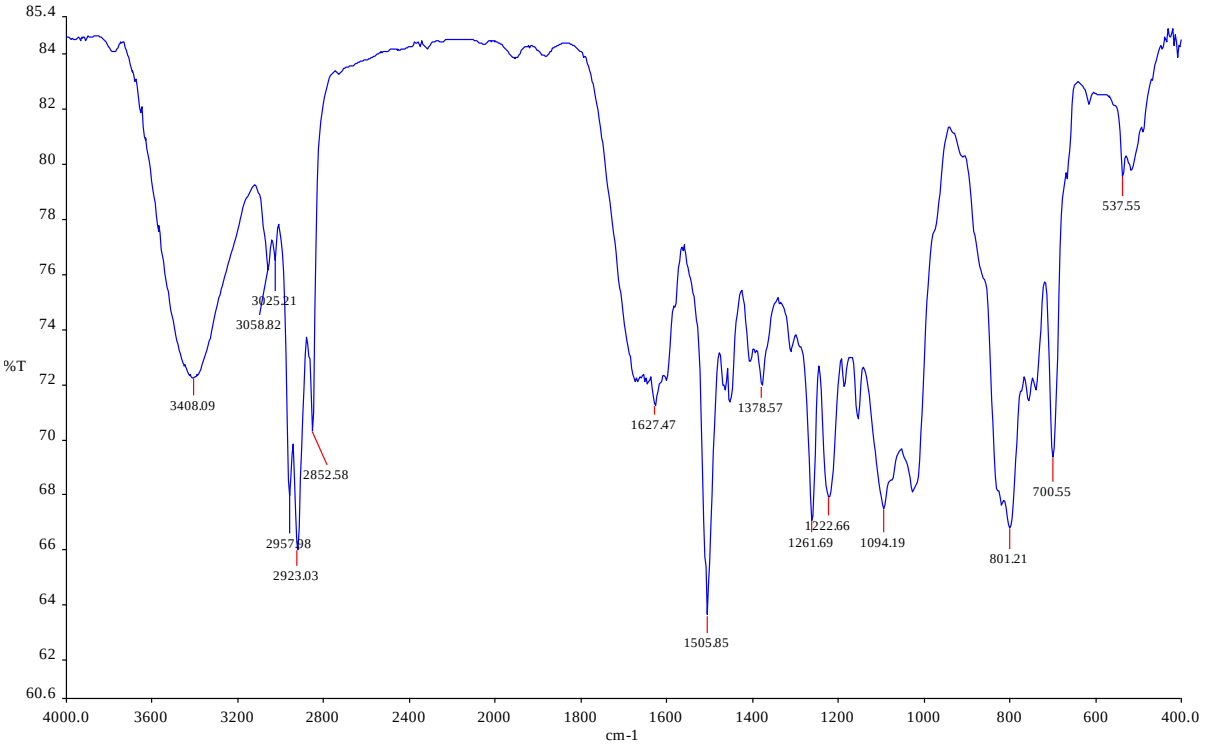


Figure 3. FTIR spectrum of Compound **3a** ($CDCl_3$, cm^{-1})

3.2. N-Benzylidene-4-nitroaniline (3b)

Yield: 93 %, M.p. 115°C, yellow solid**.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.27 (d, *J* = 8.8 Hz, 2H), 7.92 (d, *J* = 7.1 Hz, 2H), 7.61 – 7.45 (m, 3H), 7.25 (d, *J* = 8.9 Hz, 2H). (Figure 4).^{13}C NMR (400 MHz, $CDCl_3$) δ 162.95, 135.37, 132.43, 129.30, 128.99, 126.35, 125.05, 121.27, 113.37, 77.35, 77.03, 76.72. (Figure 5). FTIR ($CDCl_3$, cm^{-1}): 3482 (aromatic CH), 3369 (CH=N), 1629 (aromatic C=C), 1586, 1470, 1304, 1114, 838, 752, 697, 488 cm^{-1} (Figure 6). $C_{13}H_{10}N_2O_2$ Anal. calc. for: C,69.02; H, 4.46; N, 12.38; O, 14.14 % Found: C, 69.50; H, 4.370; N, 12.02; O, 15.10 %

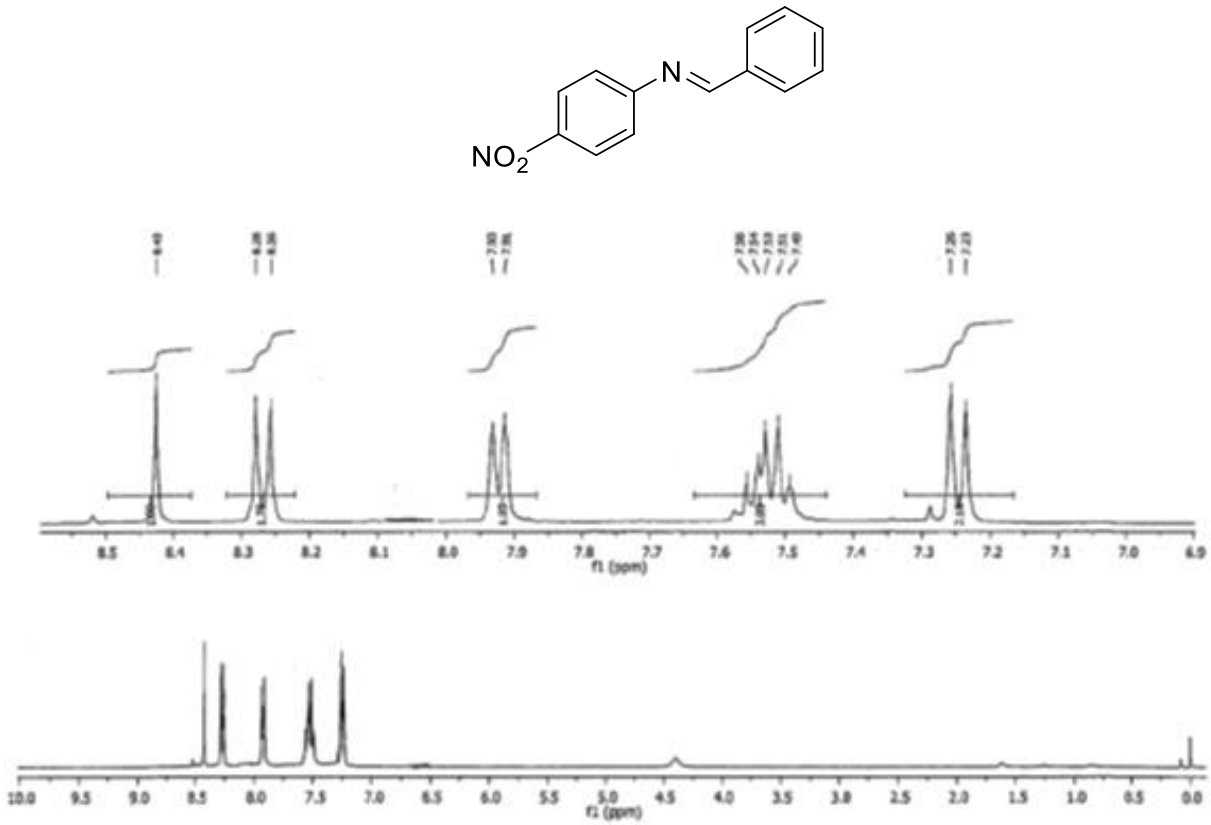


Figure 4. ^{1}H-NMR spectrum (400 MHz) of Compound **3b** ($CDCl_3$, δ ppm)

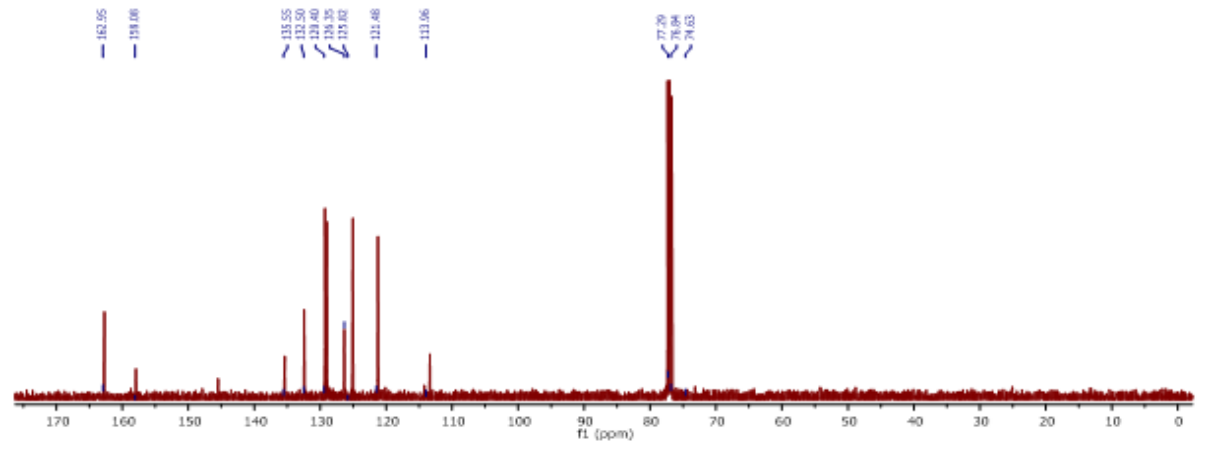


Figure 5. ^{13}C-NMR Spectrum (400 MHz) of Compound **3b** ($CDCl_3$, δ ppm)

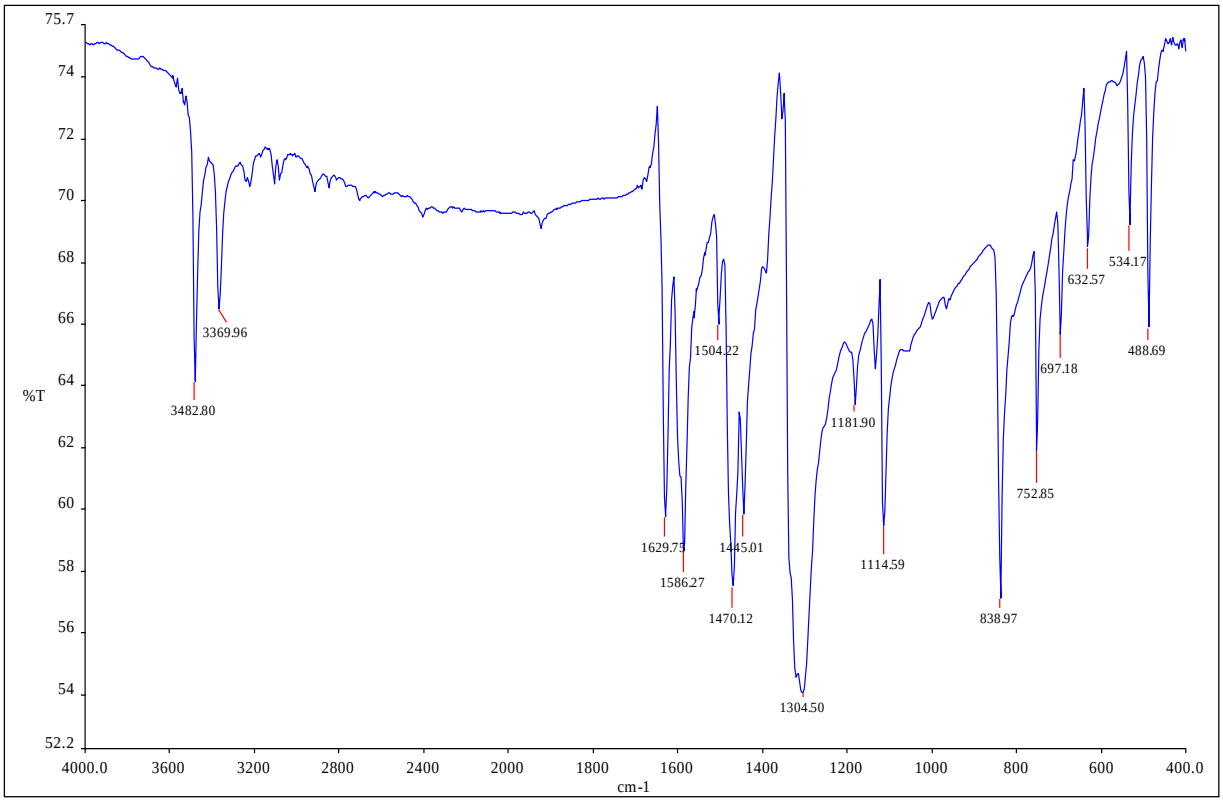


Figure 6. FTIR spectrum of Compound **3b** ($CDCl_3$, cm^{-1})

3.3. Purification Results of Carbonic Anhydrase Isoenzymes (hCA-I and hCA-II)

hCA-I and hCA-II isoenzymes were purified in one step using human blood Sepharose 4B-L tyrosine-sulphanylamide affinity chromatography. hCA-I was purified with specific activity of 2953.1 fold and 60.2 %, hCA-II purified with 797.33 specific activity, 7497.2 fold and 52.3% yield (Table 1).

Table 1. Purification of hCA I and hCA II from human erythrocytes

Purification steps	Volume	Activity	Total activity		Protein	Specific activity	Purification layer number
	(mL)	(EU/mL)	(EU)	(%)	(mg/mL)	(EU/mg)	
Hemolysate	100	44.67	4467	100	1.64×10^3	0.27	-
hCA-I	45	59.8	2691	60.2	0.075	797.33	2953.1
hCA-II	35	66.8	2338	52.3	0.033	2024.24	7497.2

The carbonic anhydrase enzyme has been purified many times from numerous different organisms and the effects of various pesticides, chemicals and drugs on enzyme activities have been investigated. In this study, hCA-I and hCA-II isoenzymes were purified by Sepharose 4B-L-tyrosine-sulphanylamide affinity chromatography and kinetic studies for schiff base (**3a, 3b**) were carried out by hydratase activity method using CO_2 as substrate. For two isoenzymes this was seen as a highly effective activator. The inhibitory effects on the activity of the isoenzymes were tested in vitro, the IC_{50} values were calculated from the % Activity- [inhibitor] plot drawn for compound (**3a, 3b**) and the results are given in (Figure 7), (Figure 8).

Chemical	**CA-I** IC50	**CA-II** IC50
$C_{13}H_{10}FN$	1,82	1,85
Acetazolamide (AAZ)	5.2	6.1

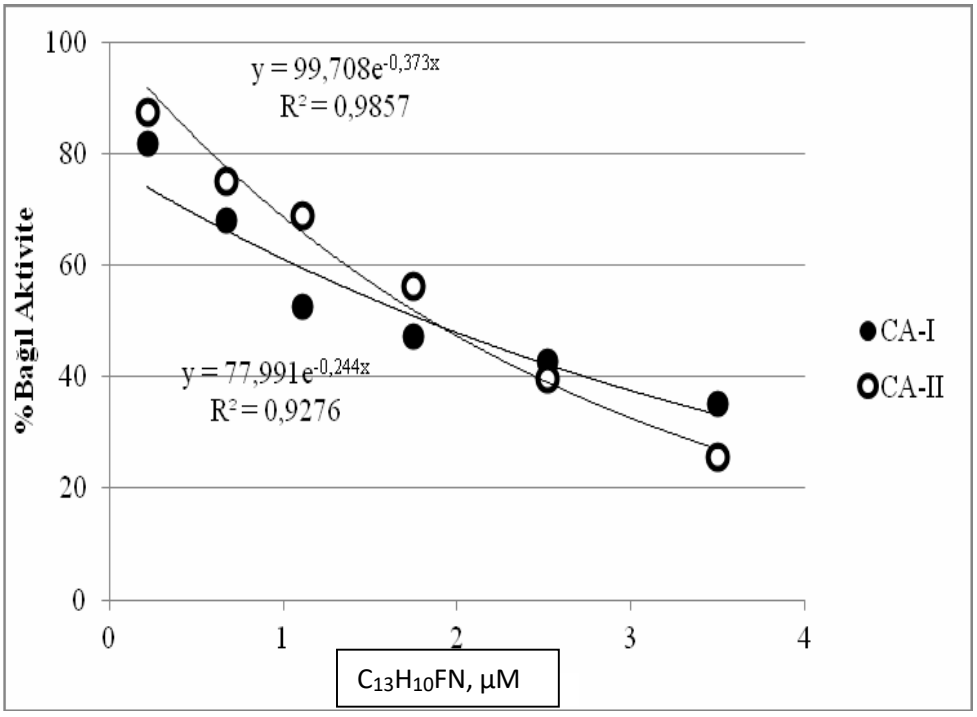


Figure 7. IC_{50} values for the in vitro inhibition of hCA I and hCA II with synthetized *N*-Benzylidene-4-fluoroaniline (**3a**)

Chemical	**CA-I** IC_{50}	**CA-II** IC_{50}
$C_{13}H_{10}N_2O_2$	1,85	2,07
Acetazolamide (AAZ)	5.2	6.1

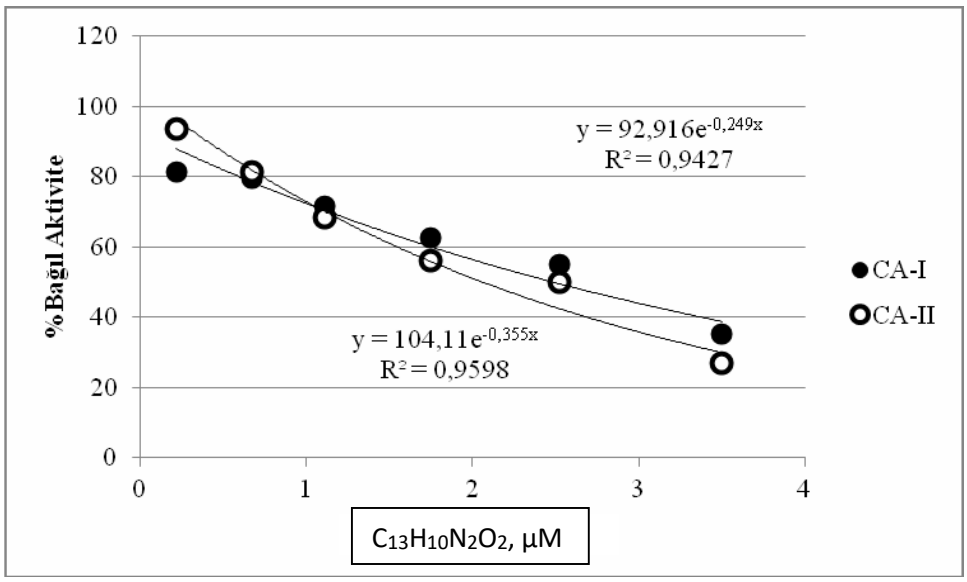


Figure 8. IC_{50} values for the in vitro inhibition of hCA I and hCA II with synthetized N-Benzylidene-4-nitroaniline (**3b**)

4. DISCUSSION

In vitro effects of $C_{13}H_{10}FN$, $C_{13}H_{10}N_2O_2$ on human activities of hCA-I and hCA-II enzyme purified from human erythrocytes revealed that inhibitory activity against acetazolamide reference compound was higher. The most effective effect on hCA1 and hCAII isoenzymes has IC_{50} values of 1.82 mM and 1.85 mM, respectively, of the $C_{13}H_{10}FN$, compound. It is thought that the newly synthesized Schiff bases show strong inhibition effect on hCAI and hCAII isoenzymes and they will serve as a reference for the formation of new inhibitors of CA enzyme [26-27].

The pharmacological effects of synthesized compounds **3a** and **3b** can be improved clinically for hCA I and II. The novel therapeutic applications of these enzyme inhibitors or activators will be directed to the design of prodrugs and drugs in the health sector.

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