Optimization of the Synthesis of Diclofenac Derivatives with Hydrazone Structure and In Vitro Evaluation of the Anti-Inflammatory Potential

ALIN FOCSA¹, ANDREEA IACOB¹*, IOANA VASINCU¹, SANDRA CONSTANTIN¹, LOREDANA ANDRIESCU¹, ALEXANDRU SAVA¹, FREDERIC BURON², SYLVAIN ROUTIER², MARIA APOTROSOAEI¹*, LENUTA PROFIRE¹

¹University of Medicine and Pharmacy “Grigore T. Popa” from Iasi, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 16 Universității Str., 700115, Iasi, România
²University of Orleans, Institute of Organic and Analytical Chemistry, Pole de Chimie, Chartres Str., 45067, Orleans, France

Abstract. The aim of the study was to optimize the synthesis of diclofenac derivative with hydrazones structure in order to obtain higher yields and purity by variation of different parameters such as: ratio between reactants, solvent, catalyst, temperature, time of reaction and method used. The anti-inflammatory effects of diclofenac derivatives were evaluated using in vitro assays: albumin denaturation and erythrocyte membrane stability. The obtained results showed that the effect of the tested derivatives is increasing with the concentration, the best results being obtained at the concentration of 125 µg/mL (albumin denaturation assay), respectively 111.11 µg/mL (erythrocyte membrane stability assay). The most active compound was 4d which showed the highest inhibition effect on albumin denaturation and an appreciable effect on erythrocyte membrane stability, in comparison with diclofenac, used as drug reference.

Keywords: diclofenac, hydrazone, optimization, anti-inflammatory effect

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the oldest and most successful drugs known in modern medicine to decrease pain and inflammation by inhibiting prostaglandins synthesis, but unfortunately their use is associated with a number of serious side effects. NSAIDs, such as diclofenac, are indicated for improvement of all degrees inflammation associated with a large number of conditions, including arthritic disorders, acute musculoskeletal disorders and other painful conditions resulted from trauma. Using NSAIDs have been reported gastrointestinal bleeding, ulceration or perforations, which can be fatal, and can occur at any time during treatment, with or without a history of serious gastrointestinal disorders. If gastrointestinal bleeding or ulceration occurs to patients who receive diclofenac, treatment will be ended [1,2].

It is important to note that cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes play an important role in inflammation. The modifying the structure of the classical NSAIDs is a common practice which was used over the years in order to improve the biological effects of parent molecule. Hydrazone derivatives represent an attractive group of compounds with a broad spectrum of pharmacological and biological characteristics [3,4]. In order to improve the pharmacological profile of diclofenac, some hydrazone derivatives were synthesized by researchers [5,6].

The aim of this study was to optimize the method for synthesis of diclofenac derivatives with hydrazone structure in order to apply it for extending the diclofenac hydrazone derivatives library. The synthesized derivatives were evaluated for antiinflammatory effects using in vitro assays.

In vitro anti-inflammatory assays are important tools to select the most active derivatives to be tested using in vivo assays, and so, the use of a high number of animals is avoided [7,8]. Proteins denaturation is a well-documented cause of inflammation and the anti-inflammatory drugs, such as

*email: andreea.panzaria@unfiasi.ro, apotrosoaei.maria@unfiasi.ro
diclofenac, salicylic acid etc. have shown dose-dependant ability to inhibit the thermally induced protein denaturation [8]. It is also known that inflammation is linked with a significant release of lysosomal components and so drugs that increase the stability of cells membrane, as human red blood cell, could reduce the inflammation process [7].

2. Materials and methods
2.1. Chemistry
Reagents and equipments
Diclofenac sodium salt, hydrazine hydrate 64%, 4-methyl-benzaldehyde, ethanol, methanol, dioxane, sulfuric acid, were purchased from Redox Lab Supplies Com S.R.L. Thin layer chromatography F254 plates and deuterated dimethyl sulfoxide (DMSO-d6) were purchased from Sigma Aldrich. Microwave irradiation was carried out using Biotage Initiator System (France), at a standard absorbance level (300 W maximum power). The melting points were measured using a Buchi Melting Point B-540 apparatus and they are uncorrected. The infrared (IR) spectra were recorded on a Thermo Nicolet AVATAR 320 AEK0200713 FT-IR Spectrometer (Canada), at a resolution of 4 cm⁻¹ after 6 scans in the 4000-500 cm⁻¹ range. The spectra were processed using the Omnic Spectra Software. The ¹H-NMR (400 MHz) and ¹³C-NMR (101 MHz) spectra were recorded with a Bruker Avance Spectrometer 400 MHz (Germany), using tetramethylsilane (TMS) as internal standard and DMSO-d6 as solvent. The chemical shifts were shown in δ values (ppm). The mass spectra were recorded using a Bruker MaXis Ultra-High Resolution Quadrupole Time-of-Flight Mass Spectrometer (Germany).

Synthesis
In order to optimize the synthesis of diclofenac derivatives different parameters were varied such as: the molar ratio between reagents, the solvent, temperature, the reaction time, the catalyst and the method used (classic or microwaves). The synthesis was monitored by thin layer chromatography (TLC) using different eluents and the structure of the derivatives was proved by infrared (IR), nuclear magnetic resonance (¹H-NMR, ¹³C-NMR) and high resolution mass spectrometry (HR-MS) [9,10].

The synthesis of the diclofenac derivatives with hydrazone structure was performed according to the Scheme 1.

Scheme 1. The synthesis of diclofenac derivatives with hydrazone structure (4a-s)

2.2. Serum albumin denaturation assay
Reagents and solvents: saline phosphate buffer, bidistilled water, methanol, DMSO, bovine serum albumin (BSA), diclofenac, diclofenac derivatives (4a-s).

Procedure: stock solutions of 1 mg/mL in DMSO were prepared using diclofenac and diclofenac derivatives (4a-s). From each stock solution a volume of 50 μL, 100 μL, 200 μL and 500 μL was measured and methyl alcohol was added to 1000 μL. Then, a sample of 30 μL from each dilution was added to 3 mL of 1% bovine serum albumin solution. The concentration of the tested derivatives in the obtained samples was 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 125 µg/mL, respectively. A mixture of 30 μL of methanol and 3 mL of 1% bovine serum albumin was used as control. The samples and the
control were incubated for 20 min at 37°C, then 5 min at 72°C, cooled at room temperature for 10 min and then 1 mL of phosphate buffered saline pH 7.2 was added. The turbidity of the samples and control was read at 416 nm against distilled water. The capacity of the tested derivatives to inhibit serum albumin denaturation was calculated using the following formula [11]:

\[
\text{Denaturation inhibition (\%)} = \left(\frac{A_c - A_s}{A_c}\right) \times 100
\]

in which:

- \(A_c\) – the absorbance value of the control;
- \(A_s\) – the absorbance value of the sample.

For each sample the effective concentration (EC\(_{50}\)) was calculated by linear regression analysis and diclofenac (1 mg/mL) was used as the reference drug. All experiments were performed in triplicate.

2.3. Erytrocyte membrane stabilization assay

Reagents and solvents: phosphate buffer (\(pH = 7.4\)), NaCl solution (0.36%, 0.85%), erythrocyte suspension (HRBC-human red blood cell, solution 10% (v/v), HRBC in 0.85% NaCl solution, \(pH\) 7.2, DMSO, diclofenac, diclofenac derivatives (4a-s).

Procedure: stock solutions of 1 mg/mL in DMSO were prepared using diclofenac and diclofenac derivatives (4a-s). From each stock solution, a volume of 100 μL, 200 μL and 500 μL was measured and 0.85% NaCl solution was added to 1000 μL. To each sample 1 mL of phosphate buffer, 2 mL of 0.36% NaCl solution and 0.5 mL of HRBC solution (10% v/v) were added. The concentration of the tested derivatives in the obtained samples was 22.22 µg/mL, 44.44 µg/mL and 111.11 µg/mL, respectively. The control was prepared by adding of 1 mL of 0.85% NaCl solution to a mixture of 1 mL of phosphate buffer, 2 mL of 0.36% NaCl solution and 0.5 mL of HRBC [12,13].

The samples and the control were incubated at 37°C for 30 min, then centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was read at 560 nm against distilled water. The capacity of the tested derivatives to preserve erythrocyte membrane expressed as stability (%), was calculated using the following formula [14]:

\[
\text{Stability (\%)} = 100 - \left(\frac{A_s}{A_c}\right) \times 100
\]

in which:

- \(A_c\) – the absorbance value of the control;
- \(A_s\) – the absorbance value of the sample.

For each sample the effective concentration (EC\(_{50}\)) was calculated by linear regression analysis and diclofenac (1 mg/mL) was used as the reference drug. All experiments were performed in triplicate.

3. Results and discussions

3.1. Chemistry

Optimization of the synthesis of ethyl 2-[2-(2,6-dichlorophenylamino)phenyl]acetate (2) was performed using different procedures reported in the literature [15,16]. The influence of different parameters, such as the ratio between diclofenac (1) and H\(_2\)SO\(_4\) as catalyst (1:0.1, 1:0.2, 1:0.25, 1:0.3), the time of reaction (between 30 min to 6 h) and the method used (classic or microwaves) on the reaction yield is presented in Table 1.

It was observed that the highest yield (85%) was obtained using the following conditions: ratio between diclofenac and H\(_2\)SO\(_4\) of 1 eq : 0.25 eq, heating at 80°C (reflux) for 3 h and ethanol as solvent (20 mL).

### Table 1. The different reaction conditions used to optimize the esterification reaction of diclofenac

| No. | Diclofenac | H\(_2\)SO\(_4\) | Solvent | \(T\) (°C) | Time (min/h) | Method used | Yield |
|-----|------------|----------------|---------|------------|--------------|-------------|-------|
| 1.  | 1 eq.      | 0.1 eq.        | ethanol | 80         | 1 h          | classic     | 55 %  |
| 2.  | 1 eq.      | 0.1 eq.        | ethanol | 80         | 3 h          | classic     | 70 %  |
Optimization of the reaction of diclofenac ethyl ester (classic or microwave) on the reaction yield is presented in Table 2. The influence of different parameters such as the ratio between ethyl 2-[2-(2,6-dichlorophenylamino)phenyl]acetohydrazide (3) was performed using different procedures reported in the literature [17-19]. The influence of different parameters such as the ratio between ethyl 2-[2-(2,6-dichlorophenylamino) phenyl]acetate (diclofenac ethyl ester) (2) and hydrazine hydrate 64% (1:1, 1:5, 1:11, 1:15, 1:20, 1:25), the temperature (80°C or 105°C), the solvent (ethanol or dioxane), the time of reaction (between 30 min to 12 h) and the method used (classic or microwave) on the reaction yield is presented in Table 2.

It was observed that the highest yield (90%) was obtained using the following conditions: ratio between diclofenac ethyl ester (2) and hydrazine hydrate 64% of 1 eq.: 20 eq., dioxane as solvent and heating at 105°C (reflux) for 6 h. Using these conditions the compound 3 was obtained in yield which is higher than the value of 72% which was reported by other researchers [5,6].

### Table 2. The different reaction conditions used to optimize the reaction of diclofenac ethyl ester with hydrazine hydrate

| No. | Diclofenac ethyl ester | Hydrazine hydrate 64% | Solvent | T (°C) | Time (min/h) | Method used | Yield |
|-----|------------------------|-----------------------|---------|--------|-------------|------------|-------|
| 1.  | 1 eq.                  | 1 eq.                 | ethanol | 80     | 2           | classic    | nd    |
| 2.  | 1 eq.                  | 5 eq.                 | ethanol | 80     | 4           | classic    | nd    |
| 3.  | 1 eq.                  | 15 eq.                | ethanol | 80     | 5           | classic    | 15 %  |
| 4.  | 1 eq.                  | 20 eq.                | ethanol | 80     | 6           | classic    | 25 %  |
| 5.  | 1 eq.                  | 20 eq.                | ethanol | 80     | 30 min      | mw         | 23 %  |
| 6.  | 1 eq.                  | 1 eq.                 | dioxane | 105    | 2           | classic    | nd    |
| 7.  | 1 eq.                  | 5 eq.                 | dioxane | 105    | 3           | classic    | 16 %  |
| 8.  | 1 eq.                  | 10 eq.                | dioxane | 105    | 4           | classic    | 25 %  |
| 9.  | 1 eq.                  | 15 eq.                | dioxane | 105    | 3           | classic    | 46 %  |
| 10. | 1 eq.                  | 20 eq.                | dioxane | 105    | 2           | classic    | 65 %  |
| 11. | 1 eq.                  | 20 eq.                | dioxane | 105    | 4           | classic    | 80 %  |
| 12. | 1 eq.                  | 20 eq.                | dioxane | 105    | 6           | classic    | 90 %  |
| 13. | 1 eq.                  | 20 eq.                | dioxane | 105    | 9           | classic    | 79 %  |

Yield: 85%; m.p: 72°C; IR (cm⁻¹): 3145, 3090, 3016 (-NH-), 2355, 1588, 1298 (CH₃), 1696 (-CO-), 897, 765, 744, (-C-Cl); ¹H-NMR (400 MHz, DMSO-d₆, δ ppm): 1.22 (3H, m, CH₃), 3.79 (2H, s, CH₂CO), 4.13 (2H, q, CH₂) 6.27 (1H, d, Ar-H3), 6.86 (1H, t, Ar-H5), 7.06 (2H, d, Ar-H4, NH), 7.20 (2H, m, Ar-H6, Ar-H4′), 7.53 (2H, m, Ar-H3′, Ar-H5′); ¹³C-NMR (101 MHz, DMSO-d₆, δ ppm): 172.00 (O=C), 143.26, 137.54, 131.36, 131.06, 129.65, 128.19, 126.36, 123.72, 121.13, 116.35 (Ar-C), 61.07 (O-CH₂), 37.63 (Ar-CH₂), 14.55 (CH₂-CH₃); HR-MS: m/z calculated for C1₈H₁₅Cl₂NO₂, [M + H]^+ 324.055261, found 324.055286; TLC (petroleum ether:ethyl acetate = 9.0:1.0 v/v) Rf=0.78.

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The physicochemical characteristics of the synthesized diclofenac hydrazones are presented in our paper [23].

Yield: 80%; m.p.: 164°C; IR (cm\(^{-1}\)): 3325 (-NH\(_2\)), 3155, 3070, 3024 (-NH-), 2361, 1582, 1288 (CH\(_2\)), 1636 (-CO-NH), 987, 771, 741, (-C-Cl); \(^1\)H-NMR (400 MHz, DMSO-d\(_6\), \(\delta\) ppm): 3.53 (2H, 2s, CH\(_2\)CO), 4.32 (2H, d, NH\(_2\)), 6.31 (1H, d, Ar-H3), 6.85 (1H, t, Ar-H5), 7.04 (1H, t, Ar-H4), 7.17 (2H, m, Ar-H6, Ar-H4'), 7.51 (2H, m, Ar-H3', Ar-H5'), 8.54 (1H, 2s, NH), 9.49 (1H, 2s, CONH); \(^1^3\)C NMR (101 MHz, DMSO-d\(_6\), \(\delta\) ppm) 171.27 (O=Cl), 143.44, 137.68, 130.81, 129.74, 129.67, 129.67, 127.71, 127.71, 125.83, 125.42, 121.18, 116.52 (Ar-C), 38.13 (Ar-CH\(_2\)); HR-MS: \(m/z\) calculated for C\(_{14}\)H\(_{13}\)Cl\(_2\)N\(_3\)O, [M + H]\(^+\) 310.050844, found 310.050844; TLC (petroleum ether : ethyl acetate = 8.0 : 2.0 v/v) \(R_f=0.1\).

Optimization of the synthesis of 2-[(2,6-dichlorophenyl)-N-[(E)-(4-methylphenyl)methylideneamino]acetamide (4i) was performed using different procedures reported in the literature [19-22]. The influence of different parameters such as the ratio between 2-[(2,6-dichlorophenyl)-methylideneamino]phenyl]acetohydrazide (diclofenac hydrazide) (3), 4-methyl-benzaldehyde and glacial acetic acid, as catalyst, the reaction temperature (65°C or 80°C), the solvent (methanol or ethanol), the time of reaction (between 30 min and 6 h) and the method used (classic or microwave) on the reaction yield is presented in Table 3.

It was observed that the highest yield (92%) was obtained using the following conditions: ratio between diclofenac hydrazide (3), 4-methyl-benzaldehyde and glacial acetic acid of 1 eq.: 1.5 eq.: 0.25 eq., ethanol as solvent and heating at 80°C for 2 h. Using these conditions, the compound 4i was obtained in a yield which is higher than the value of 65% which was reported by other researchers, which didn’t use the catalyst, and the ratio between diclofenac hydrazide and 4-methyl-benzaldehyde was 1:2 [5].

Table 3. The different reaction conditions used to optimize the reaction of diclofenac hydrazide with 4-methyl-benzaldehyde

| No. | Diclofenac acetoxydrazide | 4-methyl-benzaldehyde | Glacial acid acetic | Solvent | T (ºC) | Time (min/h) | Method used | Yield |
|-----|--------------------------|-----------------------|--------------------|--------|--------|-------------|-------------|-------|
| 1.5 | 1 eq.                    | 2 eq.                 | 0.30 eq.           | methanol | 65     | 2 h        | classic     | 77 %  |
| 1.  | 1 eq.                    | 1 eq.                 | 0.20 eq.           | methanol | 65     | 2 h        | classic     | 82 %  |
| 1.  | 1 eq.                    | 1 eq.                 | 0.20 eq.           | ethanol  | 65     | 2 h        | mw         | 89 %  |
| 1.  | 1 eq.                    | 1 eq.                 | 0.20 eq.           | methanol | 65     | 2 h        | classic     | 86 %  |

\(T (ºC)= \text{temperature; } mw= \text{microwave; nd=non-detected}\)

Using the optimized method for 2-[(2,6-dichloroanilino)phenyl]-N-[(E)-(4-methylphenyl)methylideneamino]acetamide other diclofenac hydrazones were obtained. The physico-chemical characteristics of the synthesized diclofenac hydrazones are presented in our paper [23].
3.2. Serum albumin denaturation assay

The generation of autoantigens in rheumatic and inflammatory disorders can cause tissue protein precipitation, thus leading to the aggravation of these diseases. It was proved that any compound exhibiting a protein degradation inhibition greater than 20% can be further evaluated as a potential anti-inflammatory agent. Many NSAIDs drugs such as aspirin, diclofenac, indomethacin, flufenamic acid, in addition to the ability to inhibit the synthesis of protaglandins (cyclooxygenase inhibition), also showed an appreciable capacity to prevent BSA denaturation [24].

From the analysis of the obtained results (Figure 1) it is observed that for all studied derivatives, the inhibition of bovine serum albumin denaturation increases with the concentration, the highest inhibition being obtained at 125 μg/mL. At this concentration the most active were 4d (95.36 ± 0.43%), 4o (92.83 ± 0.40%) and 4n (92.13 ± 0.37%) which were more active than diclofenac (85.67% ± 0.44%), used as a positive control.

Figure 1. The inhibition (%) of serum albumin denaturation by the diclofenac derivatives (4a-s)

From the analysis of the EC$_{50}$ values (Table 4) we noticed derivatives 4d (R = 3-OCH$_2$CH$_3$-4-OH, CE$_{50}$ = 5.234 ± 0.002) and 4g (R=4-OCH$_3$, EC$_{50}$ = 5.887 ± 0.016), for which the inhibition of bovine serum albumin denaturation is 7.40 times, respectively 6.60 times more intense than diclofenac (EC$_{50}$ = 38.776 ± 0.022). Considerable inhibition effect was also noted for derivatives 4e (R = 4-Br-2-NO$_2$, EC$_{50}$ = 7.321 ± 0.008), 4l (R = 3-Br-4-OH, EC$_{50}$=7.556 ± 0.020) and 4n (R = 2-Br-4-F, EC$_{50}$=9.237 ± 0.016), which are 5.30 times (4e), 5.10 times (4l) and 4.20 times (4n) more active than diclofenac.

Table 4. EC$_{50}$ values (µg / mL) for diclofenac derivatives with hydrazone structure (4a-s), referring to inhibition of bovine serum albumin denaturation

| No. | R             | EC$_{50}$ (µg/mL) | No. | R             | EC$_{50}$ (µg/mL) |
|-----|---------------|------------------|-----|---------------|------------------|
| 4a  | 2-NO$_2$      | 16.631 ± 0.012   | 4k  | 3,4-diF       | 37.871 ± 0.021   |
| 4b  | 4-CN          | 13.848 ± 0.017   | 4l  | 3-Br-4-OH     | 7.556 ± 0.020    |
| 4c  | 3-NO$_2$      | 23.062 ± 0.013   | 4m  | 2,5-diBr      | 37.661 ± 0.016   |
| 4d  | 3-OCH$_2$CH$_3$-4-OH | 5.234 ± 0.002 | 4n  | 2-Br-4-F      | 9.237 ± 0.016    |
| 4e  | 4-Br-2-NO$_2$ | 7.321 ± 0.008    | 4o  | 4-Br-2-F      | 9.845 ± 0.012    |
| 4f  | 2-Cl-5-CF$_3$ | 19.771 ± 0.009   | 4p  | 3-F-4-CH$_3$  | 48.653 ± 0.014   |
| 4g  | 4-OCH$_3$     | 5.887 ± 0.016    | 4q  | 4-F           | 36.181 ± 0.011   |
| 4h  | H             | 22.842 ± 0.014   | 4r  | 3-OCH$_3$-4-CH$_3$ | 16.671 ± 0.005 |
| 4l  | 4-CH$_3$      | 33.793 ± 0.019   | 4s  | 2-Br-3-OH-4-OCH | 46.464 ± 0.019   |
| 4j  | 3-CF$_3$ (4j) | 47.381 ± 0.022   | Di clof enac | 38.776 ± 0.022   |

Data are mean ± SD (n = 3, p<0.05)
3.3. Erythrocyte membrane stabilization test

It is known the stability of lysosomal membrane is crucial for limiting the inflammation. By destroying of the lysosomal membrane, the released cellular constituents activate neutrophils, bactericidal enzymes and proteases, causing destruction of cells and tissue inflammation. The erythrocyte membrane, which is similar to the lysosomal one, is commonly used as assay for assessing the anti-inflammatory effect [25-28].

From the analysis of the obtained results (Figure 2) it is observed that for all tested derivatives, the stability of the erythrocyte membrane increases with the concentration, the highest stability being obtained at the concentration of 111.11 μg/mL. At this concentration the most active were 4e (97.42 ± 0.23%), 4b (96.74 ± 0.40%), 4m (90.46 ± 0.45%) and 4f (87.73 ± 0.39%) for which were more active than diclofenac (50.61 ± 0.28%).

![Figure 2. The stability capacity (%) of the erythrocyte membrane induced by diclofenac derivatives (4a-s)](image)

**Table 5.** EC\textsubscript{50} values (μg / mL) for diclofenac derivatives with hydrazone structure (4a-s), referring to erythrocyte membrane stabilization

| No. | R           | EC\textsubscript{50} (μg/mL) | No. | R           | EC\textsubscript{50} (μg/mL) |
|-----|-------------|------------------------------|-----|-------------|------------------------------|
| 4a  | 2-NO\textsubscript{2} | 153.386 ± 0.013             | 4k  | 3,4-diF     | 146.610 ± 0.019             |
| 4b  | 4-CN        | 45.009 ± 0.012              | 4l  | 3-Br-4-OH   | 135.681 ± 0.027             |
| 4c  | 3-NO\textsubscript{2} | 104.381 ± 0.017             | 4m  | 2,5-diBr    | 48.178 ± 0.021              |
| 4d  | 3-OCH\textsubscript{2}CH\textsubscript{3}-4-OH | 75.496 ± 0.023 | 4n  | 2-Br-4-F    | 131.444 ± 0.027             |
| 4e  | 4-Br-2-NO\textsubscript{2} | 43.452 ± 0.022             | 4o  | 4-Br-2-F    | 221.268 ± 0.013             |
| 4f  | 2-Cl-5-CF\textsubscript{3} | 32.260 ± 0.006             | 4p  | 3-F-4-CH\textsubscript{3} | 77.831 ± 0.019             |
| 4g  | 4-OCH\textsubscript{3} | 96.608 ± 0.008             | 4q  | 4-F         | 131.356 ± 0.031             |
| 5h  | H           | 70.083 ± 0.021             | 4r  | 3-OCH\textsubscript{3}-4-CH\textsubscript{3} | 158.541 ± 0.034             |
| 4i  | 4-CH\textsubscript{3} | 133.249 ± 0.023             | 4s  | 2-Br-3-OH-4-OCH\textsubscript{3} | 175.268 ± 0.023             |
| 4j  | 3-CF\textsubscript{3} | 219.907 ± 0.018             |     |              |                             |

| No  | R       | EC\textsubscript{50} (μg/mL) |
|-----|---------|-------------------------------|
| 4k  | 2,5-diF | 146.610 ± 0.019               |
| 4l  | 3-Br-4-OH | 135.681 ± 0.027             |
| 4m  | 2,5-diBr    | 48.178 ± 0.021              |
| 4n  | 2-Br-4-F    | 131.444 ± 0.027             |
| 4o  | 4-Br-2-F    | 221.268 ± 0.013             |
| 4p  | 3-F-4-CH\textsubscript{3} | 77.831 ± 0.019             |
| 4q  | 4-F         | 131.356 ± 0.031             |
| 4r  | 3-OCH\textsubscript{3}-4-CH\textsubscript{3} | 158.541 ± 0.034             |
| 4s  | 2-Br-3-OH-4-OCH\textsubscript{3} | 175.268 ± 0.023             |

Data are mean ± SD (n = 3, p<0.05)

From the analysis of the EC\textsubscript{50} values (Table 5) we noticed the derivatives 4f (R=2-Cl-5-CF\textsubscript{3}, EC\textsubscript{50} = 32.260 ± 0.006) and 4e (R = 4-Br-2-NO\textsubscript{2}, EC\textsubscript{50} = 43.452 ± 0.022) for which the stabilization capacity of the erythrocyte membrane is 3.40 times, and respectively 2.50 times more intense than diclofenac (EC\textsubscript{50} = 109.684 ± 0.019). Considerable stabilization effect was also noted for derivatives 4b (R=4-CN, EC\textsubscript{50} = 45.009 ± 0.012), 4m (R = 2,5-diBr, EC\textsubscript{50} = 48.178 ± 0.021) and 4h (R =H-, EC\textsubscript{50}=70.083 ± 0.021), these being 2.40 times (4b), 2.30 times (4m) and 1.60 times (4h) more active than diclofenac.
4. Conclusions

By variation of different synthesis parameters such as: the ratio between reagents, catalyst, the solvent, the temperature, the time of reaction and the method used (classic or microwave), many chemistry procedures were developed in order to increase the yield of synthesis and the purity of diclofenac derivatives. The derivatives 4d (R=3-OCH₂CH₃-4-OH) and 4g (R=4-OCH₃), obtained by reaction of diclofenac hydrazide and 3-ethoxy-4-hydroxy-benzaldehyde and 4-methoxy-benzaldehyde respectively, showed the most intense effect on inhibition of serum albumin denaturation. In addition, compound 4d showed also a good stabilization effect on the erythrocyte membrane. An intense effect on stabilization of erythrocyte membrane, in reference to diclofenac, was also showed by derivative 4f (R = 2-Cl-5-CF₃). The obtained results support that the chemical modulation of diclofenac structure had as result increasing the anti-inflammatory effects of some hydrazones derivatives, which opens new perspectives in the treatment of inflammatory diseases.

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