ORIGINAL ARTICLE

ASSESSMENT OF *IN VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF NEW AZETIDIN-2-ONE DERIVATIVES OF FERULIC ACID

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Abstract

The *in vitro* antioxidant potential of new azetidin-2-one derivatives of ferulic acid was evaluated according to DPPH and $ABTS^{+}$ radicals scavenging assays. Their anti-inflammatory effect was also evaluated using two *in vitro* assays: bovine serum albumin denaturation and human red blood cell membrane stabilization. The results showed that the compound **1e** (R = 4-F) was the most active. The antioxidant effect of this compound was more intense than ferulic acid and comparable with ascorbic acid. Also, the anti-denaturation activity of this compound was more intense than ferulic acid and comparable with diclofenac used as positive control. Regarding the ability to protect the erythrocyte membrane it was observed that all tested compounds showed a protection capacity comparable with diclofenac and higher than ferulic acid, at the concentration of 1100 μg/mL.

Rezumat

A fost evaluat potenţialul antioxidant *in vitro* al unor noi derivaţi de azetidin-2-onă ai acidului ferulic, utilizând testele de determinare a efectului antioxidant faţă de radicalul DPPH şi radicalul cation $ABTS^{\cdot+}$. Potenţialul lor antiinflamator a fost evaluat utilizând două teste *in vitro*: inhibarea denaturării albuminei serice bovine şi testul de stabilizare a membranei eritrocitare. Rezultatele au demonstrat că cel mai activ compus este derivatul **1e** (R = 4-F). Potenţialul lui antioxidant este mai intens decât al acidului ferulic şi comparabil cu cel al acidului ascorbic. De asemenea, activitatea antidegradantă a acestui compus a fost mai intensă decât a acidului ferulic şi comparabilă cu cea a diclofenacului, utilizat ca martor pozitiv. În ceea ce priveşte capacitatea tuturor compuşilor testaţi de a stabiliza membrana eritrocitară, aceasta a fost comparabilă cu cea a diclofenacului şi mai intensă decât a acidului ferulic, la concentraţia de 1100 μg/mL.

Keywords: ferulic acid, azetidin-2-one, anti-inflammatory assay, radical scavenging assay

Introduction

Free radicals can damage the human cells or tissues and may lead to different physiological disorders such as: diabetes mellitus, inflammation, cancer, cardio-vascular and neurological disorders, aging [1, 2]. Related to the implication of oxidative stress in inflammatory diseases it is known that chronic inflammation is correlated with the increase of reactive oxygen species (ROS) in cells (neutrophils, monocytes, macrophages, eosinophils) and the decrease of antioxidant enzymes (catalase, superoxide dismutase, glutationperoxidase).

In the last years, development of new compounds with heterocyclic structure in order to reduce the oxidative stress is a major concern for researchers. In this context, special attention was focused on azetidin-2-one derivatives.

Azetidin-2-one is known as a key structural unit for important antibiotics, such as penicillins, cephalosporins, carbapenems, monobactams and nocardicine [3]. Recently, new azetidin-2-one derivatives that showed other important biological effects such as: anti-inflammatory [4], antihaemorrhagic [5], anti-tumoural [6], antiviral [7], hypolipemiant [15], anti-tubercular [9, 10], analgesic, antidiabetic [11] were developed.

On the other hand, ferulic acid is a phenolic compound widely distributed in the vegetal reign. Due to its antioxidant effect, ferulic acid may protect lipids from oxidation by ROS and could be useful in the prevention and treatment of several oxidative stress-related disorders [8, 12].

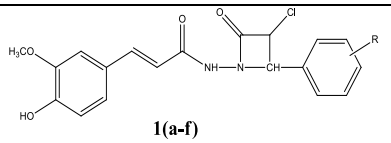
The aim of this study was the biological evaluation of new azetin-2-one derivatives of ferulic acid referring to their potential antioxidant and anti-inflammatory effects.

Materials and Methods

The structure of the tested compounds, azetidin-2-one derivatives of ferulic acid, is presented in Table I.

Table I

Azetidin-2-one derivatives of ferulic acid (**1a-f**)



1(a-f)

Compound	R	Compound	R
1a	-H	**1d**	-Cl(4)
1b	-OH(2)	**1e**	-F(4)
1c	$-NO_2$(2)	**1f**	-Br(4)

The reagents used for the antioxidant assays: $ABTS^{\cdot+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ammonium persulfate, methanol, ethanol, dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich Company and Fluka Company.

The reagents used for the anti-inflammatory assays: bovine serum albumin (BSA), 2-amino-2-hydroxy-methyl-propane-1,3-diol (Tris), diclofenac sodium, phosphate buffer (pH = 7.4), isosaline solution (NaCl 0.85%), hyposaline solution (NaCl 0.36%), acetic acid, methanol, dimethylformamide (DMFA) were purchased from Sigma Aldrich Company and Fluka Company.

DPPH radical scavenging assay. The tested compounds were dissolved in DMSO to obtain a stock solution with the concentration of 2 mg/mL, according to the procedure from the literature [13, 14] with minor modifications. Different volumes from the stock solution (50 µL, 100 µL, 150 µL, 200 µL) were diluted with methanol in order to obtain 200 µL and then were mixed with 2.8 mL of methanol solution of DPPH (0.1 mM, A_{517nm} = 1.0 ± 0.05). The mixture was incubated for 30 min, in the dark, at room temperature. The absorbance was measured at 517 nm against a blank solution (methanol). The radical scavenging ability (I%) was calculated according to the following equation:

$$I\% = (A_0 - A_t / A_0) \times 100,$$

where: A_0 is the absorbance of DPPH methanolic solution of 0.1 mM; A_t is the absorbance of the sample after 30 min [15].

For each compound the effective concentration (EC_{50}) was calculated by linear regression analysis and ascorbic acid (2 mg/mL) was used as positive control. All determinations were performed in triplicate and the values are expressed as mean ± SD.

$ABTS^{+}$ radical scavenging assay. The $ABTS^{\cdot+}$ radicals were activated by treating the solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (7 mM) with ammonium persulfate (2.45 mM) and the mixture was left at room temperature for 16 h in the dark. Before starting the experiment, the $ABTS^{\cdot+}$ solution was diluted with ethanol to obtain an absorbance value of 0.7 ± 0.02 at 734 nm. The tested compounds were dissolved in DMSO to obtain a stock solution with the concentration of 2 mg/mL. Different volumes from stock solution (5 µL, 10 µL, 20 µL, 25 µL) were diluted with DMSO up to 25 µL, to which it were added 1975 µL of $ABTS^{\cdot+}$ solution. After 6 min the absorbance was measured at 734 nm and the radical scavenging ability was calculated according to the following equation:

$$I\% = (A_0 - A_t / A_0) \times 100,$$

where: A_0 is the absorbance before adding the sample; A_t is the absorbance after 6 min of reaction. For each sample the effective concentration (EC_{50}) was calculated by linear regression analysis and ascorbic acid (2 mg/mL) was used as positive control. All determinations were performed in triplicate and the values are expressed as mean ± SD [16].

Bovine serum albumin denaturation assay. The tested compounds were dissolved in methanol in order to obtain a stock solution with the concentration of 10 mg/mL [17]. Different volumes (100 µL, 200 µL and 500 µL) were diluted with methanol in order to obtain a final volume of 1 mL. To 50 µL of each dilution there were added 5 mL bovine serum albumin (BSA) 0.2% (in Tris buffer saline, pH = 6.8). The final concentrations of samples in the test tubes were 10 µg/mL, 20 µg/mL and 50 µg/mL respectively. A mixture of 50 µL methanol and 5 mL bovine serum albumin 0.2% was used as control. The samples and the control were incubated at 37ºC for 20 min and then at 72ºC for 5 min. Finally the samples and the control were cooled for 10 min and the turbidity was measured at 660 nm in reference to Tris buffer saline solution.

The inhibition of protein denaturation (%) was calculated using the following formula:

$$\text{Inhibition of denaturation (\%)} = (A_{control} - A_{sample}/A_{control}) \times 100,$$

where: $A_{control}$ = absorbance of the control; A_{sample} = absorbance of the tested compounds.

Diclofenac (0.1 mg/mL) was used as standard anti-inflammatory drug being processed in a similar manner with the samples. All determinations were performed in triplicate and the values are expressed as mean ± standard deviation (SD) [18].

Human red blood cell (HRBC) membrane stabilization assay. The blood was collected from healthy volunteers. Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania approved the study protocol. Informed consent was obtained from all individual participants included in the study. The collected blood was mixed with equal volume of Alsever solution (dextrose 2%, sodium citrate 0.8%, citric acid 0.05%, sodium chloride 0.42%, and distilled water 100 mL) and centrifuged at 3000 rpm. The human red blood cells were washed with

isosaline solution (NaCl 0.85%) and a 10% (v/v) suspension was made with isosaline.

The tested compounds were dissolved in DMFA in order to obtain a stock solution with the concentration of 10 mg/mL. Different volumes (100 µL, 200 µL and 500 µL) were diluted with isosaline solution 0.85% to obtain a final volume of 1 mL. To this solution 1 mL of phosphate buffer (pH = 7.4), 2 mL of hypo-saline solution (0.36%) and 0.5 mL of human red blood cells (HRBC) suspension (10% v/v) were added. The final concentrations of samples in the test tube were 220 µg/mL, 440 µg/mL and 1100 µg/mL. A mixture of 1 mL of phosphate buffer (pH = 7.4), 2 mL of hyposaline solution (0.36%) and 0.5 mL of human red blood cells (HRBC) (10% v/v) was used as control. The samples and the control were incubated at 37ºC for 30 min and then centrifuged at 3000 rpm. The haemoglobin content in the supernatant solution was estimated by using a spectrophotometric method at 560 nm [19]. The erythrocyte membrane stability (%) was calculated using the following formula:

Erythrocyte membrane stability (%) = 100 - (A_{sample}/A_{blood}),

where: A_{sample} = absorbance of the tested compound; A_{blood} = absorbance of the control.

Diclofenac (0.1 mg/mL) was used as standard anti-inflammatory drug being processed in a similar manner with the samples. All determinations were performed in triplicate and the values are expressed as mean ± standard deviation (SD) [8, 20].

Statistical analysis. All data were analysed by one-way analysis of variance (ANOVA) and were expressed as mean ± standard deviation of triplicates Difference was considered to be statistically significant if $p < 0.05$.

Results and Discussion

The antioxidant activity

DPPH radical scavenging assay. The method is based on the ability of DPPH radical to react with antioxidants when its purple colour in methanol solution turns to yellow due to the formation of diphenyl-picrylhydrazyne (DPPH-H), compound with low absorbance at 517 nm. The results (Table II) support that the most active compounds are **1e** (EC_{50} = 3.81 ± 0.35 µg/mL) and **1d** (EC_{50} = 5.48 ± 0.03 µg/mL), these compounds being about 9 times and respectively 6 times more active than ferulic acid (FA, 34.21 ± 0.05 µg/mL) and comparable with ascorbic acid (EC_{50} = 5.21 ± 0.02 µg/mL) at the same concentration. A good antioxidant activity was showed also by **1f** (EC_{50} = 25.21 ± 0.28 µg/mL), its activity being comparable with ferulic acid.

Table II

The DPPH radical scavenging ability (EC_{50}, µg/mL) of azetidin-2-one derivatives (**1a-f**)

Compound	R	EC_{50} (µg/mL)	Compound	R	EC_{50} (µg/mL)
1a	-H	35.31 ± 0.70	**1d**	-Cl(4)	5.48 ± 0.03
1b	-OH(2)	47.31 ± 0.25	**1e**	-F(4)	3.81 ± 0.35
1c	-NO_2(2)	55.35 ± 0.40	**1f**	-Br(4)	25.21 ± 0.28
Ferulic acid (**FA**)		34.21 ± 0.05	**Ascorbic acid**		5.21± 0.02

Data are mean ± SD (n = 3, $p < 0.05$)

$ABTS^{\cdot+}$ radical scavenging assay. The ability of antioxidants to scavenge the radical cation $ABTS^{\cdot+}$ is monitored by the decrease of the intensity of the blue colour of the $ABTS^{\cdot+}$ species. The compounds **1e** and **1d** showed also a better scavenging activity than ferulic acid, being 1.7 times (**1e**, EC_{50} = 6.05 ± 0.02) and respectively 1.5 times (**1d**, EC_{50} = 7.23 ± 0.05 µg/mL) more active. Moreover, their activity was comparable with ascorbic acid at the same concentration (Table III). A good antioxidant activity was showed also by **1f** (EC_{50} = 10.17 ± 0.04 µg/mL), its activity being comparable with ferulic acid.

Table III

The $ABTS^{\cdot+}$ radical scavenging ability (EC_{50}, µg/mL) of azetidin-2-one derivatives (**1a-f**)

Compound	R	EC_{50} (µg/mL)	Compound	R	EC_{50} (µg/mL)
1a	-H	11.12 ± 0.05	**1d**	-Cl(4)	7.23 ± 0.05
1b	-OH(2)	26.07 ± 0.04	**1e**	-F(4)	6.05 ± 0.02
1c	-NO_2(2)	28.14 ± 0.03	**1f**	-Br(4)	10.17 ± 0.04
Ferulic acid (**FA**)		10.49 ± 0.03	**Ascorbic acid**		5.23 ± 0.06

Data are mean ± SD (n = 3, $p < 0.05$)

The anti-inflammatory activity

Bovine serum albumin (BSA) denaturation assay. Protein denaturation is one of the causes of inflammation and consequently the agents that can prevent the protein denaturation could be useful for the treatment of inflammatory diseases. It is considered that any compound that inhibits the protein denaturation more than 20% could be studied as potential anti-inflammatory agent [18]. The anti-denaturation activity of the azetidin-2-one derivatives of ferulic acid (**1a-f**) at different concentrations is presented in Figure 1. It was observed that the inhibition of BSA

denaturation is increasing with the concentration, the better anti-denaturation activity being observed at 50 µg/mL. At this concentration the most active compound was **1e** (R = 4-F, I% = 98.23 ± 0.21), for which the anti-denaturation activity was comparable to diclofenac (I% = 98.61 ± 0.01).

Also, compounds **1a** (R = H, I% = 97.38 ± 0.02), **1b** (R = 2-OH, I% = 97.38 ± 0.14), **1c** (R = 2-NO_2, I% = 97.69 ± 0.20) and **1f** (R = 4-Br, I% = 96.69 ± 0.11) showed an albumin anti-denaturation activity comparable with diclofenac (I% = 98.61 ± 0.01) and higher than ferulic acid (I% = 93.88 ± 0.14) respectively.

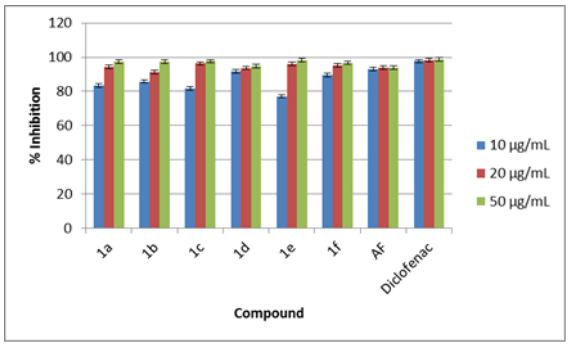


Figure 1.
The BSA denaturation inhibition (%) of azetidin 2-one derivatives (**1a-f**) at different concentrations

Human red blood cell (HRBC) membrane stabilization assay. Exposure of HRBC to hypotonic conditions results in the lysis of the membranes, with the haemolysis and oxidation of haemoglobin [19, 20]. Membrane stabilization is correlated with prevention of leakage of serum protein into the tissues and limiting the inflammatory response. The membrane stabilizing activity of the azetidin-2-one derivatives of ferulic acid (**1a-f**) at different concentrations is presented in Figure 2.

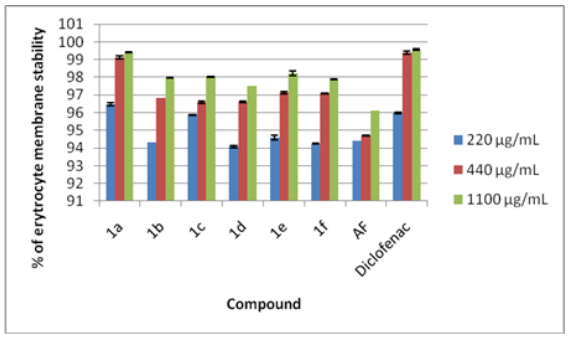


Figure 2.
The erythrocyte membrane stability (%) of azetidin 2-one derivatives (**1a-f**) at different concentrations

For all tested compounds it was observed that protection of erythrocyte membrane was higher than ferulic acid at all tested concentrations: 220 µg/mL, 440 µg/mL and 1100 µg/mL. At 1100 µg/mL all the tested compounds showed a membrane protection comparable with diclofenac (99.56 ± 0.04%) and higher than ferulic acid (96.11 ± 0.01%). The higher protection was observed for compound **1a** (R = H) at all tested concentrations.

Conclusions

The antioxidant and anti-inflammatory activities of new azetidin-2-one derivatives of ferulic acid were evaluated using *in vitro* methods. The results referring to radical scavenging activity (DPPH, $ABTS^{\cdot+}$ assays) support that the most active compound is **1e** (R = 4-F), its activity being comparable with ascorbic acid, used as positive control, and more intense than ferulic acid. A similar effect was also observed for **1d** (R = 4-Cl). Moreover, the compound **1e**, showed also a good albumin anti-denaturation effect. At the concentration of 50 µg/mL its activity was comparable with diclofenac, used as standard anti-inflammatory drug. Also, all the tested compounds showed a membrane stabilizing activity higher than ferulic acid and comparable with diclofenac, especially at 1100 µg/mL concentration.

Acknowledgement

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