Research Article

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# *In vitro* evaluation of 2-pyrazoline derivatives as DPP-4 inhibitors

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**Abstract**

**Objectives:** In this study, the synthesis of three pyrazoline derivatives and the evaluation of their inhibitory effects on dipeptidyl peptidase (DPP-4) were aimed.

**Materials and methods:** Pyrazoline-based compounds (**1–3**) were obtained *via* the reaction of 1-(2-furyl)-3-(1,3-benzodioxol-5-yl)-2-propen-1-one with 4-substituted phenylhydrazine hydrochloride. The DPP-4 inhibitory effects of compounds **1–3** were determined with a fluorometric assay using Gly-Pro-Aminomethylcoumarin as the fluorogenic substrate. The cytotoxicity of compounds **1–3** on L929 mouse fibroblast (healthy) cell line was evaluated using MTT assay.

**Results:** 1-(4-Methylsulfonylphenyl)-3-(2-furyl)-5-(1,3-benzodioxol-5-yl)-2-pyrazoline (**2**) exhibited the highest DPP-4 inhibitory activity ($IC_{50}$=5.75 ± 0.35 μM). Moreover, compound **2** exerted no significant cytotoxicity against L929 cells ($IC_{50}$=34.33 ± 7.09 μM).

**Conclusions:** Target compounds exhibited moderate DPP-4 inhibitory activity and compound **2** was identified as the most active compound.

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**Keywords:** cytotoxicity; diabetes mellitus; dipeptidyl peptidase-4; molecular docking; pyrazoline.

## Introduction

Diabetes mellitus (DM) is a long-term disease characterized by impaired glucose metabolism and vascular consequences. Insulin-dependent DM is related to immune-mediated devastation of β-cells of the pancreas. However, non-insulin-dependent DM is associated with insulin deficiency caused by β-cell dysfunction and receptor desensitization [1, 2]. An impaired glucometabolic state can affect many organs and body systems leading to serious and, possibly life-threatening complications. Many complications triggered by diabetes are widely known to cause significant morbidity and mortality rates [3]. Higher mortality rates caused a substantial reduction in life expectancy at all ages in diabetic patients [4]. Although new treatment strategies have been developed to increase the life expectancy and the quality of diabetic patients, unpredictable causes such as epidemics and pandemic infectious diseases often lead to fluctuations in survival rates. This volatility has increased dramatically with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection that has infected hundreds of millions of people across the globe and caused the death of nearly 5 million people [5]. Although the symptoms of the SARS-CoV-2 infection range in a wide spectrum, patients with chronic diseases, particularly diabetes, face a difficulty hard to deal with as a result of the epidemic [6]. Various studies have indicated a high mortality rate in T2DM patients, that are more prone to evolve a severe disease state and advancement of coronavirus disease 2019 (COVID-19) [7–9]. Furthermore, some investigators found that COVID-19 patients without a history of diabetes had new-onset hyperglycemia, insulin resistance, and β-cell dysfunction [10, 11]. Although COVID-19 is a novel infectious disease, the scientific data about this linkage is still insufficient but increasing [9, 12]. These results demonstrate that there is a bidirectional linkage between diabetes and severe COVID-19 symptoms.

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Therefore, researchers focused on promising treatment strategies and potential mechanisms of interaction between the two pandemics, diabetes mellitus and COVID-19 [3]. Dysregulated immune and inflammatory responses are the junctions of both diseases. Researchers suggest that inconspicuous alterations in the immune system and renin-angiotensin-aldosterone system (RAAS), in addition to the proinflammatory state, oxidative damage, and diminished endothelial function in diabetes can strengthen the reactions initiated by COVID-19 [12]. Free oxygen radicals and interleukin-6 (IL-6), activation of RAAS by COVID-19 trigger insulin resistance, hyperglycemia, and impaired endothelial functions. Angiotensin-converting enzyme 2 (ACE2) is the crucial enzyme of this potential pathway. Although dipeptidyl peptidase-4 (DPP-4) may operate as a linkage point for SARS-CoV-2, ACE2 is the major entrance receptor [13]. Possible links between DPP-4 and RAAS have not been thoroughly investigated but researchers point to possible genetic and functional links that play important roles in the seriousness of SARS-CoV-2, notably in diabetic individuals [14]. Elevations in DPP-4 expression at blood T cells from diabetic individuals; insulin resistance, and the upregulation of DPP-4 in diabetic animals which led to immune response dysregulation, support these findings [13].

DPP-4 has two types; the soluble form circulating in the blood and the plasma membrane-bound form and is responsible for normal glucose tolerance and insulin metabolism. DPP-4 also plays a key role in inflammatory and immune functions [13, 15, 16]. The enzyme is expressed in different tissues such as respiratory and urinary tracts, gastrointestinal system, and immune system including T cells. DPP-4 is thought to be associated with the regulation of many cytokines and chemokines activity, and incretins [17–19]. Incretins not only induce insulin secretion but also weigh down glucagon secretion in a blood-glycemia-dependent manner to normalize blood glucose levels [19]. DPP-4 reduces insulin secretion and abnormal adipose tissue metabolism *via* the degradation of incretins [20]. DPP-4 inhibitors known as gliptins are used to regulate both fasting and postprandial hyperglycemia by increasing circulating incretin levels [21].

Apart from its role in the glucose and insulin mechanism, DPP-4 might exacerbate COVID-19 transmission through the respiratory tract and propagation to other organs and systems and might participate in excessive cytokine release and immune dysregulation [18].

In addition, DPP-4 is the target of the viral spread Middle East respiratory syndrome coronavirus (MERS-CoV) and might contribute to the pathological process of COVID-19 as a coreceptor. The simple and expeditious mutational characteristics of COVID-19 may lead to the emergence of a new coronavirus that uses the DPP-4 enzyme as a gateway. In this case, DDP-4 inhibition may result in effective protection against mutant coronavirus [19, 22].

The possible role of DPP-4 in DM and COVID-19 also makes DPP-4 a valuable therapeutic target and therefore the discovery of DPP-4 inhibitors is of great importance.

There are several therapeutic applications of pyrazolines, considered as cyclic hydrazine motifs, and one of them is diabetic treatment [23, 24]. The antidiabetic effects of pyrazoline derivatives may be attributed to their inhibitory effects on DPP-4 [25, 26].

Encouraged by the aforementioned findings, herein we performed the synthesis of three pyrazoline-based compounds (**1–3**) and focused on their DPP-4 inhibitory effects and cytotoxicity. Furthermore, molecular docking was conducted for compounds **1–3** to enlighten their interactions in the active site of DPP-4.

# Materials and methods

## Chemistry

The chemicals were procured from commercial vendors and used without further purification. The melting points (M.p., °C) of the compounds were detected on an Electrothermal IA9200 melting point apparatus (Staffordshire, UK). Thin Layer Chromatography (TLC) was performed on TLC Silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) using petroleum ether-ethyl acetate solvent systems (3:1 and 1:1). Nuclear magnetic resonance (NMR, $^{1}$H and $^{13}$C) spectra were recorded on a Bruker spectrometer (Bruker, Billerica, MA, USA). Mass spectra were recorded on a Shimadzu LCMS-8040 (Shimadzu, Kyoto, Japan).

## General procedure for the preparation of the compounds

**1-(2-Furyl)-3-(1,3-benzodioxol-5-yl)-2-propen-1-one:** A mixture of 2-furyl methyl ketone (0.02 mol) and piperonal (0.02 mol) in the presence of 10% (w/v) aqueous sodium hydroxide (5 mL) in absolute ethanol (30 mL) was stirred at room temperature for 24 h. Upon completion of the reaction, the reaction mixture was poured into crushed ice. The precipitated solid was filtered, washed with water, and dried [27].

**1-(4-Substituted phenyl)-3-(2-furyl)-5-(1,3-benzodioxol-5-yl)-2-pyrazolines (1–3):** A mixture of 4-substituted phenylhydrazine hydrochloride (2 mmol), 1-(2-furyl)-3-(1,3-benzodioxol-5-yl)-2-propen-1-one (1 mmol) and glacial acetic acid (0.5 mL) in absolute ethanol (20 mL) was heated for 10 h. The precipitate was filtered and dried. The product was crystallized from ethanol.

**1-(4-Bromophenyl)-3-(2-furyl)-5-(1,3-benzodioxol-5-yl)-2-pyrazoline (1):** Yield: 82%. M.p.: 100–102 °C. $^{1}$H NMR (300 MHz, DMSO-$d_6$) $\delta$ (ppm): 3.01 (dd, $J_{AB}$=17.46 Hz, $J_{AX}$=5.79 Hz, 1H, $C_4$-$H_A$ pyrazoline), 3.80 (dd, $J_{BA}$=17.40 Hz, $J_{BX}$=12.03 Hz, 1H, $C_4$-$H_B$ pyrazoline), 5.40 (dd,

$J_{BX}$=11.97 Hz, $J_{AX}$=5.79 Hz, 1H, $C_5$-$H_X$ pyrazoline), 5.97 (d, $J$=2.10 Hz, 2H), 6.61–6.74 (m, 3H), 6.92–6.98 (m, 4H), 7.45 (d, $J$=8.76 Hz, 2H), 7.81 (t, $J$=1.56 Hz, 0.84 Hz, 0.72 Hz, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ (ppm): 44.32 ($CH_2$), 63.27 (CH), 101.59 ($CH_2$), 106.54 (CH), 109.16 (CH), 110.35 (CH), 115.36 (2CH), 119.58 (CH), 128.34 (d, $J$=5.25 Hz, 2CH), 132.02 (2CH), 135.83 (C), 136.02 (2C), 143.58 (C), 145.14 (C), 147.12 (C), 148.25 (C). MS (ESI) ($m/z$) $[M]^+$ 411.90, $[M+H]^+$ 411.91.

**1-(4-Methylsulfonylphenyl)-3-(2-furyl)-5-(1,3-benzodioxol-5-yl)-2-pyrazoline (2):** Yield: 85%. M.p.: 126–128 °C. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ (ppm): 3.08 (s, 3H), 3.12 (dd, $J_{AB}$=17.46 Hz, $J_{AX}$=4.32 Hz, 1H, $C_4$-$H_A$ pyrazoline), 3.88 (dd, $J_{BA}$=17.61 Hz, $J_{BX}$=11.94 Hz, 1H, $C_4$-$H_B$ pyrazoline), 5.55 (dd, $J_{BX}$=11.85 Hz, $J_{AX}$=4.83 Hz, 1H, $C_5$-$H_X$ pyrazoline), 5.98 (d, $J$=1.80 Hz, 2H), 6.64–6.66 (m, 1H), 6.72–6.78 (m, 2H), 6.87 (d, $J$=8.01 Hz, 2H), 7.09 (d, $J$=8.91 Hz, 2H), 7.67 (d, $J$=8.94 Hz, 2H), 7.86 (d, $J$=1.62 Hz, 1H) (Figure S1). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ (ppm): 43.44 ($CH_2$), 44.62 ($CH_3$), 61.99 (CH), 101.64 ($CH_2$), 106.41 (CH), 109.20 (CH), 112.79 (d, $J$=17.25 Hz, 2CH), 119.43 (CH), 129.04 (2CH), 129.65 (CH), 135.44 (CH), 142.70 (CH), 145.46 (C), 147.23 (C), 147.27 (2C), 147.38 (2C), 148.32 (C) (Figure S2). MS (ESI) ($m/z$) $[M+H]^-$ 408.90, $[M]^+$ 410.90, $[M+H]^+$ 411.90.

**1-(4-Sulfonamidophenyl)-3-(2-furyl)-5-(1,3-benzodioxol-5-yl)-2-pyrazoline (3) [28]:** Yield: 87%. M.p.: 136–138 °C. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ (ppm): 3.06 (dd, $J_{AB}$=17.55 Hz, $J_{AX}$=4.95 Hz, 1H, $C_4$-$H_A$ pyrazoline), 3.86 (dd, $J_{BA}$=17.58 Hz, $J_{BX}$=12.00 Hz, 1H, $C_4$-$H_B$ pyrazoline), 5.53 (dd, $J_{BX}$=11.88 Hz, $J_{AX}$=4.89 Hz, 1H, $C_5$-$H_X$ pyrazoline), 5.98 (d, $J$=2.25 Hz, 2H), 6.62–6.64 (m, 1H), 6.70–6.75 (m, 2H), 6.84–6.87 (m, 2H), 7.02–7.07 (m, 4H), 7.71 (d, $J$=8.79 Hz, 2H), 7.84 (t, $J$=1.68 Hz, 1.14 Hz, 0.54 Hz, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ (ppm): 44.31 ($CH_2$), 63.39 (CH), 101.57 ($CH_2$), 106.51 (CH), 109.07 (CH), 114.87 (2CH), 116.54 (C), 119.56 (CH), 122.72 (CH), 128.25 (d, $J$=6.75 Hz, 2CH), 129.20 (d, $J$=5.25 Hz, 2CH), 135.83 (C), 136.03 (C), 143.25 (C), 145.02 (C), 147.07 (C), 148.22 (C). MS (ESI) ($m/z$) $[M]^+$ 411.90, $[M+H]^+$ 412.90.

## Biochemistry

**Determination of DPP-4 inhibitory potency:** The experiment was performed using Cayman's DPP-4 inhibitor screening assay kit based on the manufacturer's instructions. The kit includes the fluorogenic substrate, Gly-Pro-Aminomethylcoumarin (AMC), to measure DPP-4 activity. The cleavage of the peptide bond by DPP releases the free AMC group, resulting in fluorescence that is measured at an excitation wavelength of 350–360 nm and emission wavelength of 450–465 nm.

**Cell culture and drug treatment:** L929 mouse fibroblast (ATCC® CRL-6364™) cells were cultured and drug treatments were carried out as previously described [29].

**MTT test:** The level of cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by mitochondrial succinate dehydrogenase was quantified as previously reported [30] with small modifications [29]. The stock solutions of compounds **1–3** in dimethylsulfoxide (DMSO) were prepared and diluted before use. To detect their cytotoxicity, L929 cells ($2 \times 10^4$ cells/mL) were treated with the compounds to give a final concentration in the range of 0.98–250 µM for 24 h. At the end of this period, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for a further 4 h at 37 °C. After the medium was removed and the formazan crystals were solubilized by the addition of 200 µL DMSO to each well and the absorbance was read at 540 nm with a microplate spectrophotometer (BioTek, Winooski, VT, USA). Each concentration was repeated in three wells. $IC_{50}$ values were defined as the drug concentrations that reduced absorbance to 50% of control values.

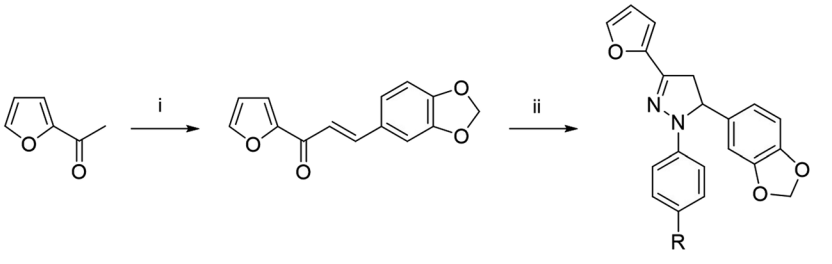
**Statistical analyses:** Statistical Package for the Social Sciences (SPSS) for Windows 15.0 was used for statistical analysis. Data was expressed as Mean ± SD. Comparisons were performed by one way ANOVA test for normally distributed continuous variables and post hoc analyses of group differences were expressed by the Tukey test.

## Molecular docking

Compounds **1–3** were docked to the active site of DPP-4, which was retrieved from Protein Data Bank (PDB) server (PDB code: 5J3J), using Schrödinger Release 2016–2 (Schrödinger, LLC, New York, NY, USA).

# Results

As depicted in Figure 1, the synthesis of compounds **1–3** was performed efficiently via the cyclization reaction of




| Compound | R |
|---|---|
| 1 | Br |
| 2 | $SO_2CH_3$ |
| 3 | $SO_2NH_2$ |

**Figure 1:** The synthetic route for the preparation of compounds **1–3**. Reagents and conditions: (i) Piperonal, 10% (w/v) NaOH, absolute ethanol, rt, 24 h; (ii) 4-substituted phenylhydrazine hydrochloride, AcOH, absolute ethanol, reflux, 10 h.

**Table 1:** DPP-4 inhibition percentages (%) of compounds **1–3**.

| Compound (100 µM) | Inhibition % (Mean ± SD) |
|---|---|
| **1** | 23.17 ± 1.89 |
| **2** | 57.44 ± 2.67 |
| **3** | 53.72 ± 2.59 |
| **Sitagliptin** | 100 |

4-substituted phenylhydrazine hydrochloride with 1-(2-furyl)-3-(1,3-benzodioxol-5-yl)-2-propen-1-one, which was obtained as described earlier [27].

The DPP-4 inhibitory effects of compounds **1**–**3** were examined at 100 µM concentration. These compounds displayed their inhibitory effects on DPP-4 with the inhibition values of 23.17 ± 1.89%, 57.44 ± 2.67%, 53.72 ± 2.59%, respectively when compared with sitagliptin (100.00%), the reference agent. *p*-Methylsulfonyl-substituted compound **2** was determined as the most promising DPP-4 inhibitor in this series as shown in Table 1.

The $IC_{50}$ values of the compounds that inhibit DPP-4 enzyme activity by more than 50% at 100 µM were determined. *p*-Methylsulfonyl-substituted compound **2** was determined as the DPP-4 inhibitor with the highest potential among the tested compounds as shown in Table 2.

The cytotoxic activities of compounds **1–3** toward L929 cells were detected using MTT method. The effective concentration of compound **2** has no cytotoxicity to L929 cells (Table 3).

Molecular docking data revealed that compounds **1**–**3** showed good affinity in the active site of DPP-4 (Figure 2). However, only compound **2** presented substrate-specific interactions. *p*-Methylsulfonyl substituent of compound **2**

**Table 2:** $IC_{50}$ values of compounds **1–3** for DPP–4 inhibition.

| Compound | $IC_{50}$ (µM) |
|---|---|
| **2** | 5.75 ± 0.35 |
| **3** | 67.5 ± 3.54 |
| **Sitagliptin** | 0.019 ± 0.001 |

**Table 3:** $IC_{50}$ values of compounds **1–3** for L929 cells after 24 h incubation.

| Compound | $IC_{50}$ (µM) |
|---|---|
| **1** | 226.67 ± 12.58 |
| **2** | 34.33 ± 7.09 |
| **3** | 66.50 ± 6.36 |

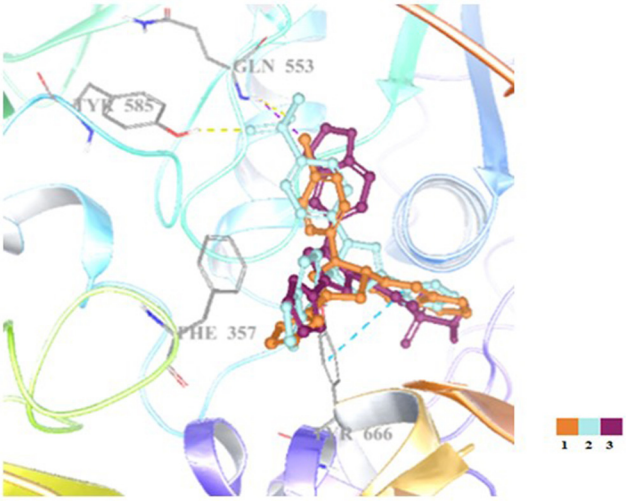



**Figure 2:** Docking poses of compounds **1–3** in the active site of DPP-4 (PDB code: 5J3J).

formed hydrogen bonds with Gln553 and Tyr585 in the active site of DPP-4 as shown in Figure 3A and B. *In silico* data indicated the significance of the *p*-methylsulfonyl group for DPP-4 inhibitory efficacy.

## Discussion

Enzymes are key molecules of biochemical processes. Regulation of enzyme activities by natural and synthetic agents is one of the important strategies in the treatment of diseases.

Enzyme inhibitors are used in many disorders ranging from hypercholesterolemia to cancer treatment. Currently, it is important to note that approximately 47% of drugs used clinically all over the world act through enzyme inhibition [31, 32]. Enzymes are common therapeutic targets for researchers and pharmaceutical companies due to their widespread role in metabolic control and high market share. Another part of this investigation is the design and synthesis of new drug molecules.

Among biologically active heterocycles, pyrazoline is a versatile motif existing in diverse agents with various therapeutic effects such as antidiabetic, anti-inflammatory, antiviral, and so on [33]. It has been reported that some pyrazoline derivatives are more effective in lowering blood glucose levels compared to insulin, which is the standard hypoglycemic agent, in the alloxan-induced diabetic rat model [34]. Although there is no information about the mechanism of the antidiabetic action in this study, there are some studies in the literature indicating that the inhibition of some enzymes may play a role in this effect. Kumar et al.

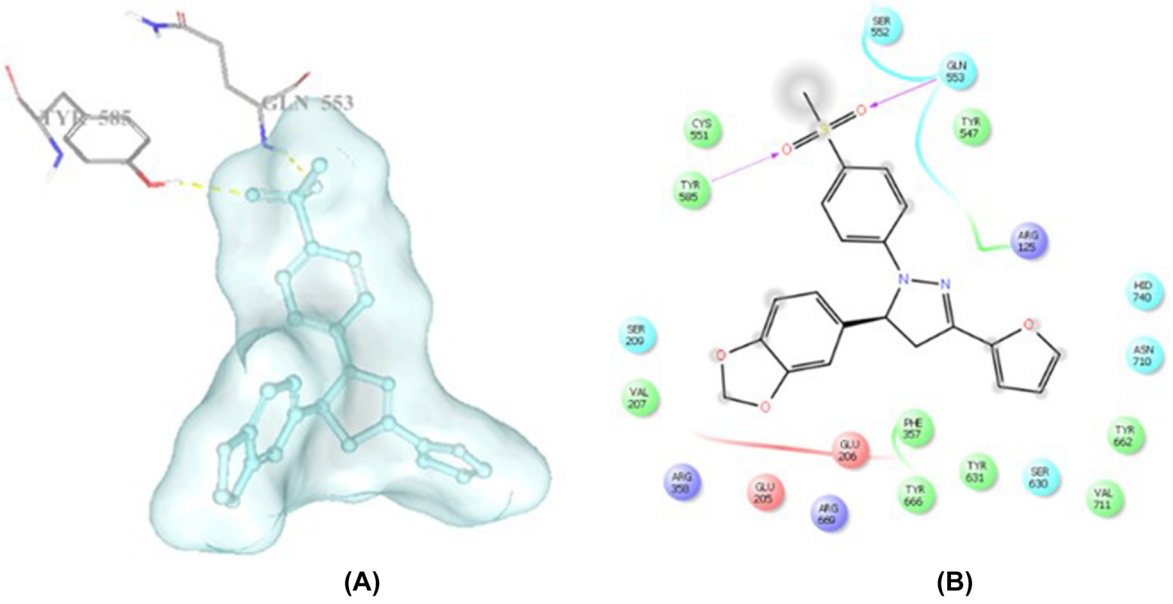



**Figure 3:** Docking pose (**A**) and interactions (**B**) of compound **2** in the active site of DPP-4 (PDB code: 5J3J).

demonstrated that some 1,2,3-triazole-pyrazoline hybrids have strong α-glucosidase inhibitory activity and some of them have higher inhibitory effect than acarbose. The researchers suggested that pyrazoline-triazole hybrids may be precursor compounds for lowering blood glucose concentration [35]. Jun et al. reported that several pyrazoline derivatives with β-amino acyl group displayed inhibitory potency on DPP-4 enzyme activity [26]. Furthermore, Ahn et al. showed the inhibitory effects of cyano-pyrazoline derivatives on DPP-4 enzyme activity in both *in vitro* and *in vivo* experiments [25].

Although DPP-4 is the target of antidiabetic agents, the enzyme has also become a part of coronavirus-related research in recent years and some authors evaluate the potential function of DPP-4 as a target of therapeutic strategies in the pathology of SARS-CoV-2 [18, 19, 22]. Moreover, the investigators suggested that vitamin D and DPP-4 inhibitor combination may play a protective role in the pathophysiology of COVID-19 through different mechanisms [36].

Besides its effect on incretin hormones, DPP-4 has a role in a wide range of biochemical processes such as the regulation of immune response, inflammation, cardiovascular function, vascular events, and viral entry into cells [37].

In conclusion, DPP-4 plays a central role in different diseases, especially diabetes and therefore the discovery of new DPP-4 inhibitors will contribute to the elucidation of the importance of this enzyme in physiological processes and the treatment of diabetes. As a keynote, it can be concluded that pyrazoline derivatives affect key biological targets including DPP-4 involved in diverse biochemical pathways, and this capability makes them promising molecules for drug discovery. The data obtained from our work indicate the potential inhibitory effects of 2-pyrazolines on DPP-4.

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**Informed consent:** Informed consent was obtained from all individuals included in this study.

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