Kragujevac J. Sci. (2025) 47 (1) 63-73
doi: 10.5937/KgJSci2501063M

UDC 547.576:.77:57.083.36
Original scientific paper

CYTOTOXIC POTENTIAL OF NOVEL *N*-FORMYL PYRAZOLINES DERIVED FROM VANILLIN

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(Received January, 14, 2025; Accepted July 04, 2025)

ABSTRACT. In the reaction of vanillin and acetone under Claisen-Schmidt conditions, dehydrozingerone, a very attractive biologically active compound, is obtained. This compound served as a starting material for the synthesis of *O*-alkyl derivatives which further react with hydrazine hydrate in formic acid, yielding a new series of *N*-formyl pyrazolines. All new products were identified and well characterized by IR, ^{1}H NMR, and ^{13}C NMR spectroscopy. An ADME study was performed to investigate the pharmacokinetic properties of the synthesized derivatives. The preliminary *in vitro* cytotoxic activity of pyrazolines against the human cervix adenocarcinoma cell line (HeLa) was evaluated using the MTT method. Among the series, compounds **3c**, **3d** and **3f** showed the most promising cytotoxic activity. Morphology changes of HeLa cells treated with selected compounds were visualized and compared with that of the control.

Keywords: vanillin, dehydrozingerone, pyrazolines, ADME, cytotoxicity, HeLa

INTRODUCTION

It is widely recognized that natural products play a key role in modern drug development, especially for antibacterial and antitumor agents. Ginger root is a very useful plant that is a significant source of many bioactive compounds. These compounds mostly have a broad spectrum of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anticancer, antidiabetic, and antiallergic (NAKAMURA and YAMAMOTO, 1983;

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DUGASANI *et al.,* 2010; CITRONBERG *et al.,* 2013; ZHANG *et al.,* 2013; KUMAR *et al.,* 2014; NILE and PARK, 2015; SEMWAL *et al.,* 2015).

One of the compounds that can be isolated from the mentioned plant is dehydrozingerone (SMITH, 1996; RATKOVIĆ *et al.,* 2016) which is also a very attractive natural product with a wide range of biological activities, mostly antitumor features (MOTOHOSHI *et al.,* 1998; ISHIDA *et al.,* 2002; ADAMS *et al.,* 2004; MAPOUNG *et al.,* 2020). Another important fact is that the enone system in this molecule makes it a good substrate for various transformations into some usable heterocyclic derivatives, such as oxazoles, pyrazoles, pyrazolines, and pyrimidines. In the last decade, pyrazolines have symbolized key structural motifs in heterocyclic chemistry and have been intensively studied as targets for potential anticancer therapeutics. These compounds have received considerable attention due to their valuable biological activities such as antimicrobial (KUMAR *et al.,* 2018), antiinflammatory (EID and GEORGE, 2018), antidepressant (TRIPATHI *et al.,* 2018), anticancer (GOMHA *et al.,* 2018; GUL *et al.,* 2018), and antioxidant (ELBORDINY *et al.,* 2018). Therefore it comes as no surprise that as privileged compounds, pyrazolines are widely incorporated into the structures of numerous important medical and biochemical agents (ANSARI *et al.,* 2017; AHSAN JAWED *et al.,* 2022).

Accordingly, as a part of our research focused on 2-pyrazolines (MUŠKINJA, 2016; RATKOVIĆ *et al.,* 2016) with biological activity, and in connection with our interest in the chemistry of certain natural products (vanillin and dehydrozingerone), in this paper we report the synthesis of some new *N*-formyl pyrazolines. A preliminary *in vitro* cytotoxicity evaluation against human cervix adenocarcinoma cells (HeLa) is also presented.

MATERIALS AND METHODS

Chemistry

All starting chemicals were commercially available and were used as received, except that the solvents were purified by distillation. IR spectra: PerkinElmer Spectrum One FT-IR spectrometer with a KBr disc, in cm^{-1}; NMR spectra: Varian Gemini 200 MHz spectrometer (200 MHz for ^{1}H and 50 MHz for ^{13}C), using $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts in ^{1}H and ^{13}C NMR spectra were reported in parts per million (ppm). Multiplicities are represented by *s* (singlet), *bs* (broad singlet), *d* (doublet), *t* (triplet), *q* (quartet), *sep* (septet), *dd* (doublet of doublets), *ddd* (doublet of doublets of doublets), *ddq* (doublet of doublets of quartets), and *m* (multiplet). The coupling constants (*J*) are given in Hertz (Hz). The melting point of the products was determined using the MelTemp1000 apparatus.

General procedure for the preparation of products 3a-f

To a stirred solution of dehydrozingerone (**2a**, 2 mmol) or its corresponding *O*-alkyl derivatives (**2b-f**, 2 mmol) in formic acid (5 mL), hydrazine monohydrate (0.6 mL) was added, and the reaction mixture was heated under reflux for 5h. The resulting solution was cooled, poured into ice-cold water, neutralized with $NaHCO_3$ (for compound **3a** after neutralizing the solution, 3M HCl was added (pH=4)), and left overnight at –20ºC. Due to the absence of precipitate, the reaction mixture was extracted with CH_2Cl_2 (3×50 mL). The organic layer was washed with water (2×50 ml), and brine (2×50 ml) and dried over anhydrous Na_2SO_4. After removing the main part of the solvent, the residue was filtered over a SiO_2 pad. The solvent was evaporated and the products are mostly obtained in the form of red-orange colored oil. Several products (**3a**, **3b, 3c**, and **3f**) were dissolved in ether and left

standing at –20ºC causing precipitate formation. The precipitates were filtered off, giving these compounds in the form of powdery substances.

5-(4-Hydroxy-3-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-1-carbaldehyde (**3a**)
Light beige powder; yield: 78%; m.p. 129-130°C; IR (KBr, cm^{-1}) ν: 3316, 1664, 1519, 1426, 1317, 1275, 1207, 1021, 821, 754; ^{1}HNMR (200 MHz, $CDCl_3$) δ: 2.08 (*s*, 3H, CH_3), 2.74 (*dd*, 1H, *J*=18.2, 5.0 Hz, $CH_{2pyrazoline}$), 3.38 (*ddq*, 1H, *J*=18.2, 11.5, 1.0 Hz, $CH_{2pyrazoline}$), 3.85 (*s*, 3H, OCH_3), 5.31 (*dd*, 1H, *J*=11.5, 5.0 Hz, $CH_{pyrazoline}$), 6.02 (*bs*, 1H, OH), 6.63-6.68 (*m*, 2H, Ar-H), 6.79-6.83 (*m*, 1H, Ar-H), 8.81 (*d*, 1H, *J*=1.0 Hz, CHO); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 15.8, 46.6, 55.8, 58.4, 108.4, 114.9, 118.1, 133.5, 145.3, 146.8, 157.5, 159.6.
5-(3,4-Dimethoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-1-carbaldehyde (**3b**)
Beige powder; yield: 76%; m.p. 105-106°C; IR (KBr, cm^{-1}) ν: 2983, 1670, 1517, 1433, 1359, 1255, 1232, 1141, 1020, 826, 752; ^{1}HNMR (200 MHz, $CDCl_3$) δ: 2.09 (*s*, 3H, CH_3), 2.75 (*dd*, 1H, *J*=18.2, 5.0 Hz, $CH_{2pyrazoline}$), 3.40 (*dd*, 1H, *J*=18.2, 11.6 Hz, $CH_{2pyrazoline}$), 3.85 (*s*, 3H, OCH_3), 3.86 (*s*, 3H, OCH_3), 5.34 (*dd*, 1H, *J*=11.6, 5.0 Hz, $CH_{pyrazoline}$), 6.69-6.77 (*m*, 2H, Ar-H), 6.83 (*d*, 1H, *J*=8.2 Hz, Ar-H), 8.82 (*d*, 1H, *J*=1.0 Hz, CHO); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 15.8, 46.5, 55.9, 58.3, 108.8, 111.5, 117.6, 133.3, 148.2, 149.3, 157.2, 159.5.
5-(4-Ethoxy-3-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-1-carbaldehyde (**3c**)
Cream powder; yield: 82%; m.p. 73-74°C; IR (KBr, cm^{-1}) ν: 2931, 1659, 1515, 1435, 1265, 1239, 1143, 1032, 812, 761; ^{1}HNMR (200 MHz, $CDCl_3$) δ: 1.44 (*t*, 3H, *J*=7.0 Hz, CH_3), 2.08 (*t*, 3H, *J*=1.0 Hz, CH_3), 2.75 (*ddq*, 1H, *J*=18.2, 4.8, 1.0 Hz, $CH_{2pyrazoline}$), 3.40 (*ddq*, 1H, *J*=18.2, 11.6, 1.0 Hz, $CH_{2pyrazoline}$), 3.85 (*s*, 3H, OCH_3), 4.07 (*q*, 2H, *J*=7.0 Hz, CH_2), 5.34 (*ddd*, 1H, *J*=11.6, 4.8, 1.0 Hz, $CH_{pyrazoline}$), 6.69-6.84 (*m*, 3H, Ar-H), 8.81 (*d*, 1H, *J*=1.0 Hz, CHO); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 14.7, 15.7, 46.5, 55.9, 58.3, 64.3, 109.1, 112.9, 117.5, 133.2, 147.9, 149.6, 157.3, 159.5.
5-(4-Isopropoxy-3-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-1-carbaldehyde (**3d**)
Red-orange oil; yield: 82%; IR (KBr, cm^{-1}) ν: 2975, 1666, 1510, 1427, 1309, 1229, 1256, 1137, 1108, 1033, 755; ^{1}HNMR (200 MHz, $CDCl_3$) δ: 1.35 (*d*, 6H, *J*=6.0 Hz, $CH(\underline{CH_3})_2$), 2.09 (*s*, 3H, CH_3), 2.75 (*dd*, 1H, *J*=18.0, 4.8 Hz, $CH_{2pyrazoline}$), 3.39 (*dd*, 1H, *J*=18.2, 11.6 Hz, $CH_{2pyrazoline}$), 3.83 (*s*, 3H, OCH_3), 4.48 (*sep*, 1H, *J*=6.2 Hz, $\underline{CH}(CH_3)_2$), 5.34 (*dd*, 1H, *J*=11.8, 5.0 Hz, $CH_{pyrazoline}$), 6.69-6.72 (*m*, 2H, Ar-H), 6.69-6.77 (*m*, 2H, Ar-H), 6.83 (*d*, 1H, *J*=8.2 Hz, Ar-H), 8.82 (*d*, 1H, *J*=1.0 Hz, CHO); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 15.9, 22.1, 46.6, 56.1, 58.4, 71.5, 109.6, 116, 117.6, 133.6, 147.1, 150.7, 157.4, 159.6.
5-(4-Butoxy-3-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-1-carbaldehyde **(3e)**
Red-orange oil; yield: 93%; IR (KBr, cm^{-1}) ν: 2934, 1667, 1514, 1425, 1309, 1233, 1256, 1138, 1030, 755; ^{1}HNMR (200 MHz, $CDCl_3$) δ: 0.96 (*t*, 3H, *J*=7.2 Hz, CH_3), 1.38-1.57 (*m,* 2H, CH_2), 1.73-1.87 (*m,* 2H, CH_2), 2.08 (*s*, 3H, CH_3), 2.75 (*dd*, 1H, *J*=18.2, 4.8 Hz, $CH_{2pyrazoline}$), 3.39 (*dd*, 1H, *J*=18.2, 11.5 Hz, $CH_{2pyrazoline}$), 3.84 (*s*, 3H, OCH_3), 3.98 (*t*, 2H, *J*=6.6 Hz, CH_2), 5.34 (*dd*, 1H, *J*=11.5, 4.8 Hz, $CH_{pyrazoline}$), 6.69-6.74 (*m*, 2H, Ar-H), 6.82 (*d*, 1H, *J*=8.6 Hz, Ar-H), 8.82 (*d*, 1H, *J*=1.0 Hz, CHO); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 13.7, 15.8, 19.1, 31.1, 46.5, 56.0, 58.3, 68.7, 109.4, 113.2, 117.6, 133.2, 148.2, 149.7, 157.2, 159.5.
5-(4-(Benzyloxy)-3-methoxyphenyl)-3-methyl-4,5-dihydro-1H-pyrazole-1-carbaldehyde **(3f)**
Cream powder; yield: 93%; m.p. 79-80°C; IR (KBr, cm^{-1}) ν: 3000, 2327, 1675, 1516, 1423, 1310, 1257, 1134, 1025, 798, 747; ^{1}HNMR (200 MHz, $CDCl_3$) δ: 2.06 (*s*, 3H, CH_3), 2.72 (*dd*, 1H, *J*=18.2, 4.8 Hz, $CH_{2pyrazoline}$), 3.37 (*dd*, 1H, *J*=18.2, 11.5 Hz, $CH_{2pyrazoline}$), 3.86 (*s*, 3H, OCH_3), 5.11 (*s*, 2H, CH_2), 5.32 (*dd*, 1H, *J*=11.5, 4.8 Hz, $CH_{pyrazoline}$), 6.64-6.72 (*m*, 2H, Ar-H), 6.82 (*d*, 1H, *J*=8.2 Hz, Ar-H), 7.28-7.44 (*m*, 5H, Ar-H), 8.81 (*d*, 1H, *J*=1.0 Hz, CHO); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 15.8, 46.5, 56.0, 58.3, 71.0, 109.4, 114.2, 117.5, 127.1, 127.7, 128.4, 133.8, 137.0, 147.8, 149.9, 157.2, 159.5.

In silico ADME calculations

The physicochemical parameters of all the compounds were predicted using DataWarrior software (SANDER, *et al.* 2015), and SwissADME web-based tool (DAINA, *et al.* 2017).

Cytotoxic activity

The HeLa cells were harvested from the culture flasks during the exponential growth phase, counted and 5×10^3 cells per well were seeded into 96-well culture plates. The cells were allowed to adhere overnight in a humidified incubator with 5% CO_2. Afterward, the supernatants were removed and the remaining cell monolayers were treated with 200 µL volumes of dilutions of the tested compounds in fresh DMEM (Dulbecco's Modified Eagle Medium), which was used as a control. Control wells were treated the same as test wells. All cells were incubated at 37°C in an atmosphere of 5% CO_2 and absolute humidity for 24 and 48h. Then, the cell culture media (with the investigated compounds) was removed, and 100 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 0.5 mg/mL) was added to each well. After 2h incubation, the MTT solution was removed, and 150 µL of DMSO was added to dissolve the formazan crystals. Absorbance (ABS) was measured with a multiplate reader (Zenyth 3100, Anthos Labtec Instruments GmbH, Austria) at 595 nm. The percentage of cytotoxic cells was calculated using the following formula (RISS *et al.,* 2013):

$$\text{Cytotoxicity (\%)} = (1 - \text{test group(ABS)}) / \text{control group(ABS)} \times 100 \quad (1)$$

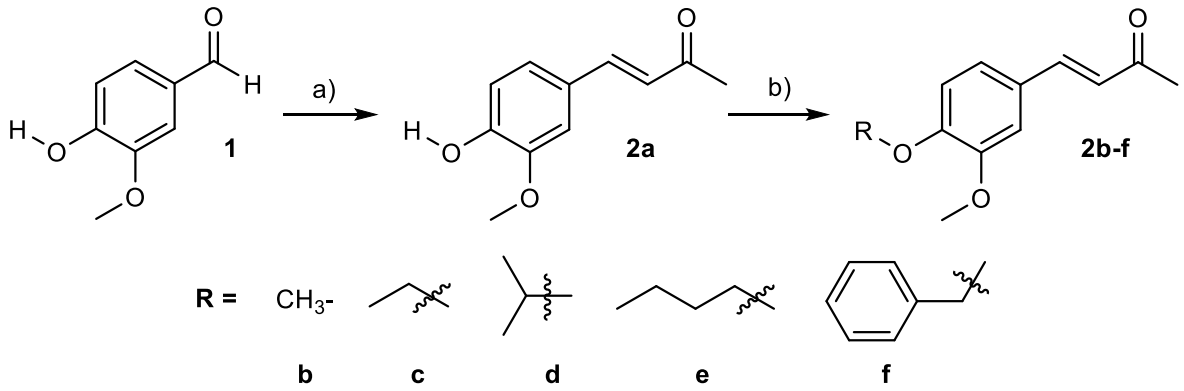
To determine and compare the cytotoxic effects of the tested substances on the morphology of treated and untreated HeLa cells, we have used a phase-contrast microscope. The HeLa cells were seeded in a 24-well plate and incubated for 48h with different concentrations of pyrazolines (100, 150, and 200 µM). Morphological changes of both experimental and control HeLa cells were visualized with phase contrast microscopy under 100× magnification on Olympus microscope (model BX51).

RESULTS AND DISCUSSION

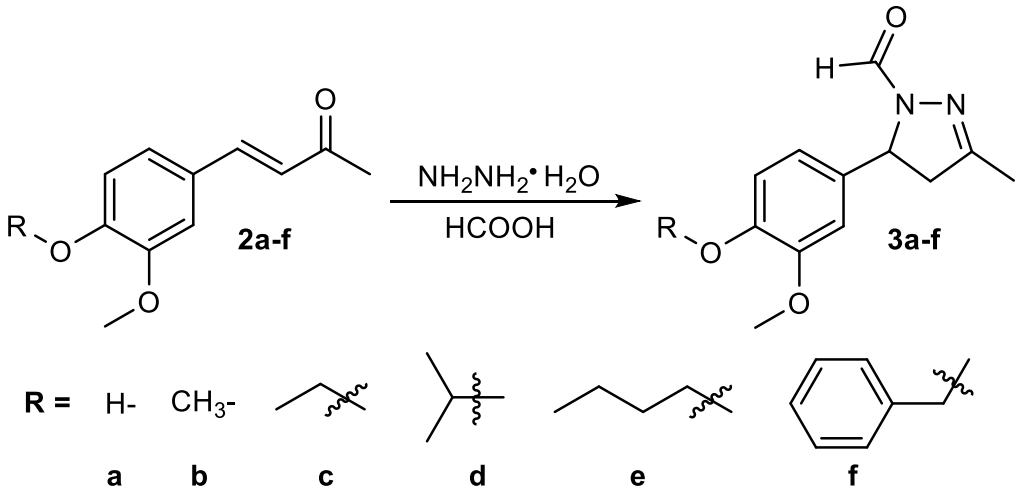
Chemistry

The synthesis of the alkyl derivatives of dehydrozingerone (**2b-f**, Scheme 1) has been previously reported by our research group (MUŠKINJA, 2016; RATKOVIĆ *et al.,* 2016). The general synthetic plan first employed the synthesis of dehydrozingerone (**2a**) using the Claisen-Schmidt condensation reaction between vanillin and acetone in basic conditions. In the further phase of the reaction, the formed dehydrozingerone (**2a**) undergoes the alkylation of the free phenolic group giving derivatives **2b-f** in very good yields (91-98%).

The synthetic route for the target compounds is outlined in Scheme 2. The starting compounds **2a-f** reacted with hydrazine monohydrate in formic acid to afford the novel pyrazolines, (5-(4-hydroxy-3-methoxyphenyl)-3-methyl-4,5-dihydro-1*H*-pyrazole-1-carbaldehyde, **3a**, and (5-(4-alkoxy-3-methoxyphenyl)-3-methyl-4,5-dihydro-1*H*-pyrazole-1-carbaldehydes, **3b-f**) in excellent yields of 76-93%. Reactions were carried out at reflux and monitored by TLC using CH_2Cl_2 as an eluent. The best yields for all synthesized compounds were achieved after 5h of reaction time.



Scheme 1. Synthesis of 4-(4-alkoxy-3-methoxyphenyl)-but-3-en-2-ones, **2a-f**. Reagents and conditions: a) NaOH, acetone; b) RX (X = Cl, Br or I), K_2CO_3, acetone.



Scheme 2. Synthesis of *N*-formyl pyrazolines **3a-f**.

The structure of the newly synthesized pyrazolines was characterized and confirmed by spectral data (IR, ^{1}H NMR, and ^{13}C NMR). The representative IR and ^{1}H NMR spectra of compound **3a** are shown in Figure 1 and Figure 2, respectively. In the IR spectrum, the presence of valence vibrations in the form of a broad signal in the region of 3316 cm^{-1} originating from the OH group is very characteristic. A sharp signal in the area of approximately 1664 cm^{-1} belongs to the C=O bond valence vibration in the CHO group. In the ^{1}H NMR spectrum (Figure 2), the methoxy protons (O-CH_3) appeared as singlets at 3.83-3.86 ppm, whereas aromatic protons (Ar-H) appeared between 6 and 7 ppm. The signal belonging to the proton from the OH group is located at 6.02 ppm, in the form of a broad singlet. The signals originating from the pyrazoline ring can be seen in the form of two dd (2.74 and 5.31 ppm) signals and one ddq (3.38) signal. Interestingly, the protons of the pyrazoline ring, including the protons of the groups directly attached to it (methyl and formyl group), gave more complex groups of signals in ^{1}H NMR than it could be expected. These splittings, caused by a very strong coupling occurring across the pyrazoline fragment, could be noticed in the spectra of all compounds, but are best resolved for derivative **3c**. For all compounds, the pyrazoline CH_2 protons positioned next to a chiral center gave two signals due to their magnetic nonequivalence. In ^{1}H NMR spectra of **3c**, these protons appeared as two ddq signals caused by long-range coupling through four bonds with methyl protons, present in the form of a corresponding triplet. In addition, the CH proton gave a ddd signal due to long-

range coupling with the CHO proton, which is present in all spectra as a doublet. The connection between these signals is confirmed by the values of the corresponding coupling constants.

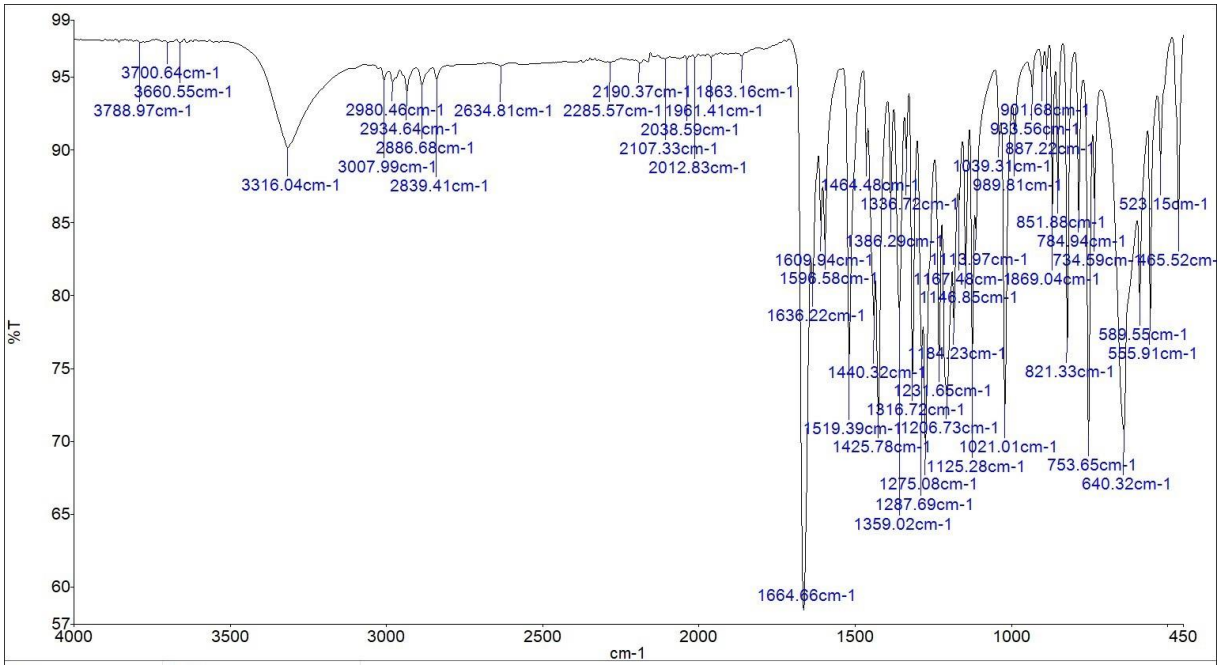


Figure 1. The IR spectrum of compound **3a**

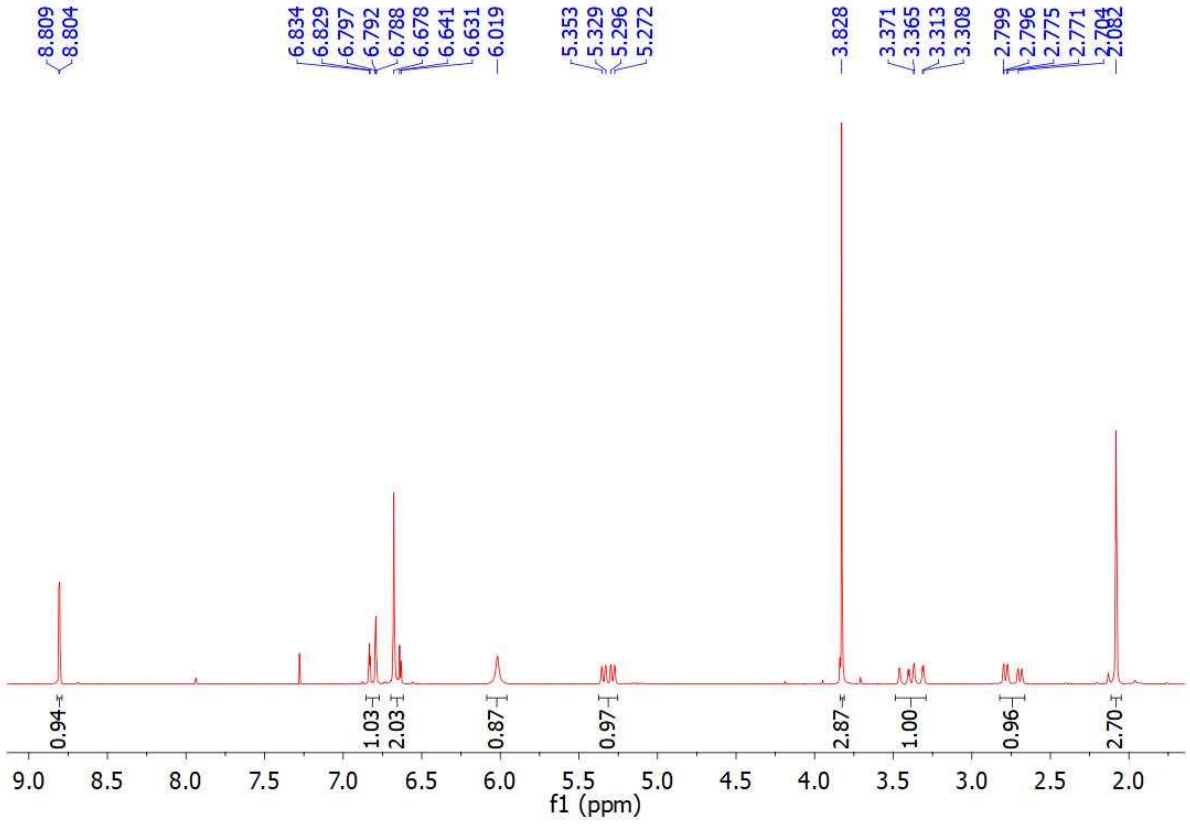


Figure 2. ^{1}H NMR spectrum of compound **3a**

In silico ADME analysis

The ADME study has a crucial role in the development and optimization of new molecules, enhancing the rate of success in drug discovery. Here, the physicochemical properties of the synthesized compounds **3a-f** were calculated and discussed compared to doxorubicin (DOX) as a standard chemotherapeutic agent (Table 1).

Table 1. The calculated values for **3a-f**.

Compd	LRo5					cLog*S*	Drug-likeness score	Bio-availability score
	Mol. wt	cLog*P*	HBA	HBD	Violation			
3a	234.254	1.2189	5	1	0	–2.113	3.2186	0.55
3b	248.281	1.4946	5	0	0	–2.427	3.2116	0.55
3c	262.308	1.9009	5	0	0	–2.727	1.8164	0.55
3d	276.335	2.2602	5	0	0	–3.105	2.8216	0.55
3e	290.362	2.8097	5	0	0	–3.267	–1.9724	0.55
3f	324.379	2.9127	5	0	0	–3.750	3.0027	0.55
DOX	543.523	0.1673	12	6	3	–4.507	6.6484	0.17

LRo5-Lipinski's rule of five; **Mol. wt**-molecular weight; **cLog*P***-calculated octanol-water partition coefficient; **HBA**-the number of hydrogen bond acceptor atoms; **HBD**-the number of hydrogen bond donor atoms; **Violation** of LRo5: **cLog*P*** > 5, **Mol. wt** > 500, **HBA** >10, **HBD** > 5; **cLog*S***-calculated water solubility (mol/L).

Overall, as can be seen in Table 1, doxorubicin exhibits three violations of Lipinski's rule of five (Mol. wt > 500, HBA > 10, and HBD > 5), while the tested pyrazolines **3a-f** meet all the requested criteria (LIPINSKI, *et al.* 1997). All compounds have much higher lipophilicity than DOX, with derivative **3f** exhibiting the highest value of cLog*P*, probably due to the presence of an additional phenyl group as a substituent with hydrophobic properties (RITCHIE and MACDONALD, 2009). However, the number of the aromatic rings has an inverse effect on the solubility of a molecule, which is known to decrease with increasing lipophilicity (RAN and YALKOWSKY, 2001). According to this, molecule **3f**, which shows the highest lipophilicity, also has the lowest solubility among the evaluated compounds. In comparison to DOX, the cLog*S* values for the whole series are still more positive, indicating their better solubility in water. Furthermore, these compounds also have optimal values of the bioavailability score, indicating a 55% probability of achieving rat bioavailability higher than 10% (MARTIN, 2005). Although the druglikeness scores for the derivatives **3a-f** are respectably lower than that of DOX, the calculated values for most of the derivatives indicate that the derivatization of these compounds could lead to promising drug candidates. In this sense, the further optimization of these molecules is necessary to enhance their properties, such as the introduction of different scaffolds, which could improve the solubility of the compounds.

Cytotoxic activity

To evaluate the biological potential of the synthesized compounds, a preliminary cytotoxicity study of pyrazolines **3a-f** against the human cervix adenocarcinoma cell line, HeLa, was performed using the MTT test. The results presented in Figure 3 show that compounds **3c**, **3d**, and **3f** exhibited the most promising effects in a dose-dependent manner compared to all tested substances. Among them, the best result was observed for compound **3f**, which shows very high activity at all applied concentrations (100, 150, and 200 μM). Similar to lipophilicity, this could be explained by the presence of an additional phenyl group in the structure of **3f**, due to its planarity and hydrophobic properties, enabling it to achieve better interactions with various active centers.

	100 µM	150 µM	200 µM
3a	11.10	57.71	52.15
3b	47.92	40.22	48.44
3c	31.26	64.82	77.02
3d	29.69	56.63	76.93
3e	0	50.73	35.13
3f	86.45	95.31	95.59

Figure 3. The cytotoxic effects of the **3a-f** against HeLa cells after 48h of treatment.

To further examine the cytotoxic effects, a phase-contrast microscopy study was conducted on the morphological changes in HeLa cells treated with compounds **3c**, **3d**, and **3f** (Figure 4). Figure 4 shows that these compounds induced the cell shape loss and a significant decrease in the number of HeLa cells, in a dose-dependent manner compared to the control group (cells not treated with tested compounds), thus supporting their cytotoxic potential.

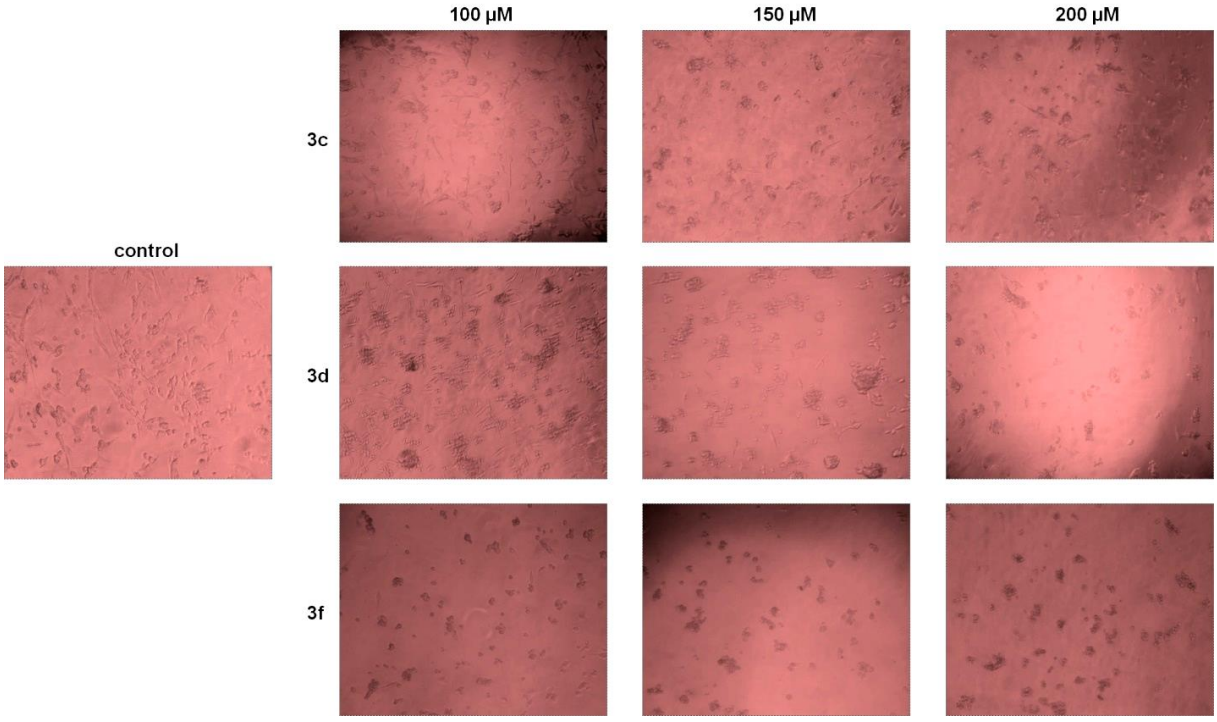


Figure 4. Morphological changes in HeLa cells induced by compounds **3c**, **3d**, and **3f**.

CONCLUSION

In the present paper, the synthesis and structural characterization of a series of new pyrazoline derivatives **3a-f**, obtained in very good yields, are reported. The ^{1}H NMR spectra revealed a very strong coupling throughout the pyrazoline scaffold, giving a complex splitting of the corresponding signals. A pharmacokinetic profile of the synthesized pyrazolines was evaluated using an ADME study, revealing their good lipophilic properties and bioavailability score. A preliminary cytotoxicity screening of molecules **3a-f** against HeLa cells showed that compounds **3c**, **3d**, and **3f** exhibit promising cytotoxic potential, with derivative **3f** having the highest activity at all concentrations. The morphological study further confirmed the cytotoxic potential of the tested compounds **3c**, **3d**, and **3f**. Based on obtained results, it could be presumed that the further optimization of these compounds would be the most promising strategy for the development of the molecules with improved physicochemical proprerties and biological potential. Having in mind the presence of the formyl group in the structure of these molecules, their further derivatization and detailed biological study are in progress.

Acknowledgments

The authors are grateful to the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant Nos. 451-03-136/2025-03/200378 and 451-03-136/2025-03/200122) for financial support.

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