Research Article

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Design, synthesis, and biological evaluation of phenyl-isoxazole-carboxamide derivatives as anticancer agents

https://doi.org/10.1515/hc-2020-0134
received April 13, 2021; accepted October 28, 2021

Abstract: The present study aimed to design and synthesize a series of phenyl-isoxazole-carboxamide derivatives and investigate their antitumor and antioxidant activities. The *in vitro* cytotoxic evaluation was conducted using the MTS assay against four cancer cell lines: hepatocellular carcinoma (Hep3B and HepG2), cervical adenocarcinoma (HeLa), breast carcinoma (MCF-7), in addition to the normal cell line (Hek293T). Besides, the antioxidant activity was evaluated using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. All obtained compounds were found to have potent to moderate activities against Hep3B and MCF-7 cancer cells lines, except compound **2e**. It was found that compound **2a** has potent activity against HeLa and Hep3B cancer cell lines with IC_{50} values of 0.91 and 8.02 μM, respectively. The IC_{50} dose range of the tested compounds against Hep3B was 5.96–28.62 μM, except for **2e**, compared with doxorubicin, which has an IC_{50} value of 2.23 μM. Also, the IC_{50} value range of the compounds against Hek293T was 112.78–266.66 μM, compared with doxorubicin, which has an IC_{50} dose of 0.581 μM. The antioxidant activity of the synthesized compounds was weak, and compound **2d** showed moderate activity against the DPPH enzyme with an IC_{50} value of 138.50 μM in comparison with Trolox, which has an IC_{50} dose of 37.23 μM.

Keywords: cancer, antioxidant, isoxazole, doxorubicin

1 Introduction

In 2020, worldwide cancer disease was the leading cause of death, which accounted for about 10 million deaths. In terms of new cancer cases, the most common types included breast, lung, colon and rectum, prostate, skin (non-melanoma), and stomach, representing 2.26, 2.21, 1.93, 1.41, 1.20, and 1.09 million cases, respectively [1,2].

Indeed, air pollution, physical inactivity, unhealthy diet, alcohol, and tobacco use are the major risk factors for cancer. In addition, some chronic infectious diseases are risk factors for cancer in low- and middle-income countries. Globally, in 2018, about 13% of patients diagnosed with cancer were associated with carcinogenic infections, including Epstein-Barr virus, hepatitis C virus, hepatitis B virus, human papillomavirus (HPV), and *Helicobacter pylori* [3,4].

Liver cancer accounts for a large portion of the global cancer incidence. In several countries, the prevalence of this disease has increased in recent years. Hepatocellular carcinoma (HCC) is the most common histologic form of liver cancer, responsible for the vast majority of diagnoses and mortality [5]. Cervical cancer is now one of the most common gynecologic cancers in the world. Thus, according to the current statistics, it is the 14th most common cancer worldwide and the third most prevalent cancer among women [6]. However, breast cancer is considered one of the most common cancers globally, with 2.09 million new cases in 2018 [7].

Despite the fact that chemotherapeutic protocols for cancer treatment have a short history of use, their effects are still growing. Chemotherapy is an important treatment

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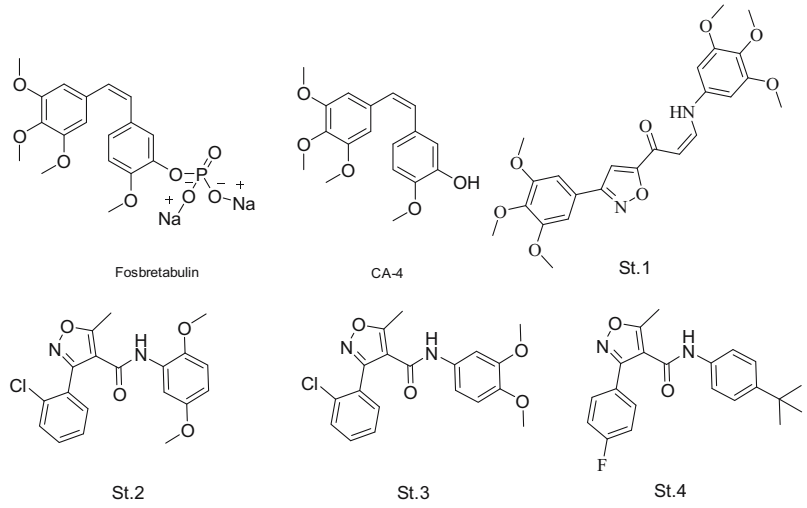
choice for most human tumors, and it can even be curative in some cases [8]. Chemotherapy's function has become much more critical in enhancing cancer patients' quality of life. This advancement has been made possible by the current research involving drug production, pharmacology, preclinical studies, quantitative response criteria, and various clinical trials that looked at the value of dose schedule and chemotherapeutic agent sequencing [9]. Chemotherapy, especially against various types of cancers, has been frequently used [10]. Even so, several products were derived from plants with varying pharmacological activities [11].

Fosbretabulin (Combretastatin A4 phosphate; CA-4P; Figure 1) is a water-soluble Combretastatin A4 prodrug approved for thyroid cancer treatment by the FDA in 2018 [12]. CA-4 is a microtubule depolymerizing factor used in vascular binding. These effects cause an increase in vascular permeability and interruption in the tumor blood flow. CA-4P monotherapy is effective and reduces cancer blood flow, according to the findings of the first clinical trials. Combretastatin A-4 phosphate and other CA-4 derivatives were synthesized and evaluated as tubulin inhibitors [13–15].

Several forms of research have attempted to combine or hybridize novel anti-cancer agents between different chemical groups like trimethoxyphenyl derivatives (part of CA-4) to innovate several compounds that have heterocyclic-trimethoxyphenyl anti-cancer agents [13,16,17].

Isoxazole molecules have a complex range of targets and biological effects. One of the latest phenomena is the production of compounds with heterocycle rings. The addition of an isoxazole ring will enhance the physical–chemical qualities of a compound. The isoxazole ring is now a common moiety in the structures of several compounds due to its characteristic therapeutic properties. The applications of isoxazole compounds in curing various ailments, particularly cancer, microbial infections, and inflammation, have gained a lot of attention. [18]. The research that combined the trimethoxyphenyl moiety with isoxazole (arylamino-isoxazolyl-2-propenone) showed cytotoxic activity against different cancer cell lines (HeLa, A549, MCF-7, and HCT116) with low IC_{50} values (Figure 1: St.1) [19], one of recently published research that has a hybridization strategy between the trimethoxyphenyl derivatives and phenyl-isoxazole confirmed that the compounds (Figure 1: St.2 and St.3) had potent activities against Hep3B cells [20].

The biologically active antioxidant agents work to slow or prevent cell damage caused by free radical molecules.



Compound	Note	IC_{50}
Fosbretabulin	Approved drug by FDA for thyroid cancer [12]	Huh7 15.02 nM [36]
CA-4 [37]	Biologically active compound but has a low water solubility [13]	HeLa 4 nMand MCF-7 10 nM [38]
St.1 [19]	Potent candidate compound	HeLa 3.69 nM and MCF-7 2.80 nM
St.2 [20]	New moderately activeagent	HeLa 32.35 μg/ml, MCF-7 63.10 μg/ml and Hep3B 23.44 μg/ml
St.3 [20]	New moderately activeagent	HeLa 15.48 μg/ml, Hep3B 23.98 μg/ml
St.4 [38]	New moderately activeagent	HepG2 41.53 μg/ml and Hep3B 9.58 μg/ml

Figure 1: Fosbretabulin, CA-4, and isoxazole derivatives and their main anticancer activities.

There are different antioxidants, some of which are endogenous; some like β-carotene, R-tocopherol, and ascorbic acid are found in natural plants and foods; and the rest can be chemically synthesized [21–24]. In our previous work, a series of isoxazole derivatives were synthesized and evaluated as antioxidant agents. One of these structures (Figure 1: St.4) has potent antioxidant activity with an IC_{50} value of 7.8 ± 1.21 μg/mL, compared with Trolox as a positive control (IC_{50}: 2.75 μg/mL) [20].

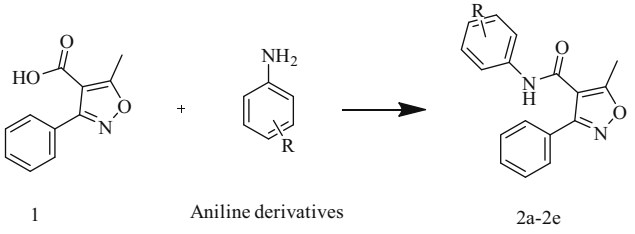
The current work aims to synthesize a series of isoxazole-carboxamide derivatives (**2a–2e**) with or without a methoxyphenyl moiety, characterization of their chemical structures with IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy, predict their bioactivity score by molinspiration analysis and evaluating their anti-proliferative activity against four cancer cell lines, liver (Hep3B, HepG2), cervical (HeLa), and breast (MCF-7) comparing with normal cell line (Hek293T), as well as antioxidant activity was evaluated using the DPPH assay (2,2′-diphenyl-1-picrylhydrazyl radical).

2 Results and discussion

2.1 Chemistry

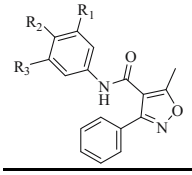
The amide derivatives (**2a–2e**) were synthesized as outlined in Scheme 1 and Table 1. The coupling to form amides **2a–2e** was afforded to employ EDCI as an activating agent and DMAP as a covalent nucleophilic catalyst. Then, the active species were reacted with the aniline derivatives [25]. Compounds **2a** and **2e** were synthesized previously by different chemical reactions, and the yield was very close to our yields values [26–28], as well as our coupling method seems to be milder without the need for heating.

All compounds were purified using column chromatography (*n*-hexane/ethyl acetate and dichloromethane/ethyl acetate solvent systems). To confirm the synthesis



Scheme 1: **1** + aniline derivatives dissolved in 15 mL of DCM, then DMAP and EDC were added under nitrogen gas, stirred for 24 h (R for **2a**: 4-*t*-butyl, for **2b**: 3,4-dimethoxy, for **2c**: 3,5-dimethoxy, for **2d**: 3,4,5-trimethoxy and for **2e**: H).

Table 1: Chemical structures of compounds

Compound structure	Compound code	R1	R2	R3
	**2a**	H	-*t*-Butyl	H
	2b	$-OCH_3$	$-OCH_3$	H
	2c	$-OCH_3$	H	$-OCH_3$
	2d	$-OCH_3$	$-OCH_3$	$-OCH_3$
	2e	H	H	H

of these five compounds, the ^{1}H-NMR peaks confirmed the current study synthesis, one singlet signal was observed in the range of 10.30–10.40 ppm for N–H, one singlet signal for 3 protons were observed around 2.58 ppm for isoxazole-C**H$_3$**, singlet signals were observed in the range of 3.64–3.75 ppm, which presents the protons of methoxy groups, while doublet triplet and multiplet signals were observed in the aromatic area. According to the ^{13}C-NMR spectrum, a C signal of carbonyl groups was found around 170 ppm. In the aromatic area, several signals were observed presenting aromatic carbons, and in the aliphatic area, different signals were observed around 35 ppm, one or two signals representing methoxy groups, and around 12 ppm one signal was observed for isoxazole-**C**H$_3$ carbon. Regarding IR-spectroscopy, strong bands were observed around 1,650/cm that represent the amide carbonyl (C═O).

2.2 Chemo-informatics properties and Lipinski's rule (RO5) of evaluated compounds

Table 2 depicts the chemical properties of a library of five compounds that were previously synthesized. The results revealed that the tested compounds have a good predicted value of molecular weight (M. Wt.) (g/mol), hydrogen bond acceptor (HBA) and donor (HBD), and log *P*. Moreover, Lipinski's rule of five (RO5) review shows that all compounds obey the RO5 rule and possess good comparable values against standard M.Wt. (<500 g/mol; range: 278.11–338.13 g/mol), HBA (<10; range of compounds: 3–6), HBD (<5; range 1) and log *P* (<5) values (2.66–4.9) [29]. Our predicted analysis indicates that all of the assessed compounds were within the standard range, demonstrating their high oral bioavailability.

2.3 Bioactivity score

The bioactivity of drugs was also assessed by measuring activity scores for various targets, including GPCR (G-protein-coupled receptor) ligands, ion channel modulators,

Table 2: Chemo-informatics properties of evaluated compounds

	2a	2b	2c	2d	2e
Molecular formula	$C_{21}H_{22}N_2O_2$	$C_{19}H_{18}N_2O_4$	$C_{19}H_{18}N_2O_4$	$C_{20}H_{20}N_2O_5$	$C_{17}H_{14}N_2O_2$
Molecular weight (g/mol)	334.419	338.363	338.363	368.389	278.311
No. of HBA	3	5	5	6	3
No. of HBD	1	1	1	1	1
Mol log *P*	4.90	2.68	3.36	2.66	2.96

kinase inhibitors, and nuclear receptor ligands [30]. The Molinspiration drug-likeness score was used to review all of the parameters. All compounds showed moderate to high activity as the nuclear receptor ligand in range of −0.12 to 0.12, which could confirm anticancer activities of these compounds; while low activities against the GPCR ligand, ion channel, a kinase inhibitor, enzyme, and protease inhibitors were predicted. A molecule with a bioactivity score in the range of 0.00–0.5 is more likely to have significant biological activities, whereas ratings in the range of −0.50 to 0.00 are moderately active, and scores of less than −0.50 are assumed to be inactive [31].

2.4 Biological evaluations

2.4.1 Cytotoxic evaluation of compounds 2a–2e

MTS assay was used to determine the cytotoxicity effect of phenyl-isoxazole-carboxamide derivatives on hepatocellular carcinoma (Hep3B and HepG2), cervical adenocarcinoma (HeLa), breast carcinoma (MCF-7), and the normal cell line (Hek293T). Five concentrations (300, 100, 50, 10, and 1 μg/mL) were used. After the calculations of the IC_{50} in μg/mL, it was converted to μM by using the molecular weight for each compound in g/mol. As shown in Table 3, it was found that compound **2a** was the most potent against the HeLa cell, with an IC_{50} value of 0.91 μM. It showed also potent activity against MCF-7 and Hep3B cells with IC_{50} values of 14.43 and 8.02 μM, respectively. All compounds except compound **2e**, showed potent-moderate activities against Hep3B, with IC_{50} values ranging from 5.96 to 28.62 μM compared with the positive control Dox, which has an IC_{50} value of 2.23 μM. Compounds **2a**, **2b**, and **2c** showed potent activity against the MCF-7 cancer cell line with IC_{50} values in the range of 4.56–17.16 μM, in comparison with Dox, which has an IC_{50} value of 0.577 μM. However, all evaluated compounds showed moderate activity against HepG2 and normal cell line Hek293T compared with Dox.

The cell viability was calculated for all compounds as well as for doxorubicin, which was utilized in our experiment as a positive control, at 10 μg/mL (Figure 2); it was clear that the MCF-7 and Hep3B cell viability percentage after the treatment with compounds **2a–2d** were under 50%, which was very close to the potency of doxorubicin, while the cell viability for the other cells including the normal cell (Hek293T) was higher than 50%.

In Figure 3a–c, the cell inhibition percentages for MCF-7, Hep3B, and Hek293T cells are presented, except compound **2e.** All compounds (**2a–2d**) showed potent anti-proliferative activity against both MCF-7 and Hep3B cancer cell lines with inhibition percentage too close to the anticancer drug doxorubicin, while the anti-proliferative activities of all compounds were weak against the normal cell line Hek293T. Our compounds showed selective inhibition properties against cancer cell lines (Hep3B and MCF-7) in comparison with normal cell lines (Hek293T).

Table 3: Cytotoxicities of compounds **2a–2e** and DOX determined in different human cancer cells

Compound/cell line	IC_{50} μM				
	HepG2	**Hep3B**	**HeLa**	**MCF-7**	**Hek293T**
2a	226.40 ± 2.21	8.02 ± 1.33	0.91 ± 1.03	14.43 ± 1.97	180.31 ± 1.88
2b	NI	28.62 ± 2.56	NI	17.16 ± 2.45	130.28 ± 2.35
2c	NI	6.93 ± 1.88	NI	4.56 ± 2.32	260.66 ± 0.89
2d	NI	5.96 ± 0.87	281.58 ± 1.76	50.43 ± 1.40	112.78 ± 1.31
2e	NI	NI	NI	NI	NI
DOX	2.94 ± 0.89	2.23 ± 1.21	1.55 ± 1.35	0.577 ± 1.21	0.581 ± 0.79

*NI: weak or negligible inhibition activities > 300 μM (p value < 0.05).

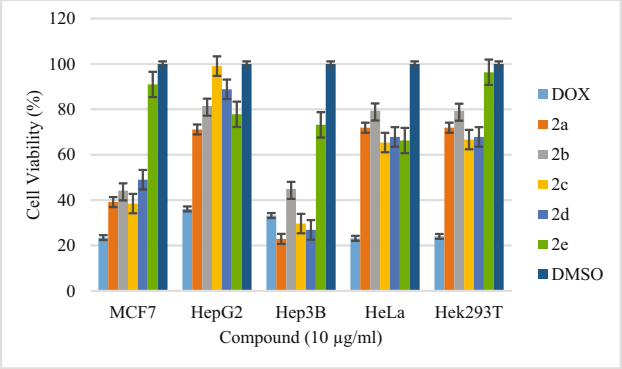


Figure 2: *In vitro* anti-proliferative activities of compounds **2a–2e,** DOX, and DMSO against MCF-7, Hep3B, HepG2, HeLa, and Hek293T cell lines at 10 µg/mL for 72 h, and the cell viability was finally determined by MTS assay.

The selectivity ratio was calculated to find the best selective compound, in comparison with the doxorubicin selectivity ratio (Figure 4). Doxorubicin was not a selective anticancer agent, while all synthesized compounds except compound **2e** (weak activity) showed the selectivity ratio, and the most selective agent was compound **2c**, which was selective 57 and 38 times on MCF-7 and the Hep3B cancer cell line, respectively, compared with the normal cell line.

The carboxamide poly-methoxyphenyl or alkyl-substituted compounds were potent anticancer agents according to various research works [8,25], and the substituted groups on the phenyl moiety seem to be essential features for anticancer activities, and one of the most potent anticancer compounds was (*E*)-*N*-(4-(*tert*-butyl)phenyl)-3-(1*H*-indol-3-yl)acrylamide, which has phenyl-substituted with a tret-butyl group [8]. Actually, in our synthesized compounds, compound **2a** with the same functional group (*t*-butyl) was one of the most active compounds against three cancer cell lines (MCF-7, HeLa, and Hep3B) with an IC_{50} range of 0.91–14.43 µM. However, the presence of poly-methoxyphenyl moiety connected to amide or carbonyl has a strong relationship with the potent anticancer effect [16,20,25]. In the synthesized compounds, it was clear that the unsubstituted phenyl, like compound 2e, has very weak anticancer activities, while the other compounds with substituted groups on the phenyl moiety, like compounds **2a–2d**, have potent activities. This can be explained by the fact that the biological target has a binding pocket that can make binding interactions possible with methoxy or *t*-butyl groups, which will inhibit the proliferation of cancer cell lines. Compound **2a** was synthesized, and targeting the synergy of cardiac transcription factors GATA4 and NKX2-5, and it was considered as one of the moderate active compounds with IC_{50}/

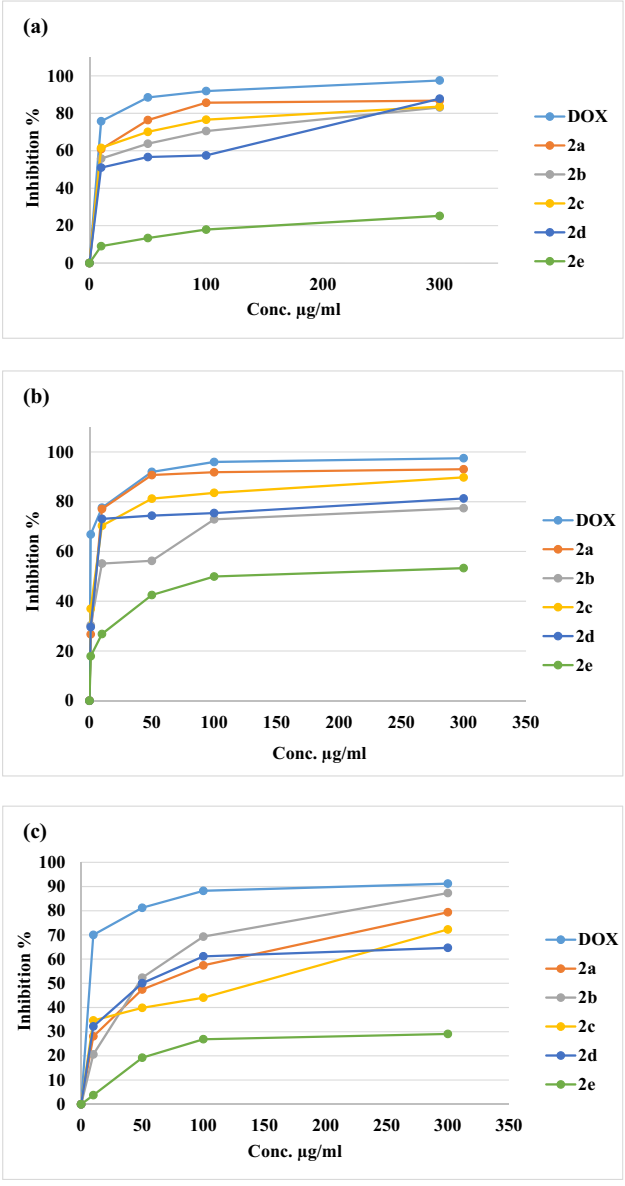


Figure 3: *In vitro* anti-proliferative activities of compounds **2a–2e** and DOX against (a) MCF-7, (b) Hep3B, and (c) Hek293T cell lines.

K_a = 18 µM [26,27,32], while our results regarding this compound showed better activity (IC_{50} < 18 µM) against three cancer cell lines (HeLa, Hep3B, and MCF-7), and these findings make our compound as a possible anticancer candidate, especially with better selectivity ratio than the well-known anticancer agent doxurubicine.

2.4.2 Antioxidant evaluation

In this research project, the assessment of the antioxidant activity of all the synthesized compounds (**2a–2e**) was

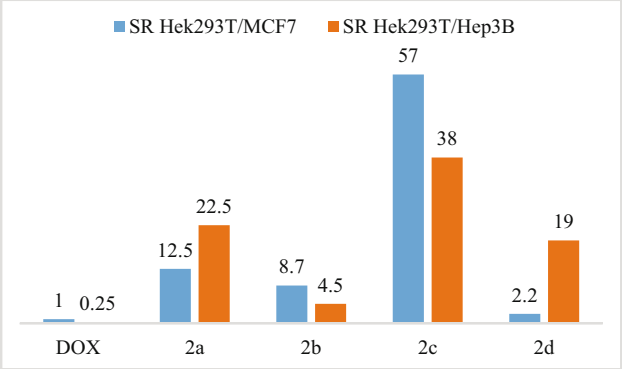


Figure 4: Comparison of the selectivity ratio (SR; normal/cancer cell) between **2a–2d** compounds and DOX.

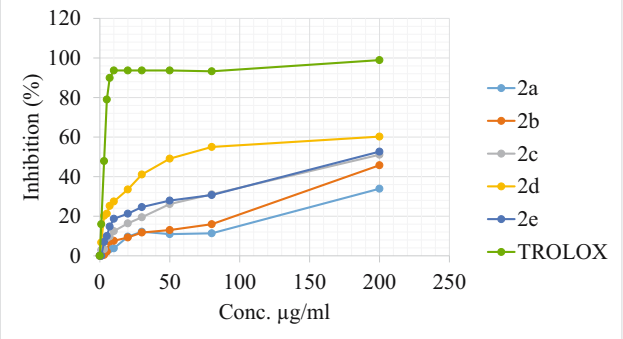


Figure 5: DPPH inhibitory activities of the synthesized compounds (**2a–2e**) and the positive control Trolox.

performed using an *in vitro* antioxidant DPPH assay, and the results were compared with the positive control (Trolox) that has a potent antioxidant effect. To evaluate the *in vitro* antioxidant activity of the synthesized compounds, the interaction with the stable free radical DPPH method was used. The DPPH method is described as a simple, convenient, and rapid method, which is independent of sample polarity for screening. These advantages make the DPPH method interesting for testing our synthesized compounds [33]. A range of concentrations of the synthesized agents was prepared and tested to calculate the IC_{50} of these structures. The antioxidant activity of the synthesized compounds was weak, as shown in Figure 5. It was clear that only compound **2d** was the most active one with an IC_{50} value of 138.50 µM, compared with Trolox (IC_{50} = 37.23 µM).

3 Conclusion

The synthesized isoxazole-carboxamide derivatives **2a–2d** illustrated potent anticancer activities against different cancer cell lines, which was related to the presence of substituted alkyl or methoxy groups on phenyl-amide. Almost all the synthesized compounds were more active against cancer cell lines Hep3B and MCF-7 than the normal cell line in comparison with the anticancer agent doxorubicin, and this makes these compounds safer than doxorubicin. Furthermore, just compound **2d** has moderate antioxidant activity. Chemoinformatics analysis showed that all synthesized derivatives **2a–2e** obeyed Lipinski's rule, which makes these structures suitable to be used orally. In future studies, we would like to synthesize more analogues of isoxazole-carboxamide derivatives as promising anticancer agents, and further *in vivo* investigations are required to approve these effects and to design suitable pharmaceutical dosage forms from these compounds.

4 Experimental

4.1 Chemistry

All chemicals were purchased from Sigma-Aldrich and Alfa Aesar. Melting points were determined with an SMP-II Digital Melting Point Apparatus and are uncorrected. IR spectra were obtained using a Perkin Elmer Spectrum 400 FTIR/FT-NIR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 on a Bruker 500J MHz-Avance III High-Performance Digital FT-NMR spectrometer at the Faculty of Science, Department of Chemistry, The University of Jordan, Jordan. Tetramethylsilane was used for calibration issues. All chemical shifts were recorded as *d* (ppm).

4.2 The general procedure of isoxazole-carboxamide synthesis 2a–2e

5-(Methyl)-3-phenyl-1,2-isoxazole-4-carboxylic acid (1.5 mmol) was dissolved in 15–20 mL of dichloromethane (DCM); then, diethylamino-pyridine (DMAP; 0.3 mmol), and EDC (1.65 mmol) were added. After that, the mixture was stirred under argon gas at room temperature for 30 min, and the aniline derivatives (1.6 mmol) were added. The reactions were monitored by TLC, while the solvent was removed under reduced pressure and dissolved again in DCM, and then extracted with 1% $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The obtained product was

purified by flash chromatography using the appropriate solvent systems (*n*-hexane/ethyl acetate or DCM/ethyl acetate) or by recrystallization utilizing an appropriate solvent system [20].

4.3 *N*-(4-*tert*-Butylphenyl)-5-methyl-3-phenyl-1,2-isoxazole-4-carboxamide (2a)

Purified by silica gel column chromatography using *n*-hexane/ethyl acetate solvent system (3:2). Melting point: 178–179°C; color and appearance: solid and off-white in color; yield: 75%; IR: 1,652/cm amide carbonyl (C═O). RF: 0.78 (*n*-hexane:ethyl acetate, 3:2). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 10.38 (s, 1H, N–H), 7.71–7.49 (7H, m, Ar–H), 7.36 (2H, d, J = 8.5 Hz, Ar–H), 2.57 (3H, s, CH_3), 1.27 (9H, s, –C–$(CH_3)_3$). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 170.19, 160.62, 160.27, 146.95, 136.53, 130.56, 129.32, 128.59, 128.17, 125.90, 119.94, 113.88, 34.54, 31.62, 12.34.

4.4 *N*-(3,4-Dimethoxyphenyl)-5-methyl-3-phenyl-1,2-isoxazole-4-carboxamide (2b)

Purified by silica gel column chromatography using *n*-hexane/ethyl acetate solvent system (1:1.8). Melting point: 149–150°C; color and appearance: solid powder white in color; yield: 69.8%; IR: 1650.38 cm^{-1} amide carbonyl (C═O). RF: 0.37 (*n*-hexane:ethyl acetate, 3:2). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 10.30 (1H, s, amide NH), 7.72–7.70 (2H, m, Ar–H), 7.52–7.50 (3H, m, Ar–H), 7.33 (1H, d, J = 2 Hz, Ar–H), 7.15 (1H, dd, J = 8.5, 2 Hz, Ar–H), 6.92 (1H, d, J = 9 Hz, Ar–H), 3.73 (6H, s, O–CH_3), 2.58 (3H, s, CH_3). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 170.51, 160.64, 160.01, 149.06, 145.96, 145.80, 142.83, 132.64, 130.56, 129.32, 128.60, 128.41, 128.20, 113.89, 112.55, 112.20, 108.23, 105.26, 56.23, 55.87, 12.38.

4.5 *N*-(3,5-Dimethoxyphenyl)-5-methyl-3-phenyl-1,2-isoxazole-4-carboxamide (2c)

Purified by silica gel column chromatography using *n*-hexane:ethyl acetate solvent system (1.2:1). Melting point: 143–145°C; color and appearance: solid powder, green in color; yield: 78%; IR: 1,652/cm amide carbonyl (C═O). RF: 0.56 (*n*-hexane:thyl acetate, 3:2). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 10.39 (1H, s, amide NH), 7.69–7.50 (5H, m Ar–H), 6.88 (2H, s, Ar–H), 6.29 (1H, s, Ar–H), 3.72 (6H, s, O–CH_3), 2.58 (3H, s, CH_3). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 170.36, 161.00, 160.66, 160.49, 140.69, 130.59, 129.34, 128.82, 128.50, 128.19, 113.80, 98.50, 96.44, 55.61, 12.39.

4.6 5-Methyl-3-phenyl-*N*-(3,4,5-trimethoxyphenyl)-1,2-isoxazole-4-carboxamide (2d)

Purified by silica gel column chromatography using *n*-hexane/ethyl acetate solvent system (2:3). Melting point: 165–167°C; color and appearance: solid powder, white incolor; yield: 77%; IR: 1648.56 cm^{-1} amide carbonyl (C═O). RF: 0.37 (*n*-hexane:ethyl acetate, 3:2). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 10.39 (1H, s, amide NH), 7.71–7.50 (5H, m, Ar–H), 7.03 (2H, s, Ar–H), 3.75 (6H, s, O-CH_3), 3.64 (3H, s, O-CH_3), 2.58 (3H, s, CH_3). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 170.28, 160.67, 160.27, 153.25, 135.22, 134.52, 130.57, 129.35, 128.54, 128.21, 113.82, 97.89, 60.57, 56.21, 12.40.

4.7 5-Methyl-*N*,3-diphenyl-1,2-isoxazole-4-carboxamide (2e)

Purified by silica gel column chromatography using *n*-hexane/ethyl acetate solvent system (1:1). Melting point: 166–168°C; color and appearance: solid powder, light brown in color; yield: 71%, IR :1673.64 cm^{-1} amide carbonyl (C═O). RF: 0.75 (*n*-hexane:ethyl acetate, 3:2). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 10.44 (1H, s, amide NH), 7.71–7.11 (10H, m, Ar–H), 2.58 (3H, s, CH_3). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 170.33, 160.67, 160.46, 139.04, 138.86, 130.59, 129.33, 129.30, 128.54, 128.19, 124.69, 124.57, 120.32, 120.21, 12.38.

4.8 Biology methods

4.8.1 Cell culture and MTS assay

Hepatocellular carcinoma (Hep3B and HepG2), cervical adenocarcinoma (HeLa), breast carcinoma (MCF-7), and normal cell line (Hek293T) were cultured in RPMI-1640

media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin antibiotics, and 1% L-glutamine. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. Cells were seeded at 2.6×10^4 cells per well in a 96-well plate. After 72 h, cells were confluent, and media was changed and cells were incubated with various concentrations of the synthesized compounds for 24 h. Cell viability was assessed by CellTilter 96® Aqueous One Solution Cell Proliferation (MTS) assay according to the manufacturer's instructions (Promega Corporation, Madison, WI). Briefly, at the end of the treatment, 20 µL of MTS solution per 100 µL of media was added to each well and incubated at 37°C for 2 h. Absorbance was measured at 490 nm [34].

4.8.2 Antioxidant evaluation

DPPH (2,2-diphenyl-1-picrylhydrazyl) was used to evaluate the antioxidant activity of all synthesized compounds (**2a–2e**). A quantity of 1.5 mg of each compound was dissolved in methanol to prepare the stock solution. A serial dilution of the previous stocks was prepared (1, 3, 5, 7, 10, 20, 30, 50, 80, and 200 µg/mL). DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above-prepared working concentration in a ratio of 1:1:1, respectively. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517 nm using a UV-Vis-spectrophotometer. The percentage (%) that was used to inhibit the DPPH free radical was determined using the following formula:

$$\text{DPPH inhibition potential } (\%) = [(C - T)/C] \times 100\%,$$

where C is the absorbance of the control (without samples) and T is the absorbance of the tested samples [35].

Acknowledgements: The authors would like to thank An-Najah National University.

Funding information: The authors state no funding involved.

Conflict of interest: The authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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