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Synthesis and Biological Evaluation of Benzodioxole Derivatives as Potential Anticancer and Antioxidant agents

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Abstract: a series of benzodioxole compounds were synthesized and evaluated for their cytotoxic activity against cervical (Hela), colorectal (Caco-2), and liver (Hep3B) cancer cell lines. Compounds **5a, 5b, 6a, 6b, 7a** and **7b** showed very weak or negligible anticancer activity with IC_{50} 3.94-9.12 mM. On the contrary, carboxamide containing compounds **2a** and **2b** showed anticancer activity. Both **2a** and **2b** reduced Hep3B secretions of α-fetoprotein (α-FP) to 1625.8 ng/ml and 2340 ng/ml, respectively, compared to 2519.17 ng/ml in untreated cells. The results also showed that compound **2a** has potent anticancer activity against Hep3B cancer cell line. Furthermore, in cell cycle analysis, compound **2a** induced arrest in the G2-M phase in value of 8.07% that was very close to the activity of doxorubicin (7.4%). These results indicate that compound **2a** has a potent and promising antitumor activity. However, benzodiazepine derivatives (**7a** and **7b)** showed moderate antioxidant activity with IC_{50} values of 39.85 and 79.95 μM, respectively compared with the potent antioxidant agent Trolox (IC_{50} = 7.72 μM).

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Introduction

Cancer is still one of the most pervasive global health care problems. The development and discovery of novel anticancer medications remain extremely important due to various factors. These factors include treatments that may cause major side effects or can be rather expensive. Alternatives that are safer biologically and more affordable are still highly desirable [1-4]. The investigations of conventional anticancer medications have been focused mainly on the identification of cytotoxic chemotherapeutic agents that could be from synthetic or natural origin [5].

According to the World Health Organization (WHO) surveys, cancer is one of the most leading causes of death around the globe and in the last few years approximately 9 million deaths were estimated annually because of this disease, and due to this reason the discovery and development of novel anticancer agents are still very necessary [4,6]. In 2018, liver cancer was predicted to be the sixth most commonly diagnosed cancer worldwide and a fourth leading cause of cancer deaths, with approximately 782,000 deaths and eight hundred thousand new cases annually [7]. Since the researchers could not find the ideal treatment for liver cancer, and because of the disadvantages of the current agents like the toxicity and side effects, chemotherapy remains one of the most promising methods in the treatment of liver cancer. Therefore, to innovate a new chemotherapeutic agent, great efforts are required to develop and find a new anticancer agent with safer doses toward cancerous cells [2,8,9].

Different natural extracts were evaluated in the last years as antitumor agents with great activity [10], and

many different naturally isolated agents, such as alkaloids and taxoids, targeted the tubulin have reached the clinic as anticancer agents [9]. Combretastatin (Figure 1) was isolated from the natural plant *Combretum caffrum* and by chemical modification, where its structure was altered to Combretastatin-A4 (CA-4), and many other CA-4 derivatives were synthesized and evaluated as tubulin inhibitors, combretastatin A-4 phosphate (fosbretabulin) figure. 1 is one of these agents which was approved by the FDA for thyroid cancer [2,11,12].

Antioxidants are biologically active agents that can prevent or slow the damage of cells caused by unstable free radical molecules produced by the body as a reaction to environmental and other pressures [13,14]. Different types of substances work as antioxidants, some of these are endogenous, which our bodies can produce, other agents can be taken from natural plants and foods like ascorbic acid (Vitamin C), R-tocopherol (Vitamin E), and β-carotene, the last type can be chemically synthesized [13-16]. In the last few decades, there has been a good number of chemically synthesized agents that have significant antioxidant activity, such as quinolinone-3-aminoamides [14], thienopyrazole, thienopyrimidine [17], and N-aryl-1,4-dihydropyridines derivatives [18]. The mechanism of some of the chemically synthesized substances, like Rebamipide and the water-soluble Vitamin E analog Trolox (Figure 2), work by scavenging free radicals, hence they exhibit effective antioxidant properties [14,19,20].

The heterocycle-containing structures have several biological effects including anticancer [2,3], anti-inflammatory [21], antioxidant [17] and analgesic [22]. Therefore, benzodiazepine (BDZs) derived molecules have various biological and pharmacological effects. In addition to their effect on the central nervous system [23,24], they also have anti-proliferative activities [25], anti-HIV [26], anti-inflammatory [27] and anti-microbial activities [28]. While the compounds containing benzodioxole e moiety have been shown to have different biological activity including anticancer, anti-tuberculosis, antimicrobial, analgesic, and anti-epileptic activities (Figure 3) [29-34]. At the same time, compounds that have both benzodioxole and methoxyphenyl which are related to the CA-4, an important moiety, has shown anti-tumor activity [3] (Figure 3).

Due to that evidence, the current work aims to synthesize benzodioxole derivatives with or without methoxyphenyl moiety and to evaluate some of their new biological activity, such as antioxidant and anticancer activity on HeLa, Caco-2, and HepB3 cancer cell lines.

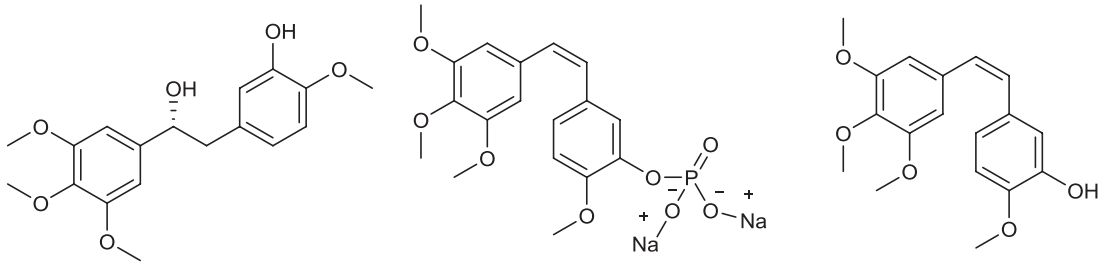


Figure 1 Combretastatin, CA-4P (fosbretabulin) and CA-4 structures

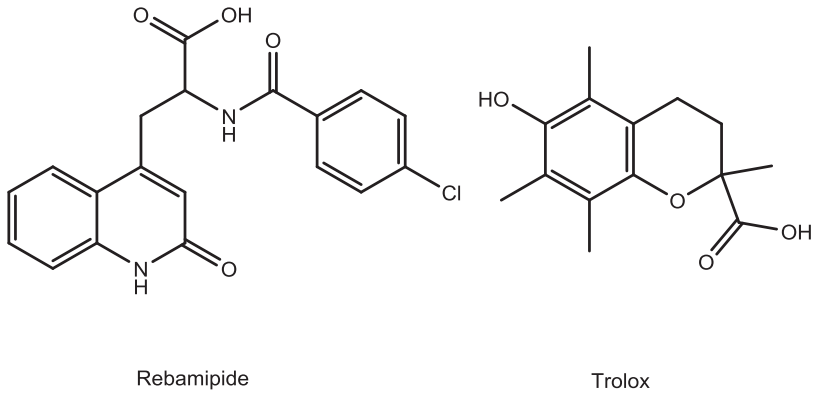


Figure 2 Chemical structures of Rebamipide and Trolox

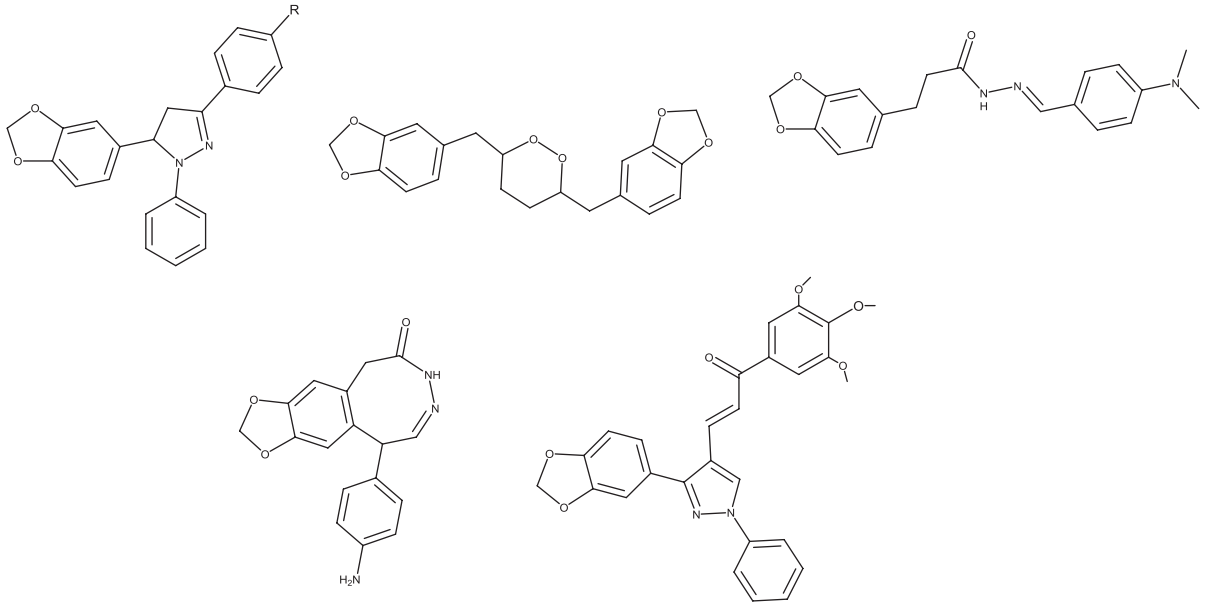
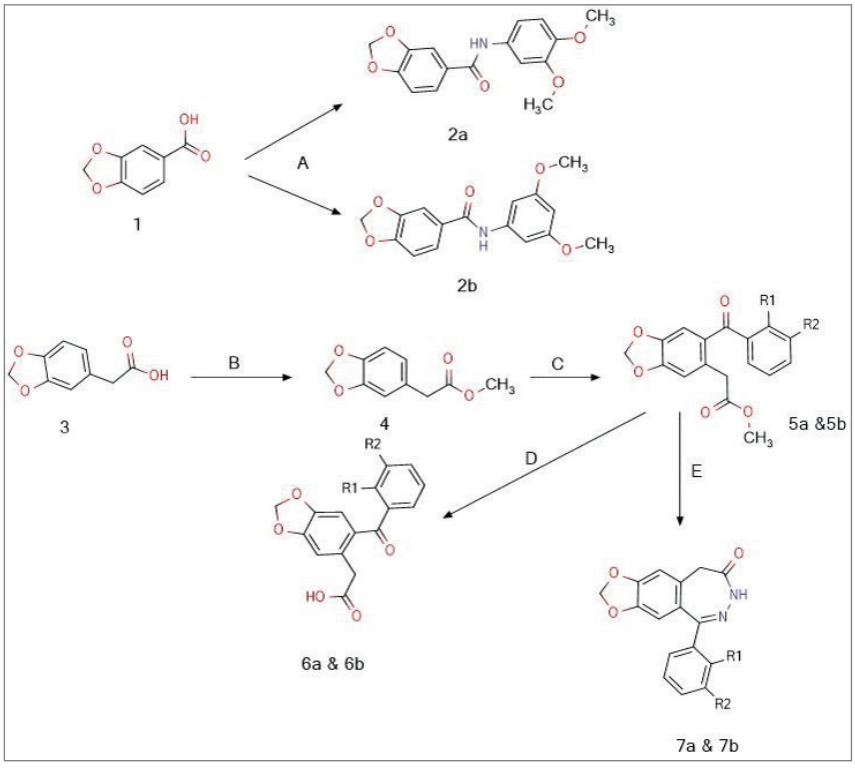


Figure 3 Structures of Benzodioxole derivatives that have biological activities



Scheme 1 The reaction steps A) Aniline derivatives, DCM, EDC, DMAP B) methanol, oxalyl chloride C) DCM, P_2O_5, aryl-carboxylic acid, D) MeOH/THF/H_2O, LiOH reflux E) NH_2NH_2, acetic acid, ethanol, reflux, for 24 h,. (for **5a**, **6a**, **7a** the R1=H, R2=Cl and for **5b**, **6b**, **7b** the R1=Cl, R2=H)

Results and Discussion

Chemistry

The amide derivatives (**2a** & **2b**), ketoester (**5a** & **5b**), acetic acid (**6a** & **6b**) and the 2,3-benzodiazepine derivatives (**7a** & **7b**) were synthesized as outlined in Scheme 1. The coupling to form amides **2a** & **2b** was afforded to employ EDCI as activating agent and DMAP as a covalent nucleophilic catalyst, then the active species were reacted with the aniline derivatives [35]. To confirm the synthesis of these two compounds, the HRMS was used and 302 g/mol

molecular mass was observed for **2a** & **2b,** after that, they were purified by using column chromatography (n-hexane: ethyl acetate solvent system 60%:40%). The ^{1}H-NMR peaks confirmed the synthesis of these compounds, one proton at 9.91 and 9.97 ppm singlet peaks for N-H, 2 protons at 6.12 and 6.13 ppm another singlet peaks for -CH_2- of benzodioxole were observed for **2a** & **2b** compounds, respectively**.** However, 6 protons were observed in the aromatic area and another 6 protons of di-methoxy substituents were founded at 3.73 ppm for both compounds. The ^{13}C-NMR spectrum showed a C signal of carbonyl at 164 ppm and signals at 55-56 ppm for methoxy's carbon were observed for both **2a & 2b** compounds.

The methyl 3,4-(Methylenedioxy) phenyl acetate (**4**) was generated by the esterification reaction of 3,4-(Methylenedioxy) phenylacetic acid (**3**) (Scheme 1). To collect high yield of ester (**4**), oxalyl chloride was used which was added dropwise to methanol solvent and stirred for 30 minutes in an ice bath. The synthesis of ester (**4)** was confirmed by TLC were the point was moved from a lower point and travel more in the mobile phase and it was colorless oil as cited before in the patent [36] and in another article [37]. The ketoesters **5a** & **5b** were synthesized by dissolving the ester (**4**) in dichloromethane with benzoic acid derivatives in the presence of an excess of phosphorus pentoxide, stirring at room temperature for approximately 16 hrs. To confirm the synthesis of ketoester **5a** & **5b** compounds, the HRMS was used and 333 g/mol molecular mass was observed**.** The ^{1}H-NMR spectrum data of these compounds showed 6 protons in the aromatic area, 2 protons at 6.14 and 6.15 ppm singlet peaks for -CH_2- of benzodioxole and 5 protons were observed in 3.49 and 3.78 ppm for $-CH_2$-CO-CH_3 for **5a & 5b**. According to the ^{13}C-NMR spectrum, C signal of carbonyl groups was found at 194 and 171 ppm for **5a,** and at 37-51 ppm two signals of aliphatic carbon were observed, and similar signals for **5b** were observed. The subsequent treatment of ketoester (**5a** & **5b)** with hydrazine hydrate in ethanol with acetic acid afforded the benzodiazepine derivatives **7a** and **7b** [24], compounds **6a** & **6b** were synthesized by hydrolysis reaction of the ester compounds **5a** & **5b** using LiOH [38]. (see Scheme 1). HRMS results confirmed the synthesis of **6a** & **6b** with molecular weight 319 g/mol, and for **7a** and **7b** with molecular weight 315 g/mol, and the ^{1}H-NMR spectrum data showed one proton with singlet peak around 12 ppm (-COOH) for **6a** and **6b,** and one proton with singlet peak around 11 ppm (-CONH-) for **7a** and **7b.** However, ^{13}C-NMR spectrum data showed C signal of carbonyl groups at 195 and 172 ppm for both **6a** and **6b,** and at 174 and 175 ppm signals of carbonyl carbon of both benzodiazepine **7a** and **7b** compounds, respectively.

Table 1 The IC_{50} different cancer cell lines (HeLa, Caco-2 and Hep3P) in Milli Molar concentrations

Compound Code	Hela Cell IC_{50} (mM)	Caco-2 Cell IC_{50} (mM)	Hep3B Cell IC_{50} (mM)
2a	0.5>	0.5>	0.5>
2b	0.5>	0.5>	0.5>
5a	7.79	5.38	5.80
5b	N/A	8.64	3.78
6a	7.29	7.29	6.07
6b	5.79	4.98	3.69
7a	5.46	5.46	3.49
7b	9.12	7.11	4.23
DOX	0.5>	0.5>	0.5>

Biological evaluations

Cytotoxic evaluation of the compounds 3a, 3b, 4a, and 4b

MTS assay was used to determine the cytotoxicity effect of benzodioxole e derivatives on HeLa, Caco-2, and Hep3B cells. As shown in Table 1, four concentrations were used (5, 2, 1, and 0.5 mM). Except amide benzodioxole derivatives compounds (**2a** & **2b),** the rest of the compounds (**5a-7b**) showed low anticancer activity with a high IC_{50} in all tested cell lines, the IC_{50} were in range of 3.94-9.12 mM. However, their cytotoxicity effect was increased as their IC_{50} values on Hep3B cell line were the lowest, and this means that all benzodioxole derivatives without amide derivatives are less cytotoxic on all investigated cells. Therefore, a positive correlation was found between benzodioxole derivatives with amide derivatives and the cytotoxicity toward cell lines, compounds **2a** and **2b** showed the lowest IC_{50}, indicating a strong cytotoxicity effect when compared with Doxorubicin, which was used as a positive control (**DOX**).

Alpha-fetoprotein results

According to results obtained from the MTS test, the benzodioxole derivatives with amide (**2a** & **2b)** have potent cytotoxicity and to further associate the inhibitory effects of **2a** and **2b** on cell proliferation, we evaluated medium levels of αFP. Both **2a** and **2b** reduced Hep3B secretions of α-FP to 1625.8±64.37 ng/ml and 2340±65.57 ng/ml, respectively, as compared to 2519.17 ±198.05 ng/ml in untreated cells indicated, less proliferation and reduced in the tumorigenicity of Hep3B following treatments (Figure 4). The results showed that the **2a** has potent anticancer

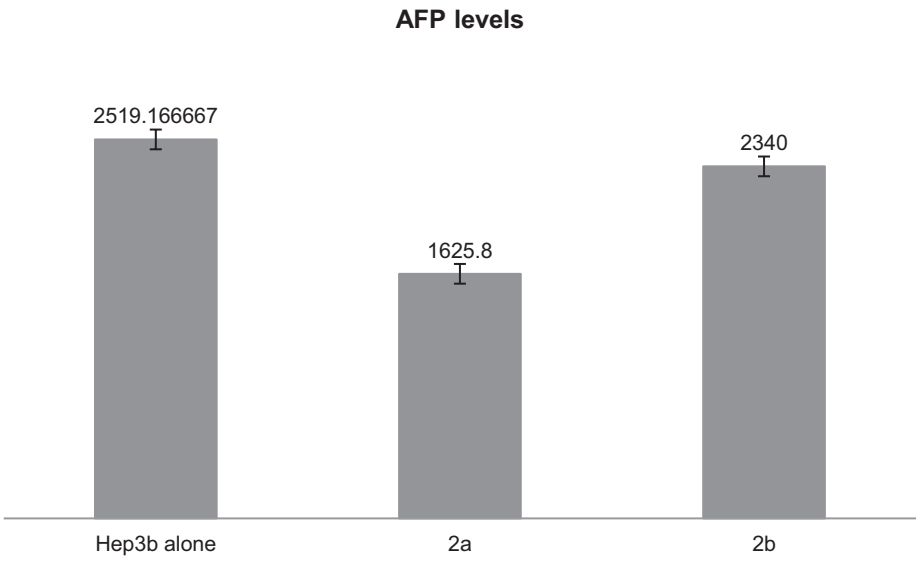


Figure 4 Cell proliferations of untreated cells (control) **2a** and **2b**

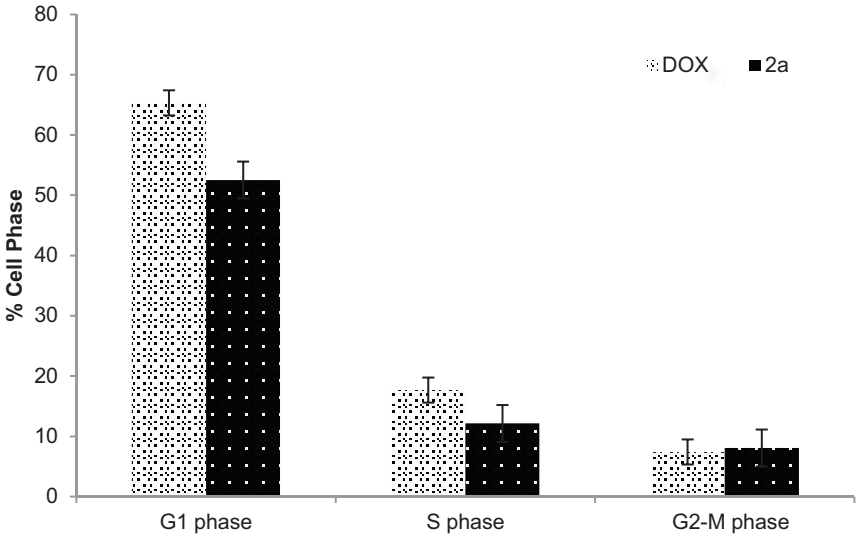


Figure 5 Cell cycle analysis of Hep3B cells after treatment with compound **2a** and **DOX** control

activity on the Hep3B cancer cell line, and compound **2b** has weak anticancer activity (Figure 4).

2a inhibited DNA cell cycle of Hep3B cells

To investigate whether **2a** could induce cell cycle perturbations in liver cancer cells, flow cytometry analyses of propidium iodide stained nuclei cells were performed. Cell cycle parameters were only investigated with compound **2a** because **2b** did not show potent activity in the last test. Figure 5 shows a significant decrease ($P<0.04$) in the fraction of cells in the G1 phase following **2a** treatments to 52.53 % as compared to 65.3 % in the **DOX**. Similarly, a reduction in the S phase fraction was noticed as it decreased from 17.6 % in **DOX** to 12.13% ($P<0.05$), and in G2-M phase we can see that the **2a** make arrest in value (8.07%) very close to that in **DOX** (7.4%). These results indicate compound **2a** as a potent inhibitor of cell cycle progression at the G2-M phase and might suggest a potential anticancer property.

Antioxidant Evaluation

In this research project, the investigation for the antioxidant activity of all the synthesized compounds (**2a-7b)** was estimated using *in vitro* antioxidant DPPH assay and

the results were compared to the well-known antioxidant compound (Trolox). To evaluate the *in vitro* antioxidant activity of the synthesized compounds, interaction with the stable free radical DPPH method was used. The DPPH method is described as a simple, convenient and rapid method independent of sample polarity for screening. These advantages made the DPPH method interesting for testing our synthesised compounds [39]. A range of concentration of the synthesized compounds were prepared and tested in order to obtain the IC_{50} of these compounds. Out of all the tested compounds, only two compounds having the benzodiazepine structures, namely compounds **7a** & **7b,** showed a moderate antioxidant activity with IC_{50} values of 39.85 and 79.95 μM, respectively compared to Trolox as positive control (IC_{50} 7.72 μM). Benzodiazepine ring with different substituent groups probably increased the antioxidant activities of this group of compounds and made a good interaction with DPPH. Therefore, the substituted groups on this scaffold improved the inhibitory activities on free radicals [40].

Conclusion

The synthesized carboxamide compounds **2a** & **2b** illustrated potent anticancer against different cancer cell lines, which was related to the presence of di-methoxy phenyl amide derivatives in benzodioxole compounds which refer to **CA-4** structure potent anticancer agent. However, the other benzodioxole compounds without amide (**5a-7b**) showed weak cytotoxicity against HeLa, Caco-2, and Hep3B cancer cell lines at high concentration with IC_{50} range of 3.94-9.12 mM. According to the α-FP tumor marker test, the **2a** compound reduced Hep3B secretions of αFP to 1625.8 ng/ml as compared to 2519.17 ng/ml in untreated cells indicated less proliferation and reduced in the tumorigenicity of Hep3B. Further investigation to the molecular effects of the synthesized derivatives, it was revealed that compound **2b** caused cell cycle arrest at the G2/M phase and it was very close to Doxorubicin value as a reference. Furthermore, the benzodiazepine compounds **7a** and **7b** have moderate antioxidant activity with IC_{50} doses of 39.85 and 79.95 μM, respectively, and their antioxidant activities occur due to their benzodiazepine structure that can make interaction with DPPH. In future studies, we can synthesize more analogs of carboxamide-benzodioxole derivatives as promising anticancer agents and more benzodiazepine analogs as anti-oxidant agents, further *in-vivo* investigations are required to approve these effects and to design suitable pharmaceutical dosage forms from these compounds.

Experimental

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/FTNIR spectrometer. 1H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d6 and were performed on two NMR instruments. The first was 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. The second was a Bruker 300 MHz-Avance III High-Performance Digital FT-NMR spectrometer at the NMR facility at the Doping and Narcotics Analysis Laboratory of the faculty of pharmacy, Anadolu University, Turkey. Tetramethylsilane was used as the internal standard. All chemical shifts were recorded as d (ppm). High resolution mass spectra data (HRMS) were collected using a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) using ESI (+) method (The instrument was coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA) at Pharmacy Faculty Gazi University Ankara-Turkey.

General procedure of N-(methoxyphenyl)benzo[d][1,3] dioxole-5-carboxamide 2a & 2b

3,4-(Methylenedioxy)benzoic acid (**1**) (1.5 mmol) was dissolved by stiring in dichloromethane (15 ml). To this mixture, DMAP (0.3 mmol) and EDC (1.8 mmol) were added and the mixture was allowed to stir under nitrogen gas at room temperature for 1 h. After that, the appropriate aniline derivative (1.8 mmol) was added and the mixture was allowed to stir for 24-78 h. The reaction was monitored by TLC for the end of the reaction, the solvent was removed under reduced pressure and dissolved again in dichloromethane, 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 system or by the crystallization utilizing appropriate solvent system.

N-(3,4-dimethoxyphenyl)benzo[d][1,3]dioxole-5-carboxamide HSD-2 (2a)

Purified by silica gel column chromatography using n-hexane: ethyl acetate solvent system (3:2). Solid product, M.P. 175-177°C, Yield 75%. 1H NMR (300 MHz, DMSO-d6) δ: 9.91 (s, 1H, N-H), 7.55 (dd, 1H, *J* = 8.1, 1.8 Hz, Ar-H), 7.50 (d, 1H, *J* = 1.8 Hz, Ar-H), 7.44 (d, 1H, *J* = 2.4 Hz, Ar-H), 7.30 (dd, 1H, *J* = 8.7, 2.4 Hz, Ar-H), 7.04 (d, 1H, *J* = 8.1 Hz, Ar-H), 6.91 (d, 1H, *J* = 8.7 Hz, Ar-H), 6.12 (s, 2H, O-C**H**$_2$-O), 3.74 (s, 3H, -OC**H**$_3$), 3.73 (s, 3H, OC**H**$_3$); 13C NMR (300 MHz, DMSO-d6) δ ppm: 164.50 (C=O), 150.36, 148.82, 147.81, 145.47, 133.20, 129.27, 123.09, 112.67, 122.25, 108.37, 108.04, 105.86, 102.24, 56.15 (O-CH_3), 55.79 (O-CH_3). IR spectrum (FT-IR/ATR) cm-1: 3346-3280 (N-H), 2941-2833 (aliphatic C-H), 1684 (C=O). HRMS (m/z): $[M+H]^+$ calcd. For $C_{16}H_{16}NO_5$ 302.1028, found. 302.1024.

N-(3,5-dimethoxyphenyl)benzo[d][1,3]dioxole-5-carboxamide HSD-4 (2b)

Purified by silica gel column chromatography using n-hexane: ethyl acetate solvent system (3:2). Solid product, M.P. 150.5-152°C, Yield 77%. 1H NMR (300 MHz, DMSO-d6): δ 9.97 (s, 1H, N-H), 7.55 (dd, 1H, *J* = 8.4, 1.8 Hz, Ar-H), 7.49 (d, 1H, *J* = 1.8 Hz, Ar-H), 7.04-7.06 (m, 3H, Ar-H), 6.24 (t, 1H, *J* = 2.4 Hz, Ar-H), 6.13 (s, 2H, O-C**H**$_2$-O), 3.72 (s, 6H, -OC**H**$_3$). 13C NMR (300 MHz, DMSO-d6): δ 164.97 (C=O), 160.79, 150.54, 147.83, 141.38, 129.12, 123.30, 115.66, 108.39, 108.14, 102.30, 98.85, 96.04, 55.56 (O-CH_3), 55.53 (O-CH_3). IR spectrum (FT-IR/ATR) cm^{-1}: 3344-3130 (N-H), 2931-2833 (aliphatic C-H), 1672 (C=O). HRMS (m/z): $[M+H]^+$ calcd. For $C_{16}H_{16}NO_5$ 302.1028, found. 302.1028.

Synthesis of methyl 2-(2H-1,3-benzodioxole -5-yl) acetate (4)

3,4-(methylenedioxy)-phenyl acetic acid (**3**) (2 g, 11.10 mmol) was dissolved in 30 ml methanol. This solution was then cooled in an ice bath at 0°C, and oxalyl chloride (1 mL, 11.70 mmol) was added drop wise; the reaction mixture was stirred for 30 min. The reaction mixture was evaporated under reduced pressure to dryness. The dried residue was dissolved with ethyl acetate, then washed with saturated sodium bicarbonate (NaHCO3) and distilled water respectively. The organic layer was separated and dried with magnesium sulfate as a drying agent. It was then filtered and evaporated using rota evaporator, the dried residue was then purified by silica gel column chromatography using n-hexane: ethyl acetate mobile system (50:50), afforded compound (**4**) was yellow oil with 95% yield.

General procedure for ketoester (5a & 5b) synthesis

To a stirred solution of dichloromethane (40 ml) and methyl 2-(2*H*-1,3-benzodioxole -5-yl) acetate compound (**4**) (666 mg, 3.42 mmol), ortho or meta chlorobenzoic acid (700 mg, 4.44 mmol) and phosphorus pentoxide (5 g) were added. Then the mixture was further stirred at room temperature for 16-20 hrs, then distilled water (60 mL) was cautiously added and the mixture extracted with ethyl acetate (2 × 60 mL). The organic layer was separated and then it was treated with 1M NaOH (60 mL), saturated sodium chloride (60 mL), and distilled water (2 × 60 mL). The organic layer was dried with magnesium sulphate as the drying agent, then was filtered and evaporated under reduced pressure, and purified by silica gel column chromatography.

Methyl 2-[6-(3-chlorobenzoyl)-2H-1,3-benzodioxole -5-yl] acetate (5a)

Purified by silica gel column chromatography using n-hexane: ethyl acetate solvent system (4:1). Crude green semi solid, Yield 70%. 1H NMR (500 MHz, DMSO-d6) :δ 7.74 (d, 1H, J = 7.5 Hz, Ar-H), 7.63 (t, 1H, J = 2 Hz, Ar-H), 7.55–7.61 (m, 2H, Ar-H), 7.07 (s, 1H, Ar-H), 6.95 (s, 1H, Ar-H), 6.15 (s, 2H, O-C**H**$_2$-O), 3.78 (s, 2H, -C**H**$_2$-CO-), 3.49 (s, 3H, -CO-O**CH**$_3$).13C NMR (400 MHz, DMSO-d6) :δ 194.65 (Ketone C=O), 171.04 (ester C=O), 149.46, 145.63, 139.64, 133.23, 132.50, 130.43, 129.80, 128.95, 128.34, 127.83, 112.19, 109.89, 102.01, 51.46 (-O-CH_3), 37.81 (-CH_2-). IR (FTIR/FTNIR-ATR):1737 cm^{-1} ester carbonyl (C=O), 1661 cm^{-1} keton carbonyl (C=O).HRMS (m/z): $[M+H]^+$ calcd. For $C_{17}H_{13}O_5Cl$ 333.0524, found 333.0517.

Methyl 2-[6-(2-chlorobenzoyl)-2H-1,3-benzodioxole -5-yl] acetate (5b)

Purified by silica gel column chromatography using hexane: ethyl acetate solvent system (3:1). Crude yellow solid powder, m.p. 80-82 °C, Yield 71%; 1H NMR (500 MHz, DMSO-d6) : δ 7.54-7.59 (m, 2H, Ar-H), 7.47 (t, 1H, J = 7.5 Hz, Ar-H), 7.40 (d, 1H, J = 7.5 Hz, Ar-H), 7.11 (s, 1H, Ar-H), 6.72 (s, 1H, Ar-H), 6.14 (s, 2H, O-C**H**$_2$-O), 3.94 (s, 2H, -C**H**$_2$-CO-),

3.59 (s, 3H, -CO-OC**H**$_3$); 13C NMR (400 MHz, DMSO-d6) : δ 194.28 (Ketone C=O), 170.92 (ester C=O), 150.68, 146.06, 138.85, 132.30, 131.65, 129.85, 129.22, 127.20, 112.94, 111.22, 102.34, 51.38 (-O-CH_3), 38.83 (-CH_2-). IR (FTIR/FTNIR-ATR): 1727 cm^{-1} ester carbonyl (C=O), 1663 cm^{-1} ketone carbonyl (C=O). HRMS (m/z): $[M+H]^+$ calcd. For C17H13O5Cl 333.0524, found 333.0523.

General procedure for 2-(6-benzoyl-2H-1,3-benzodioxole -5-yl)acetic acid (6a & 6b) synthesis

The ketoesters (150 mg, 0.45 mmol) (**5a** or **5b**) were dissolved in methanol/H_2O/THF (10/10/10 mL), then NaOH (180.3 mg, 4.5 mmol) was added. The solution was heated in an oil bath and refluxed for 3.5 h, before being cooled to room temperature. The solution was then evaporated, and the residue was made acidic by adding HCl 2 N dropwise (pH = 2). The precipitate was filtered and concentrated under vacuum to give the crude products **5a** & **5b**. The resulting residue was separated by silica gel column chromatography to yield the corresponding compounds (**6a** & **6b**).

2-[6-(3-chlorobenzoyl)-2*H*-1,3-benzodioxole -5-yl]acetic acid (6a)

Purified by silica gel column chromatography using DCM: Methanol solvent system (4.7:0.3). Crude off-white solid powder, m.p. 115-117 °C, Yield 94%. ^{1}H NMR (300 MHz, DMSO-d6) : δ 12.22 (s, 1H, COO**H**), 7.71 (d, 1H, *J* = 7.8 Hz, Ar-H), 7.55-7.63 (m, 3H, Ar-H), 7.04 (s, 1H, Ar-H), 6.91 (s, 1H, Ar-H), 6.12 (s, 2H, O-C**H**$_2$-O), 3.70 (s, 2H, -C**H**$_2$-CO-); ^{13}C NMR (300 MHz, DMSO-d6) : δ 195.31 (Ketone C=O), 172.62 (COOH), 149.81, 145.92, 140.24, 133.69, 132.95, 131.15, 131.02, 130.90, 129.57, 128.91, 112.67, 110.24, 102.43, 38.60 (-CH_2-). IR (FTIR/FTNIR-ATR):1703, 1651 cm^{-1} carbonyl (C=O). HRMS (m/z): $[M+H]^+$ calcd. For $C_{16}H_{11}O_5Cl$ 319.0368, found 319.0368.

2-[6-(2-chlorobenzoyl)-2*H*-1,3-benzodioxole -5-yl]acetic acid (6b)

Purified by silica gel column chromatography using n-hexane: ethyl acetate solvent system (1:1). Crude yellow solid powder, m.p. 155-157 °C, Yield 96%. ^{1}H NMR (500 MHz, DMSO-d6) : δ 12.18 (s, 1H, COO**H**), 7.54-7.59 (m, 2H, Ar-H), 7.47 (td, 1H, *J* = 7, *J* = 1 Hz, Ar-H), 7.40 (d, 1H, J = 7.5 Hz, Ar-H), 7.08 (s, 1H, Ar-H), 6.70 (s, 1H, Ar-H), 6.13 (s, 2H, O-CH_2-O), 3.87 (s, 2H, -CH_2-CO-);13C NMR (400 MHz, DMSO-d6): δ 194.33 (Ketone C=O), 172.09 (COOH), 150.62, 145.91, 139.06, 133.23, 131.73, 129.98, 129, 45, 129.36, 127.28, 113.04, 111.21, 102.34, 38.90 (-CH_2-). IR (FTIR/FTNIR-ATR):1704, 1658 cm^{-1} carbonyl (C=O). HRMS (m/z): $[M+H]^+$ calcd. For $C_{16}H_{11}O_5Cl$ 319.0368, found 319.0365.

General procedure for 2,3-benzodiazepine (7a & 7b) synthesis

The ketoesters (200 mg, 0.6 mmol) (**5a** or **5b**) were dissolved in ethanol (25ml), then the hydrazine hydrate (214 µL, 3.44 mmol) and acetic acid (100 µL) were added, reflux for 24 hrs, then the solvent was removed under vacuum pressure and the resulting residue was separated by silica gel column chromatography to yield the corresponding compounds (**7a** & **7b**).

7,8-Methylenedioxy-1-(3-chlorophenyl)-3,5-dihydro-2,3-benzodiazepin-4(4H)-one (7a)

Purified by silica gel column chromatography using n-hexane: ethyl acetate solvent system (1:1). Crude semi brown solid powder, m.p. 214-125.5 °C, Yield 73%. ^{1}H NMR (500 MHz, DMSO-d6) : δ 11.03 (s, 1H, N-H), 7.54-7.55 (m, 2H, Ar-H), 7.44-7.50 (m, 2H, Ar-H), 7.10 (s, 1H, Ar-H), 6.64 (s, 1H, Ar-H), 6.11 (s, 2H, O-CH_2-O), 3.41 (s, 2H); ^{13}C NMR (400 MHz, DMSO-d6) : δ 175.05 (amide C=O), 163.21, 155.85, 151.46, 145.63, 138.46, 137.07, 135.65, 134.76, 133.64, 133.08, 129.25, 113.15, 107.40, 46.55 (-CH_2-). IR (FTIR/FTNIR-ATR):1661 cm^{-1} carbonyl (C=O). HRMS (m/z): $[M+H]^+$ calcd. For $C_{16}H_{11}ClN_2O_3$ 315.0531, found. 315.0527

7,8-Methylenedioxy-1-(2-chlorophenyl)-3,5-dihydro-2,3-benzodiazepin-4(4H)-one (7b)

Purified by silica gel column chromatography using n-hexane: ethyl acetate solvent system (1:1). Crude yellow solid powder, m.p. 189.5-191.5 °C, Yield 68%. ^{1}H NMR (500 MHz, DMSO-d6) : δ 11.13 (s, 1H, N-H), 7.61-7.62 (m, 1H, Ar-H), 7.50-7.52 (m, 3H, Ar-H), 7.10 (s, 1H, Ar-H), 6.30 (s, 1H, Ar-H), 6.08 (s, 2H, O-CH_2-O), 3.47 (s, 2H);^{13}C NMR (400 MHz, DMSO-d6) : δ 174.63 (amide C=O), 162.85, 155.51, 151.74, 142.90, 136.58, 136.25, 135.85, 135.08, 132.78, 133.08, 113.15, 111.63, 107.42, 46.61(-CH_2-). IR (FTIR/FTNIR-ATR): 1658 cm^{-1} carbonyl (C=O). HRMS (m/z): $[M+H]^+$ calcd. For $C_{16}H_{11}ClN_2O_3$ 315.0531, found. 315.0533.

Biology methods

Cell culture, alpha feto-protein, apoptosis, Cell cycle and flow cytometry

Hepatocellular Carcinoma (Hep3B), cervical adenocarcinoma (HeLa) and colon cancer cells (colo205) were cultured in RPMI-1640 media and 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 x 104 cells/well in a 96-well plate. After 48 h, cells were confluent and media was changed and cells were incubated with various concentrations of the tested 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.

Hep3B was cultured in DMEM with 10% fetal bovine serum and secrete α-FP served as a marker of tumor activity. Hep3B-cells also express surface HBsAg that can be stained as a marker for Hep3B-cells. The **2a** and **2b** compounds were incubated for 24 h with Hep3B in concentration of 10 µl/ml and then αFP levels in the medium were assessed by a commercially available ELISA kit from (R&D Systems, Inc., USA). Hep3B were then harvested and trypsinised (0.05% trypsin/0.53 mM EDTA) washed and analysed for apoptosis [41].

Following cultures, harvested Hep3B cells were adjusted to 10^6/ml in staining buffer (in saline containing 1% bovine albumin; Biological Industries, Israel). To determine Hep3B purity, they were fixed with 4% paraformaldehyde for 10 minand permeabilized with 0.1% saponin in PBS for 20 min, then stained with anti-human HBsAg monoclonal-antibody (R&D systems USA) for 30 min at room-temperature.

For apoptosis and viability measurements, propidium-iodide (PI) staining of fragmented DNA and phosphatidylserine staining by annexin V-conjugated to FITC (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instruction. Apoptosis was defined as annexin-V (+) but propidium-iodide (–). Viable cells were defined as annexin-V (–) but propidium-iodide (–). In each experimental setting, unstained controls, IgG isotype controls as well as FMO controls were used [42].

Cell cycle analysis by quantitation of DNA content was performed by using the propidium-iodide. Hep3B were fixed in cold 70% ethanol for at least 30 minutes at 4°C. Then the cells were washed x2 in PBS. Spin at 2000 rpm and discard supernatant. To ensure that only DNA is stained, cells were treated with ribonuclease (50 µl of 100 µg/ml RNase), stained with 5 µl of 50 µg Propidium iodide/100ml and analyzed with the flow cytometer (Becton-Dickinson LSR II, Immunofluorimetry systems, Mountain View, CA) [42].

Antioxidant evaluation

DPPH (2,2-diphenyl-1-picrylhydrazyl) was utilized to estimate the antioxidant activity of **2a, 2b, 5a**, **5b**, **6a, 6b, 7a** and **7b** synthesized compounds. A quantity of 1 mg of each compound was dissolved in methanol to prepare stock solution. A serial dilution of the previous stocks was prepared (2, 5, 10, 20, 50, 100 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 ration of 1:1:1 respectively. After a 30 min of incubation at room temperature, the absorbance was read against a blank at 517 nm by utilizing UV-Vis-spectrophotometer. The percentage (%) that used to inhibit DPPH free radical was determined using the following formula:

DPPH inhibition potential (%) = [(C-T)/C] × 100%.

where: C is the absorbance of the control (without samples) and T is the absorbance of the tested samples.

The same procedure was repeated for Trolox which was used as a positive control. The DPPH inhibition by synthesized compounds and Trolox with various concentrations were estimated by plotting the percentages of inhibition against the concentrations of the sample, providing a 50% inhibition (IC_{50}) [43].

Statistical analyses

Determination of the antioxidant activities was carried out in triplicate for each sample. While the cytotoxic test was carried out in duplicate for each sample. The obtained results were presented as means ± standard deviation (SD). Statistical analysis was performed employing GraphPad Prism software version 6.01. Comparison between three groups or more were analyzed via one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test.

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