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Synthesis, antioxidant and anticancer screenings of berberine–indole conjugates

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Abstract A variety of heterocyclic nitrogen cores in the form of indole moieties were linked to the natural isoquinoline alkaloid molecule berberine to achieve anticipated antioxidant and anticancer properties. An efficient synthetic pathway afforded final compounds **5a**–**j**, which were tested in vitro for antioxidant potency using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt radical (ABTS) bioassays, and for anticancer activity using sulforhodamine B (SRB) assay against HeLa and Caski cancer cell lines. Moreover, the toxic nature of the resultant molecules was investigated using Madin–Darby canine kidney cells. The therapeutic indices of **5a**–-**j** were more appreciable against the Caski than HeLa cell line, in which compounds with electron-releasing alkyl or alkoxy functional group on indole entity as well as azaindole derivative performed well. In addition, these compounds were well endowed with antioxidant properties, in addition to the equal antioxidant effect of the compound with electron-withdrawing chlorine atom within indole entity. Adequate confirmation of the structure of the final analogues was achieved using Fourier-transform infrared (FT-IR), ^{1}H nuclear magnetic resonance (NMR), and mass spectroscopy and elemental (CHN) analysis.

Keywords Berberine · Indole · Antioxidant · Anticancer · Cervical cancer · Natural product derivatives · Alkaloids

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Introduction

The metabolic activities of the human body demand energy generation for growth and development, with oxygen playing an essential role in the performance of these biological functions, for example, catabolism of carbohydrates, proteins, and fats. However, oxygen has a disadvantageous effect too, because it has some toxic effects on living cells due to its involvement in production of reactive oxygen species (ROS) [1–3]. When performing normal metabolism, the human body can generate ROS such as hydroxyl, alkoxyl, and lipid peroxyl radicals, nitric oxide, and peroxynitrite [4]. On the other hand, these can also be created via the explosion of biomolecules produced by chemicals and ionizing radiation, thus making it more likely for the body to suffer from diseases such as cancers, inflammation, cardiovascular diseases (CVDs), and other illnesses [5]. The functions of essential cell components such as proteins, nucleic acids, lipids, and carbohydrates can be altered by ROS, which are also the factor responsible for exposing the body to a variety of diseases. Moreover, damage to cells caused by ROS is believed to play a central role in the aging process and disease progression. Production of ROS in larger amounts can be considered as oxidative stress [6], which can be controlled by the action of antioxidant molecules. Compounds that can scavenge free radicals play an important role in the progress of these infective conditions.

Nowadays, the most severe disease to which humans are exposed is cancer, being one of the leading causes of mortality worldwide [7]. In fact, the global burden of cancer continues to increase, largely because of aging, which is caused by oxidative stress. In 2012, according to GLOBOCAN estimates, there were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million people living with cancer. Also, 65 % of cancer deaths and 57 % of newly diagnosed cancer cases occur in developing regions of the world [8]. The cell damage exerted by ROS depends on the equilibrium between ROS and endogenous antioxidant species. In the absence of an antioxidant defense system, oxidative stress causes nicks in the DNA repair system, causing DNA oxidation that generates 8-hydroxy-2′-deoxyguanosine, a product that can generate mutations in DNA, thereby causing cancer [9]. Hence, cancer and oxidative stress can be correlated, and accordingly, compounds that reduce oxidative stress, i.e., antioxidants, can be regarded as important molecules that also help to treat cancer [10]. It is worthwhile to assume that compounds bearing functional groups demonstrating potential antioxidant effects could be studied further to investigate their efficacy as chemopreventive and chemotherapeutic agents. To further support this statement, researchers have reported several molecules with antioxidant and chemopreventive properties, including compounds bearing phenolic hydroxyl group(s) [11, 12, 13] and compounds containing heterocyclic systems such as imidazole [14, 15], thiazole [16], and thiadiazole [17, 18].

Natural products have played a key role in medicine throughout our evolution. In fact, natural products are still used today in various regions of the world to relieve pain, or are wrapped (for example, in leaves) to heal wounds. Over the past decade, natural products have been the most studied for drug design and development through the invention of molecular biology and combinatorial chemistry, which

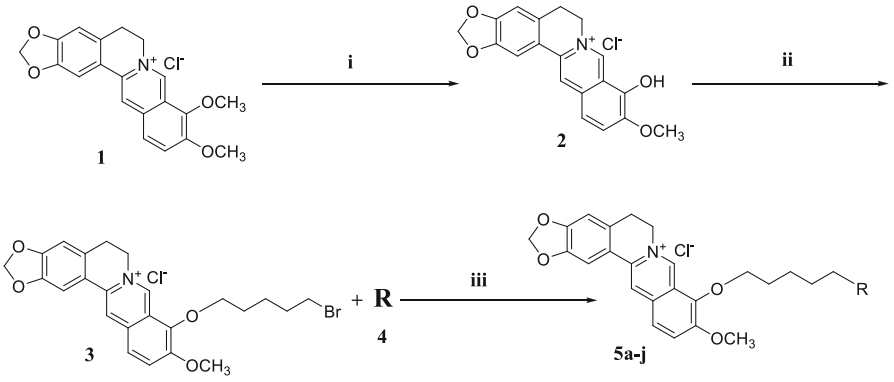
assist with the rationalization of drugs. More recently, natural products have been investigated in clinical trials or provided leads for compounds that have entered clinical trials, particularly for anticancer and antimicrobial agents [19–21]. Between 1981 and 2010, 34 % of the medicines approved by the Food and Drug Administration (FDA) were based on natural product small scaffolds or their derivatives [22–25].

Berberine is an isoquinoline quaternary alkaloid present in many therapeutic plants such as *Hydrastis canadensis*, *Berberis aristata*, *Coptis chinensis*, *C. rhizome*, *C. japonica*, *Phellodendron amurense*, *P. chinense* Schneid, and other local varieties used all over the world in conventional therapeutic practice. Plants that contain berberine have been used in protection against and therapy for many illnesses, such as gastrointestinal infections, abdominal pain and diarrhea, hyperglycemia, hyperlipidemia, metabolic syndrome, polycystic ovary syndrome, obesity, fatty liver, and coronary artery disorders [26]. Berberine has been associated with many medicinal properties, for example, anticancer [27] and antioxidant actions [28]. Derivatization of berberine has generated molecules exhibiting potential cytotoxic properties against HeLa, SVKO3, and Hep-2 [29]. The anticancer efficiency of berberine seems to result from its powerful interaction with inhibition of topoisomerase enzymes, and nucleic acids and telomerases, and binding to quadruplex structures [30–32]. Its connection with biomacromolecules, especially DNA and RNA, at the molecular level and structural information relating to such complexes are key issues to determine its biological features. Research on the DNA and RNA executed factors of berberine, its analogues, and relevant alkaloids has been substantially examined by many research groups [33–38]. Analysis of the anticancer action of berberine, in particular, has attracted extensive interest, with quite excellent results being obtained [39]. The berberine framework represents a naturally exciting molecule as well as an eye-catching natural lead substance for development of various derivatized variations exhibiting appropriate actions with other pharmacologically important heterocyclic cores. The indole core has been studied in therapeutic chemistry and is considered to be a potent scaffold [40–43]. Therefore, linkage of indoles to an intended biocore has been a subject of concentrated analysis over the decades [44–50]. Indole derivatives represent an important class of healing compounds in the therapeutic era, demonstrating e.g. anticancer [51], antioxidant [52], and anti-human immunodeficiency virus (HIV) [53, 54] actions; in fact, many indole derivatives are considered to be the most potent scavengers of free radicals [55]. Prompted by the above considerations, and given the need for new antitumor agents, we considered it of interest to prepare combinations of the berberine moiety with the indole nucleus.

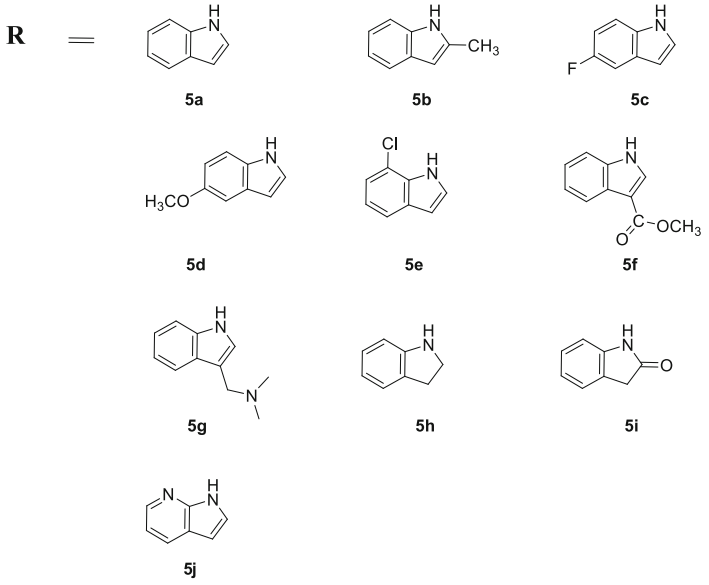
Results and discussion

Chemistry

Scheme 1 summarizes the chemical strategies adopted to obtain **5a–j** efficiently. Berberrubine (**2**) was furnished in good yield upon treatment of berberine



Reagents &conditions: i. 190°C, 20-30 mm Hg 40 min: **ii.** CH_3CN, 1,5-dibromopentan, reflux, 6h: **iii.** K_2CO_3, DMF, reflux, 6-8 h.



Scheme 1 Synthesis of indole-linked berberine derivatives

hydrochloride in a vacuum oven at temperature of 190 °C and pressure of 20–30 mmHg [56]. Berberrubine was further alkylated using dibromoalkanes in dry acetonitrile [57] to construct the final intermediate compound **3**. The intended indole entities were introduced into the berberine core via reaction with intermediate **3** in dimethylformamide (DMF) for 6–8 h to produce **5a–j** in reasonable yields.

Analytical data in terms of FT-IR, ^{1}H NMR, and mass spectrometry and determination of elemental proportions (CHN analysis) confirmed adequate production of the title compounds. Within the aromatics, C–H stretching exhibited

its characteristic bands at around 3037–3061 cm^{-1} in the FT-IR spectra of **5a–j**, including C=C characteristic bands near 1622–1531 cm^{-1}. A sharp peak corresponding to C–O–C linkage was found at around 1121–1077 cm^{-1}, and similarly for halogen linkage at 757–781 cm^{-1}. The structure of berberine was confirmed through assignment of its proton atoms through ^{1}H NMR spectra (**5b**) as corresponding to the H-8, H-13, H-1, H-12, H-4, and H-11 positions, resonating in the form of singlet peaks at 9.61, 8.43, 7.62, 7.44, 7.40, and 6.51 ppm, respectively, as well as a singlet peak observed at 6.05 ppm due to the presence of $–OCH_2O$ of berberine ring. H-6 and H-5 proton atoms of the berberine ring exhibited peaks in the form of triplets at 4.94 ppm and 2.47 ppm in the ^{1}H NMR spectra of **5b** as well as a triplet corresponding to the characteristic alkyl chain proton (H-15) observed at 4.22 ppm. Furthermore, a triplet signal at 3.20 ppm, broad singlet at 2.40 ppm, as well as multiplet signals at 2.19 ppm were due to alkyl chain protons H-19, H-17, and H-18 as well as H-16, respectively, while proton atoms present on aromatic moieties attached to the indole or in the indoline or azaindole entity exhibited corresponding signals as multiplets in the range of 7.32–6.61 ppm. Mass spectrometric data were in accurate accordance, as observed from the M^{+} ion values for the final compounds **5a–j**. All of the novel compounds gave C, H, N analyses within 0.4 percent points of the theoretical value, i.e., within an acceptable range.

Evaluation of biological activities

Antioxidant activity

The free radical scavenging capability of the new berberine–indole conjugates **5a–j** was determined using multiple antioxidant bioassays based on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical (ABTS). Overall, the title analogues presented positively enhanced radical scavenging effects when compared with the parent berberine, with 50 % inhibition concentration (IC_{50}) values ranging from 17.09 ± 0.29 to 26.38 ± 0.16 μg/mL and 9.09 ± 0.11 to 19.50 ± 1.16 μg/mL in DPPH and ABTS bioassay, respectively. In addition, it was found that neat indole entity failed to present significant antioxidant efficacy; however, the presence of different electron-withdrawing and electron-donating substituents on it significantly enhanced the potency of the resultant indoles connected to the berberine core. The title compounds served as beneficial antioxidant drug-like molecules when comparing their potencies with that of the control drug ascorbic acid with IC_{50} values of 15.59 ± 0.32 and 6.77 ± 0.02 μg/mL in DPPH and ABTS bioassay, respectively. An analogue with electron-withdrawing chlorine atom (**5e**) appeared to be the most potent radical scavenger in DPPH assay, with IC_{50} level of 17.09 ± 0.29 μg/mL, slightly lower than the control drug ascorbic acid but twice as potent as berberine. Another analogue with indoline ring (**5h**) demonstrated almost equal antioxidant power to **5e** with IC_{50} of 19.48 ± 0.39 μg/mL. Title analogues with electron-releasing alkyl functionality, e.g., compound **5b** with methyl and **5g** with dimethyl functional groups, exerted equal antioxidant efficacies

with IC_{50} values of 19 μg/mL. Azaindole-based compound **5j** presented an IC_{50} level around 19.79 ± 0.84 μg/mL, being equal in potency to compounds with alkyl functional groups. Final analogues with alkoxy (**5d**) and fluorine (**5c**) functional groups as well as one with oxindole moiety (**5i**) exhibited IC_{50} values around 20 μg/mL, being considered as moderate radical scavenging agents in the present study according to the DPPH assay. The results of the ABTS bioassay confirmed that compound **5b** with methyl indole functional group demonstrated the greatest antioxidant power with an IC_{50} level of 9.09 ± 0.11 μg/mL, being ten times more potent than the parent molecule berberine with antioxidant effects at the IC_{50} level of 90.71 ± 1.77 μg/mL according to the ABTS assay. This compound demonstrated activity close to that of the control drug ascorbic acid with antioxidant power at the IC_{50} level of 6.77 ± 0.02 μg/mL in the ABTS assay. Another three compounds with electron-releasing alkoxy (**5d**) and electron-withdrawing chlorine atom (**5e**) as well as one with indoline entity (**5i**) showed radical scavenging potential similar to **5b** with IC_{50} values of 9.40 ± 0.33, 9.64 ± 0.36, and 9.56 ± 0.53 μg/mL, respectively, in the ABTS assay. The remaining analogues can be regarded as antioxidant agents with good to moderate power against ABTS radical when comparing their potency with the control drug ascorbic acid; however, they can be regarded as very potent when compared with berberine in the same bioassay. Overall, all the presented analogues demonstrated enhanced potency as DPPH and ABTS radical inhibitors compared with the parent molecule berberine (Table 1).

Anticancer activities

The in vitro action of the berberine–indole conjugates against two cervical cancer cell lines, namely HeLa and Caski, was determined and the results are presented in Tables 2 and 3. The sulforhodamine B (SRB) colorimetric bioassay was adopted to investigate the anticancer action of the title compounds. In general, the compounds

Table 1 Screening results of DPPH and ABTS radical scavenging activity of berberine derivatives **5a–j**

Compound	IC_{50} (μg/mL) ± SD[a]	
	DPPH[b]	ABTS[c]
5a	26.38 ± 0.16	12.09 ± 0.62
5b	19.90 ± 0.34	9.09 ± 0.11
5c	20.55 ± 0.89	14.02 ± 0.87
5d	22.27 ± 0.62	9.40 ± 0.33
5e	17.09 ± 0.29	9.64 ± 0.36
5f	20.37 ± 0.12	19.50 ± 1.16
5g	19.48 ± 0.39	10.71 ± 0.38
5h	17.25 ± 0.47	9.56 ± 0.53
5i	20.58 ± 0.53	17.34 ± 0.69
5j	19.79 ± 0.84	16.46 ± 0.57
Berberine	41.87 ± 1.63	90.71 ± 1.77
Ascorbic acid	15.59 ± 0.32	6.77 ± 0.02

[a] Antioxidant activities are shown as IC_{50} values in μg/mL. All assays were carried out in triplicate, and the results are expressed as average ± standard deviation

[b] DPPH = 2,2-diphenyl-1-picrylhydrazyl

[c] ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

Table 2 Anticancer activity of synthesized compounds against HeLa cancer cells and their toxicity

Compound	IC_{50} (μg/mL) ± SD[a] HeLa	CC_{50} (μg/mL) ± SD[b] MDCK	TI[c]
5a	7.461 ± 0.12	303.1 ± 2.02	40.62
5b	7.145 ± 0.06	300.9 ± 1.25	42.11
5c	7.275 ± 0.07	310.3 ± 0.89	42.65
5d	7.215 ± 0.11	316.7 ± 1.05	43.89
5e	7.189 ± 0.18	302.4 ± 0.77	42.06
5f	7.358 ± 0.13	302.7 ± 0.66	41.14
5g	7.555 ± 0.11	310.5 ± 1.59	41.10
5h	7.582 ± 0.06	306.4 ± 0.09	40.41
5i	7.443 ± 0.11	302.5 ± 0.13	40.64
5j	7.279 ± 0.02	315.3 ± 1.41	43.32
Berberine	5.575 ± 0.18	144.1 ± 1.12	25.85

[a] Anticancer activities shown as IC_{50} values in μg/mL. All assays were carried out in triplicate, and the results are expressed as average ± standard deviation

[b] CC_{50}, 50 % cytotoxicity concentration

[c] TI, therapeutic index

Table 3 Anticancer activity of synthesized compounds against Caski cancer cells and their toxicity

Compound	IC_{50} (μg/mL) ± SD[a] Caski	CC_{50} (μg/mL) ± SD[b] MDCK	TI[c]
5a	6.639 ± 0.33	303.1 ± 2.02	45.65
5b	6.324 ± 0.58	300.9 ± 1.25	47.58
5c	7.667 ± 0.53	310.3 ± 0.89	40.47
5d	7.725 ± 0.32	316.7 ± 1.05	41.00
5e	7.893 ± 0.22	302.4 ± 0.77	38.31
5f	8.340 ± 0.26	302.7 ± 0.66	36.29
5g	7.886 ± 0.41	310.5 ± 1.59	39.37
5h	6.971 ± 0.29	306.4 ± 0.09	43.95
5i	7.277 ± 0.44	302.5 ± 0.13	41.57
5j	6.827 ± 0.26	315.3 ± 1.41	46.18
Berberine	5.870 ± 0.39	144.1 ± 1.12	24.55

[a] Anticancer activities are shown as IC_{50} values in μg/mL. All assays were carried out in triplicate, and the results are expressed as average ± standard deviation

[b] CC_{50}, 50 % cytotoxicity concentration

[c] TI, therapeutic index

exhibited reasonable levels of cancer cell inhibitory effects against the HeLa and Caski cell lines in terms of IC_{50} levels when compared with the parent molecule berberine. However, the title compounds exhibited a better level of cytotoxic nature when compared with berberine, furnishing therapeutic indices almost twice as potent as those of the parent molecule berberine. The title analogues exhibited IC_{50} values of 7.145 ± 0.06 to 7.582 ± 0.06 μg/mL and 6.324 ± 0.58 to 8.340 ± 0.26 μg/mL against the HeLa and Caski cell lines, respectively. In addition, the cytotoxicity level against Madin–Darby canine kidney (MDCK) cell lines was observed to lie in the range of 300.9 ± 1.25–316.7 ± 1.05 μg/mL for the title analogues **5a**–**j**, resulting in therapeutic indices of 40.41–43.89 and 36.29–47.58 against the HeLa and Caski cervical cancer cell lines, respectively. Overall, it can be stated that changing the nature of the substituent or functional group present on the indole ring led to significant changes or variation in the anticancer potency of the resultant molecules.

The final analogue **5d** with electron-releasing alkoxy functional group present on the 5th position of indole ring attached to the berberine core demonstrated the most potent anticancer effects with IC_{50} level of 7.215 ± 0.11 μg/mL and low cytotoxicity towards healthy MDCK cells (CC_{50} of 316.7 ± 1.05 μg/mL), thereby furnished a potent chemotherapeutic index of 43.89. This compound was one of the most potent anti-HeLa cell line inhibitory agents among all those tested and was much more potent than the parent molecule berberine with a therapeutic index of 25.85. Moreover, another compound **5j** with 7-azaindole moiety connected to the berberine core with a pentane aliphatic chain demonstrated anti-HeLa cell line inhibitory effects almost equal to those of compound **5d**, with IC_{50} level of 7.279 ± 0.02 μg/mL, and low cytotoxicity towards healthy MDCK cells (CC_{50} of 315.3 ± 1.41 μg/mL), thereby furnishing a chemotherapeutic index of 43.32. These two molecules were identified as the most potent inhibitors of the HeLa cervical cancer cell line. However, another three title derivatives, namely **5b**, **5c**, and **5e**, with either halo or alkyl substituent exhibited IC_{50} values around 7 μg/mL and therapeutic indices of around 42, close to those of the two potent derivatives mentioned above. Hence, based on these data, it is worth stating that more than half of the total number of studied molecules presented powerful anticancer effects, while the remaining analogues exhibited TIs of 40.41–41.14, still being much higher than that of berberine. Furthermore, once again compound with electron-releasing alkyl functional group exhibited the most potent cancer cell growth inhibitory effects against the Caski cell line, with IC_{50} level of 6.324 ± 0.58 μg/mL, whereas the cytotoxicity was very reasonable at 300.9 ± 1.25 μg/mL, leading to a therapeutic index of 47.58. The potency of this analogue against the Caski cell line was almost twice that of berberine at 24.55 in terms of the therapeutic index. Similar to the activity of the title analogues against the HeLa cell line, in the case of the Caski cell line, compound **5j** with azaindole functionality displayed a significant level of anticancer potential with IC_{50} level of 6.827 ± 0.26 μg/mL and cytotoxicity of 315.3 ± 1.41 μg/mL, thereby presenting a TI value of 46.18. The final compound with neat indole moiety was found to be inactive in other bioassay targets, whereas in the case of activity against the Caski cell line, compound **5a** with indole moiety connected to the berberine core exhibited a therapeutic index of 45.65 and was considered to be excellently active compared with berberine itself. The remaining analogues exhibited therapeutic indices of 36–41 and can be regarded as good to moderate anticancer agents, suggesting that further structural refinements are required to obtain more potent cancer therapeutics.

Experimental

Materials and methods

Highest quality chemicals and reagents were used in this study without prior purification. A Veego VMP-D open capillary electronic apparatus was utilized to obtain the melting points of the synthesized compounds, which are uncorrected. A Shimadzu 8400-S FT-IR spectrophotometer (KBr pellets) and Varian 500 MHz

model spectrometer [with dimethyl sulfoxide (DMSO) as solvent and tetramethylsilane (TMS) as internal standard] were used to obtain the FT-IR and ^{1}H NMR spectra of the title compounds. Thin-layer chromatography (TLC) was carried out using appropriate mobile-phase systems of silica gel-G coated microscope glass slides (2 × 7.5 cm), with TLC spots being observed in an ultraviolet (UV) light chamber. FT-IR bands are presented in cm^{-1}, while ^{1}H NMR spectral results are furnished in ppm downfield from TMS with s (singlet), d (doublet), m (multiplet), and br s (broad singlet) patterns. Elemental analyses (C, H, N) were done using a Heraeus Carlo Erba 1180 CHN analyzer.

General procedure for synthesis of berberrubine (2)

Berberine hydrochloride (10 g, 0.01 mol) was placed in a 50-mL round-bottomed flask. The reaction system was maintained at reduced pressure (20–30 mmHg) using an oil pump and warmed to 190 °C, followed by reaction for 40 min. The vacuum pump was turned off after the temperature had decreased to room temperature. The reaction product was purified using silica gel column chromatography ($CHCl_3$:-CH_3OH 15:1 and 10:1, eluting until no compound was seen in the eluent) to acquire compound **2** as a brownish-red amorphous powder (6.6 g, 85 %).

Synthesis of bromopentylberberrubine (3)

A solution of **2** (5 g, 0.01 mol) and 1,5-dibromopentane (0.01 mol) in dry acetonitrile was heated at reflux temperature for 6 h, then diethyl ether was added. The resulting solid was filtered and then subject to anion exchange to chloride form to give compound **3**.

General procedure for preparation of derivatives 5a–j

The substituent indole derivatives (0.01 mol) were added to a magnetically stirred solution of compound **3** and anhydrous K_2CO_3 in dry DMF (25 mL). The reaction mixture was heated at 80 °C for 6–8 h, and the reaction was monitored by TLC. The resulting solid was filtered at room temperature and subjected to anion exchange to chloride form. The crude product was chromatographed on an Al_2O_3 column, and eluted with $CHCl_3$/CH_3OH (9:1, v/v) to give the proposed compound.

*9-O-3-(1-(1H-Indole)pentylberberine (**5a**)*

Light-yellow solid. Yield: 67 %. M.p. 273–275 °C. IR (KBr) cm^{-1}: 3011 (C–H, Ar), 1605–1540 (C=C, Ar), 1251–1060 (C–O–C). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.75 (s, 1H, H-8), 8.62 (s, 1H, H-13), 7.73 (s, 1H, H-1), 7.58 (s, 1H, H-12), 7.34 (s, 1H, H-4), 7.25–6.76 (m, 6H, indole), 6.66 (s, 1H, H-11), 6.11 (s, 2H, –OCH_2O), 5.05 (t, 2H, J = 6.3, H-6), 4.39 (t, 2H, J = 6.5, H-15), 4.08 (s, 3H, OCH_3), 3.12 (t, 2H, J = 6.4, H-19), 2.61 (t, 2H, J = 7.4, H-5), 2.31 (br s, 4H, H-17, H-18), 2.11 (m, 2H, H-16). Anal. Calcd. for $C_{32}H_{31}ClN_2O_4$: C, 70.77; H, 5.75; N, 5.16. Found: C, 70.86; H, 5.64; N, 5.33.

*9-O-3-(1-(2-Methyl-1H-indole)pentylberberine (**5b**)*

Light-yellow solid. Yield: 61 %. M.p. 256–258 °C. IR (KBr) cm^{-1}: 3092 (C–H, Ar), 1613–1580 (C=C, Ar), 1180–1048 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.61 (s, 1H, H-8), 8.43 (s, 1H, H-13), 7.62 (s, 1H, H-1), 7.44 (s, 1H, H-12), 7.40 (s, 1H, H-4), 7.21–6.61 (m, 5H, indole), 6.51 (s, 1H, H-11), 6.05 (s, 2H, $-OCH_2O$), 4.94 (t, 2H, $J = 6.5$, H-6), 4.22 (t, 2H, $J = 6.3$, H-15), 4.12 (s, 3H, OCH_3), 3.20 (t, 2H, $J = 6.6$, H-19), 2.47 (t, 2H, $J = 7.1$, H-5), 2.40 (br s, 4H, H-17, H-18), 2.19 (m, 2H, H-16), 2.05 (s, 3H, Ar-CH_3). Anal. Calcd. for $C_{33}H_{33}ClN_2O_4$: C, 71.15; H, 5.97; N, 5.03. Found: C, 71.02; H, 6.02; N, 5.14.

*9-O-3-(1-(5-Fluoro-1H-indole)pentylberberine (**5c**)*

Light-yellow solid. Yield: 56 %. M.p. 263–265 °C. IR (KBr) cm^{-1}: 3028 (C–H, Ar), 1609–1517 (C=C, Ar), 1233–1035 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.84 (s, 1H, H-8), 8.54 (s, 1H, H-13), 7.84 (s, 1H, H-1), 7.56 (s, 1H, H-12), 7.29 (s, 1H, H-4), 7.19–6.81 (m, 5H, indole), 6.70 (s, 1H, H-11), 6.09 (s, 2H, $-OCH_2O$), 5.07 (t, 2H, $J = 6.4$, H-6), 4.35 (t, 2H, $J = 6.3$, H-15), 4.03 (s, 3H, OCH_3), 3.15 (t, 2H, $J = 6.5$, H-19), 2.55 (t, 2H, $J = 7.3$, H-5), 2.37 (br s, 4H, H-17, H-18), 2.21 (m, 2H, H-16). Anal. Calcd. for $C_{32}H_{30}ClFN_2O_4$: C, 68.50; H, 5.39; N, 4.99. Found: C, 68.35; H, 5.27; N, 5.09.

*9-O-3-(1-(5-Methoxy-1H-indole)pentylberberine (**5d**)*

Light-yellow solid. Yield: 58 %. M.p. 244–246 °C. IR (KBr) cm^{-1}: 3085 (C–H, Ar), 1621–1556 (C=C, Ar), 1244–1022 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.65 (s, 1H, H-8), 8.63 (s, 1H, H-13), 7.75 (s, 1H, H-1), 7.41 (s, 1H, H-12), 7.38 (s, 1H, H-4), 7.28–6.74 (m, 5H, indole), 6.68 (s, 1H, H-11), 6.12 (s, 2H, $-OCH_2O$), 4.96 (t, 2H, $J = 6.5$, H-6), 4.26 (t, 2H, $J = 6.4$, H-15), 4.04 (s, 3H, OCH_3), 3.91 (s, 3H, Ar-OCH_3), 3.23 (t, 2H, $J = 6.3$, H-19), 2.62 (t, 2H, $J = 7.5$, H-5), 2.44 (br s, 4H, H-17, H-18), 2.18 (m, 2H, H-16). Anal. Calcd. for $C_{33}H_{33}ClN_2O_5$: C, 69.16; H, 5.80; N, 4.89. Found: C, 69.31; H, 5.92; N, 4.96.

*9-O-3-(1-(7-Chloro-1H-indole)pentylberberine (**5e**)*

Light-yellow solid. Yield: 63 %. M.p. 277–279 °C. IR (KBr) cm^{-1}: 3034 (C–H, Ar), 1619–1561 (C=C, Ar), 1192–1019 (C–O–C), 786 (C–Cl). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.76 (s, 1H, H-8), 8.45 (s, 1H, H-13), 7.65 (s, 1H, H-1), 7.59 (s, 1H, H-12), 7.27 (s, 1H, H-4), 7.15-6.82 (m, 5H, indole), 6.55 (s, 1H, H-11), 6.01 (s, 2H, $-OCH_2O$), 5.01 (t, 2H, $J = 6.3$, H-6), 4.37 (t, 2H, $J = 6.5$, H-15), 4.05 (s, 3H, OCH_3), 3.15 (t, 2H, $J = 6.4$, H-19), 2.45 (t, 2H, $J = 7.4$, H-5), 2.34 (br s, 4H, H-17, H-18), 2.25 (m, 2H, H-16). Anal. Calcd. for $C_{32}H_{30}Cl_2N_2O_4$: C, 66.55; H, 5.24; N, 4.85. Found: C, 66.42; H, 5.11; N, 4.72.

*9-O-3-(1-(Methyl 1H-indole-3-carboxylate)pentylberberine (**5f**)*

Light-yellow solid. Yield: 54 %. M.p. 269–271 °C. IR (KBr) cm^{-1}: 3071 (C–H, Ar), 1715 (C=O), 1612–1566 (C=C, Ar), 1184–1041 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.87 (s, 1H, H-8), 8.58 (s, 1H, H-13), 7.77 (s, 1H, H-1), 7.47 (s, 1H, H-12), 7.35 (s, 1H, H-4), 7.31–7.05 (m, 5H, indole), 6.73 (s, 1H, H-11), 6.08 (s, 2H, $-OCH_2O$), 5.09 (t, 2H, $J = 6.4$, H-6), 4.28 (t, 2H, $J = 6.5$, H-15), 4.07 (s, 3H, OCH_3), 3.97 (s, 3H, Ar-OCH_3), 3.21 (t, 2H, $J = 6.3$, H-19), 2.58 (t, 2H, $J = 7.3$, H-5), 2.41 (br s, 4H, H-17, H-18), 2.16 (m, 2H, H-16). Anal. Calcd. for $C_{34}H_{33}ClN_2O_6$: C, 67.94; H, 5.53; N, 4.66. Found: C, 67.82; H, 5.68; N, 4.77.

*9-O-3-(1-(1-(1H-Indol-3-yl)-N,N-dimethylmethanamine)pentylberberine (**5g**)*

Light-yellow solid. Yield: 57 %. M.p. 253–255 °C. IR (KBr) cm^{-1}: 3046 (C–H, Ar), 1625–1574 (C=C, Ar), 1254–1049 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.63 (s, 1H, H-8), 8.66 (s, 1H, H-13), 7.81 (s, 1H, H-1), 7.54 (s, 1H, H-12), 7.24 (s, 1H, H-4), 7.21–7.01 (m, 5H, indole), 6.67 (s, 1H, H-11), 5.96 (s, 2H, $-OCH_2O$), 4.98 (t, 2H, $J = 6.4$, H-6), 4.36 (t, 2H, $J = 6.3$, H-15), 4.11 (s, 3H, OCH_3), 3.55 (m, 2H, $-CH_2-N-$), 3.19 (t, 2H, $J = 6.5$, H-19), 2.64 (t, 2H, $J = 7.2$, H-5), 2.35 (br s, 4H, H-17, H-18), 2.24 (m, 2H, H-16), 2.12 (s, 3H, Ar-CH_3),1.90 (s, 3H, Ar-CH_3). Anal. Calcd. for $C_{35}H_{38}ClN_3O_4$: C, 70.05; H, 6.38; N, 7.00. Found: C, 70.16; H, 6.49; N, 6.93.

*9-O-3-(1-(Indoline)pentylberberine (**5h**)*

Light-yellow solid. Yield: 51 %. M.p. 278–280 °C. IR (KBr) cm^{-1}: 3067 (C–H, Ar), 1607–1552 (C=C, Ar), 1177–1037 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.78 (s, 1H, H-8), 8.49 (s, 1H, H-13), 7.69 (s, 1H, H-1), 7.45 (s, 1H, H-12), 7.37 (s, 1H, H-4), 6.95–6.65 (m, 4H, indoline), 6.58 (s, 1H, H-11), 6.10 (s, 2H, $-OCH_2O$), 5.08 (t, 2H, $J = 6.3$, H-6), 4.25 (t, 2H, $J = 6.4$, H-15), 3.98 (s, 3H, OCH_3), 3.75–3.31 (m, 4H, indoline), 3.26 (t, 2H, $J = 6.4$, H-19), 2.43 (t, 2H, $J = 7.5$, H-5), 2.33 (br s, 4H, H-17, H-18), 2.15 (m, 2H, H-16). Anal. Calcd. for $C_{32}H_{33}ClN_2O_4$: C, 70.51; H, 6.10; N, 5.14. Found: C, 70.39; H, 6.02; N, 5.25.

*9-O-3-(1-(2-Oxindole)pentylberberine (**5i**)*

Light-yellow solid. Yield: 60 %. M.p. 249–251 °C. IR (KBr) cm^{-1}: 3056 (C–H, Ar), 1614–1563 (C=C, Ar), 1210–1044 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.67 (s, 1H, H-8), 8.56 (s, 1H, H-13), 7.85 (s, 1H, H-1), 7.53 (s, 1H, H-12), 7.22 (s, 1H, H-4), 7.29–6.63 (m, 6H, indole), 6.71 (s, 1H, H-11), 5.98 (s, 2H, $-OCH_2O$), 4.95 (t, 2H, $J = 6.5$, H-6), 4.38 (t, 2H, $J = 6.5$, H-15), 4.06 (s, 3H, OCH_3), 3.17 (t, 2H, $J = 6.3$, H-19), 2.57 (t, 2H, $J = 7.4$, H-5), 2.42 (br s, 4H, H-17, H-18), 2.22 (m, 2H, H-16). Anal. Calcd. for $C_{32}H_{31}ClN_2O_5$: C, 68.75; H, 5.59; N, 5.01. Found: C, 68.86; H, 5.47; N, 5.12.

*9-O-3-(1-(7-Azaindol)pentylberberine (**5j**)*

Light-yellow solid. Yield: 55 %. M.p. 281–283 °C. IR (KBr) cm^{-1}: 3083 (C–H, Ar), 1629–1576 (C=C, Ar), 1188–1025 (C–O–C). ^1H NMR ($CDCl_3$, 500 MHz): δ 9.82 (s, 1H, H-8), 8.60 (s, 1H, H-13), 7.68 (s, 1H, H-1), 7.49 (s, 1H, H-12), 7.36 (s, 1H, H-4), 7.32–6.67 (m, 5H, azaindol), 6.56 (s, 1H, H-11), 6.04 (s, 2H, $-OCH_2O$), 5.02 (t, 2H, $J = 6.4$, H-6), 4.27 (t, 2H, $J = 6.3$, H-15), 3.96 (s, 3H, OCH_3), 3.22 (t, 2H, $J = 6.5$, H-19), 2.60 (t, 2H, $J = 7.3$, H-5), 2.36 (br s, 4H, H-17, H-18), 2.13 (m, 2H, H-16). Anal. Calcd. for $C_{31}H_{30}ClN_3O_4$: C, 68.44; H, 5.56; N, 7.72. Found: C, 68.33; H, 5.68; N, 7.61.

DPPH free radical scavenging assay

Free radicals exercise deleterious effects on biological systems and foods, and hence radical scavenging activities are very useful. Various chemical reactions that occur in biological systems usually furnish free radicals that are responsible for damage to biological building blocks such as DNA, lipids, etc. Reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl is the basis of the DPPH antioxidant bioassay. It has an odd electron which exhibits a maximum absorption band at 517 nm (deep-violet color) in ethanol. The DPPH bioassay is a widely used and accepted method for investigating the free radical scavenging efficacy of an intended compound. Such substances donate a hydrogen atom when mixed with DPPH, thereby producing the reduced congener diphenyl picrylhydrazine (nonradical) with loss of the violet color.

In the present study, the DPPH bioassay was adopted to screen the berberine-based compounds for their in vitro antioxidant potency. The results of this bioassay screening are presented in the form of the percentage radical scavenging antioxidant activity (RSA %) for each substance. Investigation of the DPPH radical scavenging activity was carried out according to the methodology described by Brand-Williams et al. [58]. The stable free radical 2,2-diphenyl-1-picrylhydrazyl was allowed to react with the berberine-based scaffolds in methanol solvent with 20 μL quantities of the title compounds mixed with 180 μL DPPH in MeOH. The title compounds donated hydrogen on mixing, thereby reducing the DPPH and causing a color change from deep violet to light yellow at 517 nm after 25 min of reaction, measured using a UV–visible spectrophotometer (PerkinElmer). Blank reading was also performed using a mixture of methanol (20 μL) and sample (180 μL DPPH). Ascorbic acid served as the control drug in this assay, in solution prepared by mixing methanol (20 μL) and DPPH radical solution (180 μL). The result of this bioassay was determined as the RSA % value (radical scavenging activity in percentage) according to Mensor et al. [59] as described by the equation

$$\%\text{Scavenging} = \frac{\text{Absorbance of blank} - \text{Absorbance of test}}{\text{Absorbance of blank}} \times 100.$$

A plot between the concentration of the test compound and the % scavenging value was used to determine the IC_{50} value in the presence of ascorbic acid as standard.

ABTS radical scavenging assay

The $ABTS^{\cdot+}$ radical cation scavenging efficacy of the test compounds was determined according to the previously described method [60]. Mixing of an equal amount of 7 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) stock solution with 2.45 mM potassium persulfate stock solution produces the $ABTS^{\cdot+}$ cation. The mixture was kept in the dark at temperature of 0 °C for 12 h, and ABTS solution was diluted with MeOH to give a UV absorption value of 0.700 (± 0.20) at 734 nm. The 1000 μL stock solutions of the title compounds **5a**–**j** were established by dissolving them in MeOH, with further dilutions furnishing 100, 10, 1, and 0.1 μL quantities of the samples. We mixed 180 μL solutions of compounds to be evaluated and 20 μL of ABTS solution in 96-well plates in the dark and then incubated for 10 min to measure the UV absorption at 734 nm. A mixture of 180 μL ABTS and 20 μL methanol was used for control determination, whereas ascorbic acid was used as a reference drug. The UV absorption data represent the radical scavenging rates, giving the corresponding IC_{50} values for the test compounds.

The scavenging capability for $ABTS^{\cdot+}$ radical was calculated using the equation

$$\%\text{Scavenging} = \frac{\text{Absorbance of blank} - \text{Absorbance of test}}{\text{Absorbance of blank}} \times 100.$$

In vitro anticancer bioassay

Cell cultures The test compounds **5a**–**j** were checked for their in vitro anticancer potential against cervical cancer cell lines HeLa and Caski, and Madin–Darby canine kidney (MDCK) cells purchased from the American Type Culture Collection (ATCC). All cell lines were well maintained in a humidified cell culture incubator in presence of 5 % CO_2 at temperature of 32 °C. Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotic–antimycotic solution (100×) were used for HeLa, Caski, and MDCK cell growth, respectively. DMEM, RPMI-1640, trypsin–ethylenediaminetetraacetic acid (EDTA), antibiotic–antimycotic solution 100×, and FBS were purchased from Welgene (150-Seongseo Industrial Complex, Bukro, Dalseogu, Daegu, 704–948 Republic of Korea).

In the 96-well plates, both cancer cell lines HeLa and Caski, and MDCK were seeded at concentration of 2×10^4 cells per well. Cancerous cells were allowed to grow for 1 day initially, after which the 96-well plates were washed twice with phosphate-buffered saline (PBS). DMEM and RPMI-1640 medium containing trypsin–EDTA were used to dilute HeLa, Caski, and MDCK cells to 5×10^3 level, which was used for infection followed by placing of 10 μL compound and 90 μL cell solution onto 96-well plates in which HeLa, Caski, and MDCK cells were grown the previous day. Test compounds at concentrations of 0.1, 1, 10, and 100 μL were used in 96-well plates for analysis, with three replicates of observations. Infected plates were incubated in a CO_2 incubator for 48 h. After incubation, the medium was removed followed by washing twice with PBS buffer. Thereafter,

70 % acetone was added to fix the cells, which were incubated for 1 h at temperature of 4 °C. After incubation, the solvent was removed, and the plates were dried in an oven at temperature of 60 °C. The dried plates were incubated overnight with 100 μL SRB (0.4 mg/L), followed by SRB removal and washing thrice with 1 % acetic acid, and dried again under hot-air oven at 60 °C. Microscopic observation was carried out to determine the morphology of the cells, after which the SRB stain was dissolved with 10 mM Tris base followed by incubation overnight [61]. Spectrophotometric data were recorded at 510 nm to calculate the 50 % inhibition concentration (IC_{50}), 50 % cytotoxic concentration (CC_{50}), and therapeutic index (TI) values.

Conclusions

Alkyl chain as an aliphatic linker was utilized to connect two pharmacologically diverse natural products as well as a heterocyclic core in a compact system in the form of berberine and indole, respectively. This design was expected to present enhanced antioxidant and anticancer potential in DPPH, ABTS, and SRB bioassays. Berberine–indole conjugation was successfully achieved via efficient organic transformation, and the compounds were adequately characterized. DPPH and ABTS bioassays suggested that all the title analogues successfully demonstrated increased radical scavenging efficacies compared with the parent berberine molecule, and reasonable antioxidant power when compared with the control drug ascorbic acid. A compound with alkyl and halo functional group in the form of methyl and chlorine atoms exhibited a significant level of antioxidant effects in scavenging DPPH and ABTS radicals. However, the aza functional group was found to be essential in delivering anticancer effects against both cervical cancer cell lines studied in the present work. Furthermore, the presence of electron-releasing alkyl or alkoxy functional group was found to be beneficial to exert anticancer potential against HeLa and Caski cell lines. These compounds exhibited a higher level of therapeutic index as anticancer agents when compared with berberine. Hence, in the present study, four different molecules were identified as potent scaffolds combining natural and heterocyclic cores for future drug discovery studies.

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Compliance with ethical standards

Conflict of interest Authors report no conflict of interest.

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