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ORIGINAL RESEARCH

Synthesis of berberine-piperazine conjugates as potential antioxidant and cytotoxic agents

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Abstract Piperazine derivatives bearing different electron-withdrawing and electron-donating functional groups were linked to the well-known isoquinoline alkaloid derivative, berberine via efficient organic transformations. The entire target berberine-based analogues were examined for their in vitro antioxidant potency using 2,2-diphenyl-1-picryl-hydrazyl and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid bioassays, and anticancer activities using sulforhodamine B assay against HeLa and CaSki cervical cancer cell lines in addition to the cytotoxicity using Madin-Darby canine kidney non-cancer cell lines and, ascorbic acid and berberine used as a control for antioxidant and anticancer activities, respectively. Bioassay results revealed that newer compounds were more active against CaSki and HeLa cell lines with therapeutic indices better than that of parent berberine and showed tolerable cytotoxicity to the normal cells. A final analogue **5a** with 4-methylpiperazine substituent indicated most significant anticancer potency with a therapeutic index of 58.53 (HeLa) and 48.76 (CaSki), followed by those bearing *meta*-chloropiperazine rings with a therapeutic index of 41.83 (HeLa) and 47.35 (CaSki), respectively.In addition, newly synthesized analogues exerted a significant radical scavenging activity against 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid cation with IC_{50} values of 8.917 µg/mL, and were good to moderate scavengers of 2,2-diphenyl-1-picrylhydrazyl radical with IC_{50} values of 25.40 µg/mL. Synthesized compound was characterized using several techniques, fourier transform infrared spectroscopy, ^{1}H nuclear magnetic resonance, ^{13}C nuclear magnetic resonance, mass spectroscopy and elemental (CHN) analyses.

Keywords Berberine · Piperazine · Antioxidant · Cervical cancer

Introduction

Oxygen species plays a vital role to perform essential biological functions in the human body as energy generation for growth, metabolic activities, development and catabolism of energy sources. Though they are beneficial for the body, their disadvantages also appear in terms of toxic agents for living cells. Oxygen may play a key role in producing reactive oxygen species (ROS) found to occur during important functions of the body like metabolism and radiation through chemicals. ROS are responsible for generating disease conditions like cancers, inflammation and cardiovascular harms (Kataki et al., 2012; Manikandan et al., 2004) through their interaction with biomolecules. Thus, a human being will always be in need of consuming antioxidants regularly to get relieved out of these diseases that emerge through ROS. Researchers have developed various types of antioxidant molecules either purely synthetic or prepared and inspired by natural product (NP) fragments. They are found to have interaction with free radicals, thereby destroying their side chain transformations well in advance before the damage occurs in the vital

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biomolecules (Ames et al., 1993) and known to have potential effects as antioxidant drugs to counter diseases caused by ROS functions like carcinogenesis, inflammation, atherogenesis and aging in aerobic organisms (Tyagi et al., 2005; Karegoudar et al., 2008). Cancer is the most threatening disease that appears nowadays throughout the world. Particularly, cervical cancer is among the world's most dangerous types of cancer for females, accounting for over 2,70,000 deaths yearly, 85 % of which happen in developing countries (WHO, Internet). In the end, ROS are capable of damaging biomolecules like DNA, proteins and lipids, thus resulting in the development of cancer. It is, therefore, evident that the treatment of the above-mentioned pathophysiological conditions could benefit from the use of drugs that exert antioxidant and anticancer activity.

NPs have been the primary sources of chemical diversity for pharmaceutical discoveries over the past century. NPs and their analogues can interact with many specific targets within the cell, thereby exhibiting curable effects against varieties of diseases (Mishra and Tiwari, 2011). NP synthesis provides an essential validation of newly developed reaction technologies leading to the development of new chemotherapeutic agents. More than 2,50,000 entries of natural substances were reported in the last few years (Dictionary of Natural Products, the internet). Polyketides, carbohydrates, flavonoids, terpenes-terpenoids, steroids, alkaloids, amino acids, peptides are present in nature with a long-lasting diversity (James, 2003). Moreover, NPs have received immense attention in the research of drug discovery. To develop their derivatives containing new classes of active drug molecules that provide decreased drug level of resistance has also been mostly accepted these days (Breinbauer et al., 2002; Paul et al., 2008). Among the most beneficial classes of plant-derived compounds, alkaloids are the most diverse plant secondary metabolites. More specifically, the quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids compounds, namely berberine has received enormous importance as a drug molecule associated with plenty of pharmacological actions as antimicrobial, antileukemic, antiulcerous, and enzyme-inhibiting (Verpoorte, 1998; Bodiwala et al., 2011), anti-inflammatory (Kuo et al., 2004), anti-diarrhea (Yin et al., 2002), glucose-lowering (Leng et al., 2004), cholesterol-lowering (Peng et al., 2007), neuroprotective (Cui et al., 2009), antidepressant (Kulkarni and Dhir, 2007), Alzheimers disease-ameliorating (Asai et al., 2007), antihyperlipidemic, antibiotics (Zuo et al., 2014), pancreatic lipase (Mohammad et al., 2013) and anticancer effects (Orfila et al., 2000; Liu et al., 2014). It is found in plants such as *Berberis, Berberis aristata, Hydrastis canadensis, Phellodendron amurense, Coptis chinensis, Tinospora cordifolia*, and to a smaller extent in *Argemone mexicana* and *Eschscholzia californica* (Lo et al., 2013).

Due to the aforementioned useful activities possessed by berberine derivatives, we synthesized some novel piperazine-based berberine analogues and evaluated them for their pharmacological applications as anticancer agents (Patel et al., 2012) and antioxidants (Andonovaa et al., 2014).

Materials and methods

Highest quality chemicals and reagents were used in this study without prior purification (Sigma). Melting points were determined on Veego Open capillary electronic apparatus (**model: VMP-D**, Veego Instrument Corporation, Mumbai, India) to obtain melting points of the synthesized compounds and were uncorrected. Bruker spectrophotometer (KBr pellets) were used to obtain fourier transform infrared spectroscopy (FT-IR) and were provided in cm^{-1}.^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded with Tetramethylsilane as the internal standard on a Varian 400 spectrometer in $CDCl_3$ or dimethyl sulfoxide (DMSO). Chemical shifts (δ) were presented in ppm and coupling constants (J) in Hz. ^{1}H NMR spectral data were reported in the following order: chemical shift, multiplicity (s: singlet, d: doublet, t: triplet, m multiplet, br s: broad singlet patterns). Mass spectrometry (MS) were recorded on an Agilent liquid chromatography–mass spectrometry 1100 instrument with Varian 500-MS selective detector. Flash column chromatography on silica gel (200–300 mesh) was used for the routine purification of reaction products. Thin-layer chromatography (TLC) was performed out using appropriate mobile phase systems silica gel-G coated microscopic glass slides (2 × 7.5 cm), and TLC spots were seen in ultraviolet (UV) light chamber. Elemental analyses (C, H, N) were conducted using a Heraeus Carlo Erba 1180 CHN analyzer.

General procedure for the synthesis of berberrubine (2)

Berberine chloride (10 g, 0.01 mmol) was included in a 50 mL round bottom flask and the reaction system was maintained at reduced pressure 20–30 mmHg applying an oil pump and warmed to 190 °C followed by reacting for 40 min to afford dark-wine solid, which was recrystallized with anhydrous ethanol twice than the product was purified via silica gel column chromatography ($CHCl_3$/CH_3OH:15:1 and 10:1, eluting) to acquire a brownish red amorphous powdered compound (8.5 g, 85 %).

Synthesis of bromopentyl berberrubine (3)

In a flask charged with dry acetonitrile, compound 2 (8 g, 0.029 mmol) and 1,5-dibromopentan (3.38 mL, 0.029 mmol) were warmed at reflux temperature for 6 h, and then

diethyl ether was added. The resulting solid was filtered and then subject to anion-exchange into chloride form to give compound 3. Yield: 61 %, m.p. 195–197 °C; IR (KBr) cm^{-1}: 3065 (C–H, Ar), 1615–1565 (C=C, Ar), 1115–1050 (C–O–C); ^1H NMR($CDCl_3$, 400 MHz): δ 9.86 (s, 1H, H-8), 8.49 (s,1H, H-13), 7.86 (s, 1H, H-1), 7.54 (s, 1H, H-12), 7.54 (s, 1H, H-4), 6.84 (s, 1H, H-11), 6.03 (s, 2H,-OCH_2O), 4.93 (t, 2H, $J = 6.3$, H-6), 4.35 (t, 2H, $J = 6.5$, H-15), 4.03 (s, 3H, -OCH_3), 3.19 (t, 2H, $J = 6.4$, H-19), 2.58 (t, 2H, $J =$ 7.4, H-5), 2.43 (br s, 4H, H-17, H-18), 2.21 (m, 2H, H-16); high resolution mass spectrum (HRMS) *m/z* $[M\text{-}Cl]^+$ calcd. for $[C_{24}H_{25}BrNO_4]^+$: 471.36; found: 471.75; Anal. Calcd. for $C_{24}H_{25}BrClNO_4$: C, 56.88; H, 4.97; N, 2.76. Found: C, 56.75; H, 5.07; N, 2.54.

General procedure for the preparation of derivatives (5a–j)

In a flask charged with dimethylformamide (DMF) (25 mL) then add substituent piperazine (0.01 mmol), compound 3 (0.01 mmol) and anhydrous K_2CO_3 (0.012 mmol). The reaction mixture was heated at 80 °C for 6–8 h, and TLC monitored the reaction. The resulting solid was filtered at room temperature and subjected to anion exchange into chloride form. The crude product was chromatographed on an Al_2O_3 column, eluted with $CHCl_3/CH_3OH$ (9:1, v/v) to give the proposed compound.

9-O-3-(1-(4-Methylphenyl)piperazine)pentylberberine ***(5a)***

Light yellow solid, yield: 55 %. m.p. 234–236 °C. IR (KBr) cm^{-1}: 3031 (C–H, Ar), 1610–1553 (C=C, Ar), 1117–1080 (C–O–C). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.81 (s, 1H, H-8), 8.47 (s, 1H, H-13), 7.86 (s, 1H, H-1), 7.53 (s, 1H, H-12), 7.43 (s, 1H, H-4), 7.34–7.23 (m, 4H, Ar–H, piperazine H-27, H-28, H-30, H-31), 6.75 (s, 1H, H-11), 6.02 (s, 2H, –OCH_2O), 4.98 (t, 2H, $J = 6.4$, H-6), 4.31 (t, 2H, $J = 6.6$, H-15), 4.13 (s, 3H, OCH_3), 3.81 (br s, 4H, piperazine, H-23, H-25), 3.46 (br s, 4H, piperazine, H-20, H-22), 3.18 (t, 2H, $J = 6.4$, H-19), 2.54 (t, 2H, $J = 7.5$, H-5), 2.43 (br s, 4H, H-17, H-18), 2.15 (m, 2H, H-16), 1.90 (s, 3H, Ar–CH_3); ^{13}C NMR (DMSO, 400 MHz): δ 153.2, 152.9, 149.4, 147.7, 143.6, 142.5, 139.8, 136.3, 133.0, 132.1, 131.2, 129.9, 123.4, 121.7, 120.6, 119.5, 116.8, 110.3, 107.0, 103.1, 73.2, 59.9, 56.4, 54.7, 52.6, 50.5, 28.8, 27.3, 25.0, 22.1, 21.3. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{35}H_{40}N_3O_4]^+$: 566.71; found: 566.48. Anal. calcd. for $C_{35}H_{40}ClN_3O_4$: C, 69.81; H, 6.70; N, 6.98. Found: C, 69.75; H, 6.82; N, 6.72.

9-O-3-(1-(2-Chlorophenyl)piperazine)pentylberberine ***(5b)***

Light yellow solid, yield: 64 %. m.p. 241–243 °C. IR (KBr) cm^{-1}: 3025 (C–H, Ar), 1604–1560 (C=C, Ar), 1104–1091 (C–O–C), 760 (C–Cl). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.58 (s, 1H, H-8), 8.62 (s, 1H, H-13), 8.03 (s, 1H, H-1), 7.51 (s, 1H, H-12), 7.48 (s, 1H, H-4), 7.38–7.15 (m, 4H, Ar–H, piperazine H-27, H-28, H-29, H-30), 6.95 (s, 1H, H-11), 6.16 (s, 2H, –OCH_2O), 4.82 (t, 2H, $J = 6.3$, H-6), 4.47 (t, 2H, $J = 6.5$, H-15), 4.14 (s, 3H, OCH_3), 3.86 (br s, 4H, piperazine, H-23, H-25), 3.56 (br s, 4H, piperazine, H-20, H-22), 3.43 (t, 2H, $J = 6.4$, H-19), 2.73 (t, 2H, $J = 7.6$, H-5), 2.47 (br s, 4H, H-17, H-18), 2.18 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.9, 152.2, 149.7, 147.4, 143.5, 142.6, 139.3, 136.8, 133.1, 132.0, 131.9, 129.2, 123.7, 121.4, 120.5, 119.6, 116.3, 110.8, 107.1, 103.0, 73.9, 59.2, 56.7, 54.4, 52.5, 50.6, 28.3, 27.8, 25.1, 22.0. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}ClN_3O_4]^+$: 587.13; found: 587.36. Anal. calcd. for $C_{34}H_{37}Cl_2N_3O_4$: C, 65.59; H, 5.99; N, 6.75. Found: C, 65.73; H, 6.11; N, 6.46.

9-O-3-(1-(3-Chlorophenyl)piperazine)pentylberberine ***(5c)***

Light yellow solid, yield: 58 %. m.p. 228–230 °C. IR (KBr) cm^{-1}: 3038 (C–H, Ar), 1617–1547 (C=C, Ar), 1126–1067 (C–O–C), 747 (C–Cl). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.78 (s, 1H, H-8), 8.68 (s, 1H, H-13), 7.88 (s, 1H, H-1), 7.62 (s, 1H, H-12), 7.51 (s, 1H, H-4), 7.30–7.04 (m, 4H, Ar–H, piperazine H-27, H-28, H-29, H-31), 6.92 (s, 1H, H-11), 6.12 (s, 2H, –OCH_2O), 4.89 (t, 2H, $J = 6.2$, H-6), 4.51 (t, 2H, $J = 6.4$, H-15), 4.12 (s, 3H, OCH_3), 3.72 (br s, 4H, piperazine, H-23, H-25), 3.51 (br s, 4H, piperazine, H-20, H-22), 3.31 (t, 2H, $J = 6.3$, H-19), 2.71 (t, 2H, $J = 7.8$, H-5), 2.45 (br s, 4H, H-17, H-18), 2.11 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.6, 152.1, 149.8, 147.3, 143.0, 142.9, 139.2, 136.5, 133.4, 132.7, 131.6, 129.1, 123.8, 121.3, 120.0, 119.9, 116.2, 110.5, 107.4, 103.7, 73.6, 59.1, 56.8, 54.3, 52.0, 50.9, 28.2, 27.5, 25.4, 22.7. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}ClN_3O_4]^+$: 587.13; found: 587.29. Anal. calcd. for $C_{34}H_{37}Cl_2N_3O_4$: C, 65.59; H, 5.99; N, 6.75. Found: C, 65.44; H, 6.07; N, 6.91.

9-O-3-(1-(4-Chlorophenyl)piperazine)pentylberberine ***(5d)***

Light yellow solid, yield: 67 %. m.p. 251–253 °C. IR (KBr) cm^{-1}: 3043 (C–H, Ar), 1622–1566 (C=C, Ar), 1104–1075 (C–O–C), 783 (C–Cl). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.75 (s, 1H, H-8), 8.67 (s, 1H, H-13), 8.06 (s, 1H, H-1), 7.67 (s, 1H, H-12), 7.45 (s, 1H, H-4), 7.39–7.06 (m, 4H, Ar–H, piperazine H-27, H-28, H-30, H-31), 6.79 (s, 1H, H-11), 6.08 (s, 2H, –OCH_2O), 4.86 (t, 2H, $J = 6.1$, H-6), 4.38 (t, 2H, $J = 6.2$, H-15), 4.02 (s, 3H, OCH_3), 3.75 (br s, 4H, piperazine, H-23, H-25), 3.49 (br s, 4H, piperazine, H-20, H-22), 3.23 (t, 2H, $J = 6.5$, H-19), 2.58 (t, 2H, $J = 7.5$, H-5), 2.39 (br s, 4H, H-17, H-18), 2.21 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.3, 152.6, 149.5, 147.8, 143.7, 142.0, 139.9, 136.1, 133.2, 132.4, 131.3, 129.6, 123.5,

121.8, 120.7, 119.0, 116.9, 110.1, 107.2, 103.4, 73.3, 59.6, 56.5, 54.8, 52.7, 50.0, 28.9, 27.1, 25.2, 22.4. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}ClN_3O_4]^+$: 587.13; found: 587.02. Anal. calcd. for $C_{34}H_{37}Cl_2N_3O_4$: C, 65.59; H, 5.99; N, 6.75. Found: C, 65.42; H, 5.89; N, 6.57.

9-O-3-(1-(2-Fluorophenyl)piperazine)pentylberberine ***(5e)***

Light yellow solid, yield: 53 %. m.p. 218–220 °C. IR (KBr) cm^{-1}: 3019 (C–H, Ar), 1617–1564 (C=C, Ar), 1116–1093 (C–O–C), 760 (C–Cl). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.82 (s, 1H, H-8), 8.77 (s, 1H, H-13), 7.84 (s, 1H, H-1), 7.57 (s, 1H, H-12), 7.61 (s, 1H, H-4), 7.26–7.13 (m, 4H, Ar–H, piperazine H-27, H-28, H-29, H-30), 6.76 (s, 1H, H-11), 6.15 (s, 2H, $–OCH_2O$), 4.83 (t, 2H, $J = 6.4$, H-6), 4.53 (t, 2H, $J = 6.5$, H-15), 4.17 (s, 3H, OCH_3), 3.79 (br s, 4H, piperazine, H-23, H-25), 3.53 (br s, 4H, piperazine, H-20, H-22), 3.16 (t, 2H, $J = 6.6$, H-19), 2.48 (t, 2H, $J = 7.3$, H-5), 2.33 (br s, 4H, H-17, H-18), 2.25 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.7, 152.4, 149.1, 147.5, 143.3, 142.2, 139.0, 136.9, 133.6, 132.8, 131.7, 129.4, 123.1, 121.5, 120.3, 119.3, 116.0, 110.9, 107.6, 103.8, 73.7, 59.4, 56.1, 54.5, 52.3, 50.2, 28.0, 27.9, 25.6, 22.8. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}FN_3O_4]^+$: 570.67; found: 570.34. Anal. calcd. for $C_{34}H_{37}ClFN_3O_4$: C, 67.37; H, 6.15; N, 6.93. Found: C, 67.51; H, 6.32; N, 6.78.

9-O-3-(1-(4-Fluorophenyl)piperazine)pentylberberine ***(5f)***

Light yellow solid, yield: 55 %. m.p. 234–236 °C. IR (KBr) cm^{-1}: 3033 (C–H, Ar), 1611–1553 (C=C, Ar), 1128–1072 (C–O–C), 773 (C–Cl). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.73 (s, 1H, H-8), 8.53 (s, 1H, H-13), 8.04 (s, 1H, H-1), 7.69 (s, 1H, H-12), 7.47 (s, 1H, H-4), 7.33–7.12 (m, 4H, Ar–H, piperazine H-27, H-28, H-30, H-31), 6.84 (s, 1H, H-11), 6.18 (s, 2H, $–OCH_2O$), 4.95 (t, 2H, $J = 6.3$, H-6), 4.35 (t, 2H, $J = 6.6$, H-15), 4.23 (s, 3H, OCH_3),3.73 (br s, 4H, piperazine, H-23, H-25), 3.42 (br s, 4H, piperazine, H-20, H-22), 3.12 (t, 2H, $J = 6.4$, H-19), 2.53 (t, 2H, $J = 7.5$, H-5), 2.48 (br s, 4H, H-17, H-18), 2.03 (m, 2H, H-16). ^{13}C NMR (DMSO, 400 MHz): δ 153.8, 152.0, 149.3, 147.9, 143.1, 142.7, 139.4, 136.6, 133.5, 132.2, 131.8, 129.0, 123.3, 121.9, 120.1, 119.7, 116.4, 110.6, 107.5, 103.2, 73.8, 59.0, 56.3, 54.9, 52.1, 50.7, 28.4, 27.6, 25.5, 22.2. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}FN_3O_4]^+$: 570.67; found: 570.85. Anal. calcd. for $C_{34}H_{37}ClFN_3O_4$: C, 67.37; H, 6.15; N, 6.93. Found: C, 67.21; H, 6.03; N, 6.75.

9-O-3-(4-Trifluoromethoxyphenyl)piperazine) pentylberberine ***(5g)***

Light yellow solid, yield: 51 %. m.p. 244–246 °C. IR (KBr) cm^{-1}: 3037 (C–H, Ar), 1613–1546 (C=C, Ar), 1121–1076 (C–O–C).^1H NMR ($CDCl_3$, 400 MHz): δ 9.67 (s, 1H, H-8), 8.61 (s, 1H, H-13), 8.11 (s, 1H, H-1), 7.61 (s, 1H, H-12), 7.55 (s, 1H, H-4), 7.32–7.11 (m, 4H, Ar–H, piperazine H-27, H-28, H-30, H-31), 6.81 (s, 1H, H-11), 6.05 (s, 2H, $–OCH_2O$), 4.85 (t, 2H, $J = 6.1$, H-6), 4.41 (t, 2H, $J = 6.4$, H-15), 4.04 (s, 3H, OCH_3), 3.68 (br s, 4H, piperazinee, H-23, H-25), 3.39 (br s, 4H, piperazinee, H-20, H-22), 3.18 (t, 2H, $J = 6.5$, H-19), 2.44 (t, 2H, $J = 7.6$, H-5), 2.41 (br s, 4H, H-17, H-18), 2.17 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.1, 152.5, 149.0, 147.6, 143.4, 142.3, 139.7, 136.2, 133.8, 132.9, 131.1, 129.5, 123.0, 122.4, 121.6, 120.4, 119.3, 116.7, 110.2, 107.8, 103.9, 73.1, 59.5, 56.0, 54.6, 52.4, 50.3, 28.7, 27.2, 25.8, 22.9. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{35}H_{37}F_3N_3O_5]^+$: 636.68; found: 636.84. Anal. calcd. for $C_{35}H_{37}ClF_3N_3O_5$: C, 62.54; H, 5.55; N, 6.25. Found: C, 62.68; H, 5.41; N, 6.41.

9-O-3-(4-Trifluoromethylphenyl)piperazine)pentylberberine ***(5h)***

Light yellow solid, yield: 58 %. m.p. 221–223 °C. IR (KBr) cm^{-1}: 3017 (C–H, Ar), 1605–1563 (C=C, Ar), 1108–1086 (C–O–C). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.63 (s, 1H, H-8), 8.52 (s, 1H, H-13), 8.07 (s, 1H, H-1), 7.59 (s, 1H, H-12), 7.49 (s, 1H, H-4), 7.29–7.17 (m, 4H, Ar–H, piperazine H-27, H-28, H-30, H-31), 6.91 (s, 1H, H-11), 6.02 (s, 2H, $–OCH_2O$), 4.81 (t, 2H, $J = 6.2$, H-6), 4.46 (t, 2H, $J = 6.3$, H-15), 4.16 (s, 3H, OCH_3), 3.78 (br s, 4H, piperazine, H-23, H-25), 3.55 (br s, 4H, piperazine, H-20, H-22), 3.32 (t, 2H, $J = 6.3$, H-19), 2.49 (t, 2H, $J = 7.3$, H-5), 2.37 (br s, 4H, H-17, H-18), 2.06 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.5, 152.8, 149.6, 147.0, 143.2, 142.4, 139.1, 136.7, 133.9, 132.3, 131.5, 129.8, 125.2, 123.6, 121.0, 120.2, 119.4, 116.1, 110.7, 107.9, 103.3, 73.5, 59.8, 56.6, 54.0, 52.2, 50.4, 28.1, 27.7, 25.9, 22.3. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{35}H_{37}F_3N_3O_4]^+$: 620.68; found: 620.51. Anal. calcd. for $C_{35}H_{37}ClF_3N_3O_4$: C, 64.07; H, 5.68; N, 6.40. Found: C, 63.98; H, 5.53; N, 6.63.

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

Light yellow solid, yield: 61 %. m.p. 251–253 °C. IR (KBr) cm^{-1}: 3024 (C–H, Ar), 1618–1573 (C=C, Ar), 1533 (N–O stretch), 1347 (N–O stretch), 1117–1080 (C–O–C). ^1H NMR ($CDCl_3$, 400 MHz): δ 9.78 (s, 1H, H-8), 8.71 (s, 1H, H-13), 7.90 (s, 1H, H-1), 7.65 (s, 1H, H-12), 7.57 (s, 1H, H-4), 7.37–7.01 (m, 4H, Ar–H, piperazine H-27, H-28, H-29, H-30), 6.88 (s, 1H, H-11), 6.14 (s, 2H, $–OCH_2O$), 4.87 (t, 2H, $J = 6.3$, H-6), 4.37 (t, 2H, $J = 6.5$, H-15), 4.07 (s, 3H, OCH_3), 3.96 (br s, 4H, piperazine, H-23, H-25), 3.59 (br s, 4H, piperazinee, H-20, H-22), 3.17 (t, 2H, $J = 6.2$, H-19),

2.61 (t, 2H, $J = 7.5$, H-5), 2.44 (br s, 4H, H-17, H-18), 2.19 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.0, 152.3, 149.2, 147.1, 143.9, 142.8, 139.5, 136.4, 133.7, 132.6, 131.0, 129.3, 123.2, 121.1, 120.9, 119.8, 116.5, 110.4, 107.7, 103.6, 73.0, 59.3, 56.2, 54.1, 52.9, 50.8, 28.5, 27.4, 25.7, 22.6. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}N_4O_6]^+$: 597.68; found: 597.45. Anal. calcd. for $C_{34}H_{37}ClN_4O_6$: C, 64.50; H, 5.89; N, 8.85. Found: C, 64.33; H, 5.76; N, 8.67.

9-O-3-(1-(4-Nitrophenyl)piperazine)pentylberberine ***(5j)***

Light yellow solid, yield: 63 %. m.p. 244–246 °C. IR (KBr) cm^{-1}: 3041 (C–H, Ar), 1603–1564 (C=C, Ar), 1542 (N–O stretch), 1331 (N–O stretch), 1108–1091 (C–O–C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.76 (s, 1H, H-8), 8.63 (s, 1H, H-13), 8.08 (s, 1H, H-1), 7.58 (s, 1H, H-12), 7.63 (s, 1H, H-4), 7.39–7.09 (m, 4H, Ar–H, piperazine H-27, H-28, H-30, H-31), 6.90 (s, 1H, H-11), 6.17 (s, 2H, $–OCH_2O$), 4.90 (t, 2H, $J = 6.4$, H-6), 4.56 (t, 2H, $J = 6.4$, H-15), 4.03 (s, 3H, OCH_3), 3.91 (br s, 4H, piperazine, H-23, H-25), 3.54 (br s, 4H, piperazine, H-20, H-22), 3.28 (t, 2H, $J = 6.5$, H-19), 2.71 (t, 2H, $J = 7.5$, H-5), 2.52 (br s, 4H, H-17, H-18), 2.23 (m, 2H, H-16); ^{13}C NMR (DMSO, 400 MHz): δ 153.4, 152.7, 149.9, 147.2, 143.8, 142.1, 139.6, 136.0, 133.3, 132.5, 131.4, 129.7, 123.9, 121.2, 120.8, 119.1, 116.6, 110.0, 107.3, 103.5, 73.4, 59.7, 56.9, 54.2, 52.8, 50.1, 28.6, 27.0, 25.3, 22.5. HRMS *m/z* $[M–Cl]^+$ calcd. for $[C_{34}H_{37}N_4O_6]^+$: 597.68; found: 597.83. Anal. calcd. for $C_{34}H_{37}ClN_4O_6$: C, 64.50; H, 5.89; N, 8.85. Found: C, 64.32; H, 5.98; N, 8.94.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

Reduction of a stable free radical, 2,2-diphenyl-1-picrylhydrazyl is the base of the DPPH antioxidant bioassay. It has an odd electron that shows a maximum absorption band of 517 nm (deep violet color) in ethanol. The DPPH bioassay is the widely used and acceptable method for evaluating the free radical scavenging action of the tested compounds. Such substances donate a hydrogen atom when mixed with the DPPH, thereby introducing its reduced congener, diphenylpicrylhydrazine (non-radical) with the loss of violet color.

In the present study, DPPH bioassay was adopted to screen the berberine-based compounds for their in vitro antioxidant profiles. The results of this bioassay investigation were introduced in the form of the percentage of radical scavenging antioxidant activity (RSA %) of each substance. The investigation of the DPPH radical scavenging activity was operated according to the methodology described by Brand-Williams et al. (1995) with some modifications (Mistry et al., 2016). A stable free radical, DPPH, was allowed to react with test compounds in methanol as 20 µg/mL (100, 10, 1 and 0.1) quantities of title compounds were mixed up with 180 µg/mL of DPPH in methanol. Titled compounds donated hydrogen during the mixing thereby introduced the reduction of DPPH and hence a change in the color was observed from deep violet to light yellow at 517 nm after 25 min of reaction in a UV–visible spectrophotometer (Perkin Elmer). The blank reading was also performed using the mixture of methanol (20 µg/mL) and sample (180 µg/mL of DPPH). Ascorbic acid served as a control drug in this assay, and its solution was prepared by mixing methanol (20 µg/mL) and DPPH radical solution (180 µg/mL). The results of this bioassay, RSA % (the radical scavenging activity in percentage) was determined according to Mensor et al. (2001) as described in the below equation.

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

A plot of concentration of test compounds and % scavenging introduced IC_{50s} in the presence of an ascorbic acid as standard.

2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging assay

The $ABTS^{\bullet+}$ radical cation scavenging efficacies of the test compounds was determined according to the method described earlier (Re et al., 1999) with some modifications (Mistry et al., 2016). Mixing of an equal amount of 7 mM $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) stock solution with 2.45 mM potassium persulfate stock solution produces the $ABTS^{\bullet+}$ cation. The mixture was kept in a dark place at 0 °C temperature for 12 h and ABTS solution was diluted with MeOH so that it gives UV absorption value of 0.700 (±0.200) at the 734 nm. The 1000 µL stock solutions of titled compounds **5a–j** was established upon dissolving in methanol and further dilutions furnishes 100, 10, 1 and 0.1 µg/mL quantities of test samples. In all 180 µg/mL solutions of compounds to be evaluated and 20 µg/mL of the ABTS solution were mixed in 96-well plates in a dark place and then incubated for 10 min to measure UV absorption at 734 nm. A mixture of 180 µg/mL ABTS and 20 µg/mL methanol was used as a control determination, while ascorbic acid was used as a reference drug. The UV absorption data represented the radical scavenging rates that give the corresponding IC_{50s} for the test compounds.

The scavenging capability of $ABTS^{\bullet+}$ radical was calculated using the following equation:

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

In vitro anticancer bioassay

Cell cultures

The test compounds **5a–j** were checked for their in vitro anticancer action against cervical cancer cell line HeLa, CaSki and non-cancer Madin-Darby canine kidney (MDCK) cells which were purchased from American Type Culture Collection. All the cell lines were well maintained in a humidified cell culture incubator in the presence of 5 % of CO_2 at 37 °C temperature for cell growth. Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium supplemented with 10 % of fetal bovine serum (FBS) and 1 % of Antibiotic-Antimycotic Solution (100×) were used for HeLa, CaSki and MDCK cell growth, respectively. DMEM, RPMI-1640, trypsin–EDTA, Antibiotic-Antimycotic Solution 100× and FBS were purchased from Welgene (150-Seongseo Industrial complex Bukro, Dalseogu, Daegu, 704–948 Republic of Korea).

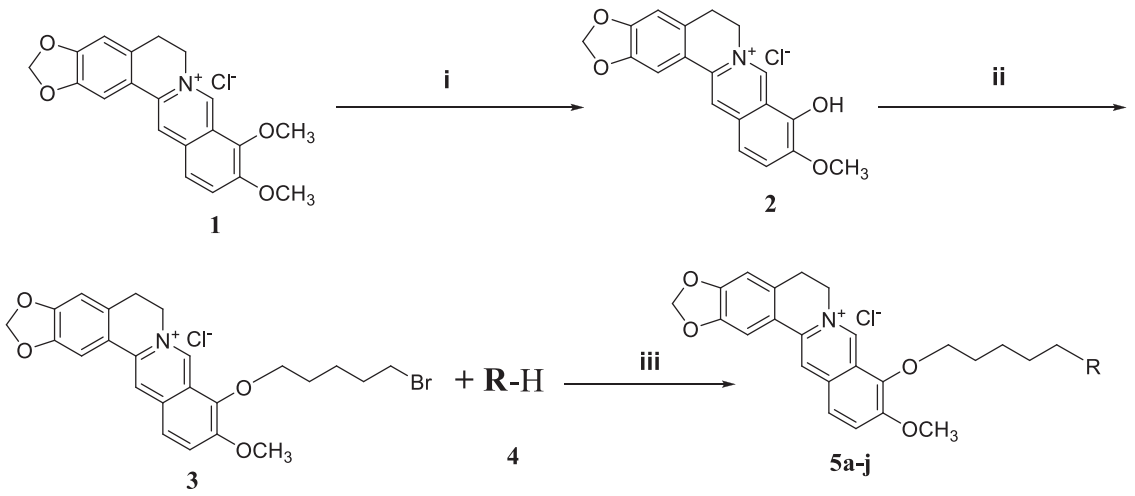
In the 96-well plates, both the cancer cell lines HeLa, CaSki and a non-cancer cell line MDCK were seeded, and plates were concentrated as 2×10^4 cells per well plate. Cancerous cells were allowed to grow for 1 day initially and after that the 96-well plates were washed twice with phosphate buffer saline (PBS). DMEM and RPMI-1640 medium containing trypsin–EDTA were used to dilute HeLa, CaSki and MDCK cells upto 5×10^3 level which was used for the infection followed by pacing of 10 µg/mL of compound quantities and 90 µg/mL of cell solution onto the 96-well plates in which HeLa, CaSki and MDCK cells were grown the previous day. In all 0.1, 1, 10 and 100 µg/mL concentrations of the test compounds were used in 96-well plates for the analysis with three replicates of observations. Infected plates were incubated in a CO_2 incubator for 48 h. After incubation, the medium was taken out and cleaned twice with PBS buffer. After that, 70 % of acetone was added to fix the cells and were incubated for 1 h at 4 °C. After incubation, the solvent was removed, and plates were dried in an oven at 60 °C. The dried plates were overnight incubated 100 µg/mL of sulforhodamine B (SRB; 0.4 mg/L) followed by SRB removal and washing thrice with 1 % of acetic acid and dried again under hot air oven at 60 °C. Microscopic observation was performed to identify the morphology of the cells and to follow this observation, the SRB strain was dissolved with 10 mM of Tris base and incubated overnight (Adaramoye et al., 2011; Mistry et al., 2016). Spectrophotometric data were recorded at 510 nm to calculate the inhibitory concentration of 50 % (IC_{50}), cytotoxic concentration of 50 % (CC_{50}) and therapeutic index (TI).

Results and discussion

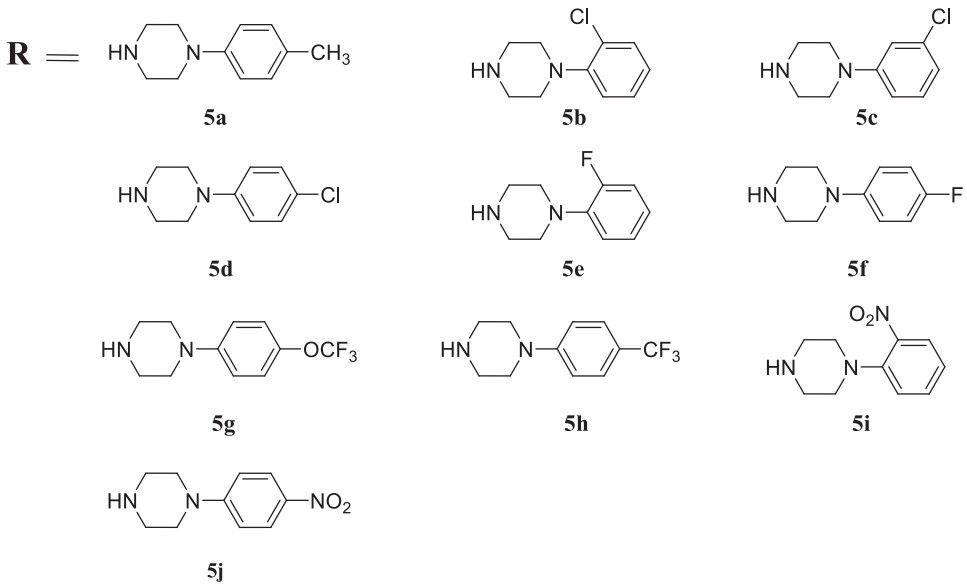
Chemistry

Scheme 1 represents the synthetic pathway followed to generate berberine-piperazine derivatives (**5a–j**). At first, demethylation of berberine hydrochloride itself under vacuum oven at 190 °C temperature and 20–30 mmHg pressure introduced berberrubine (**2**) with 85 % of yield (Iwasa et al., 1996). Alkylation was followed in the further step in the presence of dibromoalkanes in dry acetonitrile (Mistry et al., 2015; Mistry et al., 2016a; Mistry et al., 2016b) yielding final intermediate **3**. In the last step, different piperazine moieties were coupled with intermediate **3** in DMF for 6–8 h to construct **5a–j** with (51–67 %) reasonable yields.

The correct formation of the structure of compounds **5a–j** was confirmed utilizing spectroscopic techniques such as FT-IR, ^{1}H NMR, mass spectrometry and elemental analysis (CHN). Absorption bands at 3043–3017 cm^{-1} for the FT-IR of **5a–j** confirmed the presence of C-H stretching for aromatics, whereas C=C band of aromatic ring observed in the range 1622–1546 cm^{-1}. The C-O-C band indicated a sharp peak nearby 1128–1067 cm^{-1} along with sharp chlorine signal at 790–740 cm^{-1}. In the ^{1}H NMR spectra (**5a**), proton atoms of the berberine ring resonated at 9.81, 8.47, 7.86, 7.53, 7.43, 6.75 ppm for H-8, H-13, H-1, H-12, H-4, H-11, respectively, in the form of singlet along with singlet peak observe at 6.02 ppm due to the presence of -OCH_2O of berberine. Also, a triplet was indicated at 4.98 and 2.54 ppm due to H-6 and H-5 proton atoms of the berberine ring. An alkyl chain proton (H-15) falls at around 4.31 ppm in the form of the triplet. Furthermore, a triplet signal at 3.18 ppm, a broad singlet at 2.43 ppm, as well as multiplet signals at 2.15 ppm, were due to the alkyl chain protons H-19, H-17 and H-18, as well as H-16, respectively. The proton atoms belonging to the methoxy functional group were found to resonate at 4.13 ppm, whereas methyl protons of the piperazine ring were observed to have their presence around 1.90 ppm. The protons (H-23, H-25, H-20 and H-22) of piperazine ring were resonated as a broad singlet at around 3.81 and 3.46 ppm. Moreover, aromatic rings linked to the piperazine ring appeared to have corresponding signals as multiples in the range 7.34–7.23 ppm. The ^{13}C NMR data observed for compound **5a** further confirmed the correct formation of the synthesized structure



Reagents &conditions: i. 190°C, 20-30 mm Hg 40 min: **ii.** CH_3CN, 1,5-dibromopentan, reflux, 6h: **iii.** K_2CO_3, DMF, 80°C, 6-8 h.



Scheme 1 Synthesis of piperazine-linked berberine derivatives

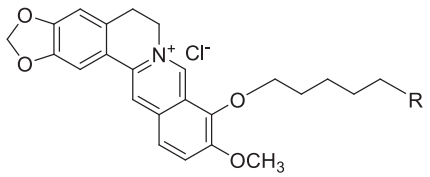
of the compounds. The high-resolution mass spectral data of compounds **5a–j** exhibited molecular ion peaks at their respective molecular weights, which confirmed their formation. All of the novel compounds gave C, H and N analyses within 0.4 percent points from the theoretical values, i.e., in an acceptable range.

Evaluation of biological activities

Antioxidant activities

Final analogues **5a–j** was examined for their in vitro anti-oxidant potencies using DPPH and ABTS bioassay and results are summarized in Table 1. It was noticed that the anti-oxidant activity of the piperazine based berberine a scaffolding varies considerably based upon on the backbone structures and functional groups linked with the berberine through a pentyl chain. Overall derivatives of berberine had used enhanced efficacy in both the anti-oxidant bioassays in evaluation to parent analog berberine. Different electron-withdrawing functional groups such as chloro, fluoro and nitro, as well as electron-donating functional group such as methyl were present on the piperazine moieties. Title analogues **5a–j** exhibited 17.25–25.40 µg/mL of IC_{50} levels in DPPH free radical scavenging assay in which the highest scavenger activity seen in compound **5c** is probably due to the existence of *meta*-chloro piperazine entity with 17.25 µg/mL of IC_{50}. In addition, for all of the remaining analogues IC_{50} level varied from 21 to 22 µg/mL, which can be regarded to be antioxidant action when

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



Compounds	[a]IC_{50} μg/mL ± SD	
	DPPH	ABTS
5a	25.40 ± 1.03	7.512 ± 1.95
5b	25.28 ± 3.12	7.267 ± 1.34
5c	17.25 ± 0.79	7.470 ± 0.76
5d	21.51 ± 2.15	4.778 ± 0.86
5e	21.87 ± 4.17	6.010 ± 1.88
5f	21.82 ± 1.21	6.543 ± 2.24
5g	22.40 ± 0.68	6.737 ± 1.65
5h	24.19 ± 2.07	7.184 ± 1.18
5i	22.37 ± 3.07	6.803 ± 2.04
5j	22.72 ± 1.06	8.917 ± 0.78
Berberine	34.29 ± 2.75	82.17 ± 4.02
Ascorbic acid	11.52 ± 1.23	5.528 ± 2.04

ABTS 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid, *DPPH* 2,2-diphenyl-1-picrylhydrazyl

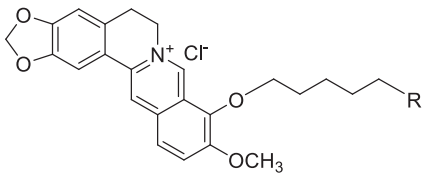
[a] Antioxidant activities are shown as IC_{50} values in μg/mL. All assays were carried out in triplicate, and the results expressed as anaverage ± standard deviation

compared to the potency of the parent compound berberine at 34.29 μg/mL and control drug ascorbic acid at 11.52 μg/mL. The least active scaffold was holding the methyl group at 25.40 μg/mL of IC_{50}. Overall, it can be stated that the presence of chlorine atom was the most beneficial part of the enhanced potency of the title compounds in DPPH assay. Secondly, the said potency of compounds **5a–j** was further verified via examining in ABTS bioassay, in which newer scaffolds demonstrated a significant level of antioxidant efficacies with IC_{50s} ranged from 4.778 to 8.917 μg/mL. The most active free radical scavenger was compound **5d** with 4.778 μg/mL of the IC_{50} level, hence again confirming the key role of chlorine atom(s). Remaining compounds exhibited IC_{50s} (6.010–7.512 μg/mL) near to that of control drugs ascorbic acid at 5.528 μg/mL, and excellent potencies when compared to the parent compound berberine with 82.17 μg/mL of IC_{50}.

Anticancer activities

In the present work, compounds **5a–j** were checked for their in vitro anticancer efficacies using a SRB bioassay and the results are summarized in Table 2 and Table 3. The results

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

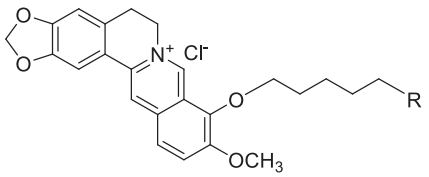


Compounds	[a]IC_{50} μg/mL ± SD HeLa	CC_{50} μg/mL ± SD MDCK	TI
5a	5.595 ± 0.02	327.5 ± 0.05	58.53
5b	7.532 ± 0.06	315.1 ± 1.14	41.83
5c	7.534 ± 0.06	318.5 ± 3.35	42.28
5d	5.526 ± 0.03	236.8 ± 3.75	42.85
5e	7.632 ± 0.09	304.9 ± 0.87	39.95
5f	5.599 ± 0.02	232.9 ± 2.72	41.60
5g	7.542 ± 0.11	216.9 ± 2.40	28.76
5h	7.180 ± 0.07	206.7 ± 2.55	28.79
5i	7.657 ± 0.13	261.4 ± 0.49	34.14
5j	7.691 ± 0.07	197.9 ± 2.10	25.73
Berberine	5.611 ± 0.01	153.8 ± 1.16	27.41

CC_{50} cytotoxicity concentration of 50 %, *TI* therapeutic index

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

Table 3 Anticancer activity of synthesized compounds against CaSki cancer cell and their toxicity



Compounds	[a]IC_{50} μg/mL ± SD CaSki	CC_{50} μg/mL ± SD MDCK	TI
5a	6.716 ± 0.05	327.5 ± 0.05	48.76
5b	6.654 ± 0.09	315.1 ± 1.14	47.35
5c	6.878 ± 0.24	318.5 ± 3.35	46.31
5d	5.742 ± 0.07	236.8 ± 3.75	41.24
5e	6.439 ± 0.08	304.9 ± 0.87	47.35
5f	5.989 ± 0.09	232.9 ± 2.72	38.89
5g	6.602 ± 0.15	216.9 ± 2.40	32.85
5h	6.515 ± 0.05	206.7 ± 2.55	31.73
5i	6.769 ± 0.13	261.4 ± 0.49	38.62
5j	6.739 ± 0.25	197.9 ± 2.10	29.37
Berberine	5.951 ± 0.06	153.8 ± 1.16	25.84

CC_{50} cytotoxicity concentration of 50 %, *TI* therapeutic index

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

of anticancer screening suggested that such analogues are potent inhibitors of the growth of cervical cancer cell lines HeLa and CaSki with IC_{50} levels of around 5.595–7.691 μg/mL and 5.742–6.878 μg/mL, as well as CC_{50} levels of around 197.9–327.5 μg/mL, respectively. Overall, tested compounds demonstrated the low cytotoxic nature towards the non-cancer MDCK cell line and as a result presented remarkable TIs of around 25.73–58.53 and 29.37–48.76, better than that of a parent compound berberine with 27.41 and 25.84 of TI, respectively, against HeLa and CaSki. An analogue **5a** with piperazine entity bearing electron-releasing methyl functional group displayed 5.595 ± 0.02 μg/mL of IC_{50} and 327.5 ± 0.05 μg/mL of CC_{50} level, presenting the highest anticancer potential against HeLa cell line with 58.53 of TI. Moreover, the same analogue had the highest efficacy against CaSki cell line with 6.716 ± 0.05 μg/mL of IC_{50} and 327.5 ± 0.05 μg/mL of CC_{50} and 48.76 of TI. The inhibitory potential of cervical cancer cell lines was followed by a group of final analogues bearing piperazine moiety with electron-withdrawing chlorine functionality as compound **5b**, **5c** and **5d** exhibited TIs of 41.83, 42.28 and 42.85, as well as 47.35, 46.31 and 41.24 against HeLa and CaSki cell lines, respectively. Overall, in regard with the TI, title compounds were having higher inhibitory potential against CaSki cell lines when compared to HeLa. Compound **5f** with *para*-fluorophenylpiperazine moiety and **5e** with *ortho*-fluorophenylpiperazine moiety emerged with equipotent anticancer effects as their chloro precursors with TI of 41.60 and 47.35 against HeLa and CaSki cell lines, respectively. Compounds with *para*-trifluoromethoxy (**5g**), *para*-trifluoromethyl (**5h**) and *ortho* and *para*-nitro (**5i** and **5j**) functional groups have the least activities observed in the bioassay with TI levels 25–38 against both the cell lines, but still it was equal or higher than that of berberine itself. Concerning the activity regarding the functional group attached to the piperazine ring, the order falls in the way alkyl > chloro > fluoro > nitro.

Conclusion

In summary, isoquinoline alkaloid natural analogue berberine has been utilized to furnish a new class of substituted piperazine-based scaffolds. Efficient synthesis of final analogues was verified using different spectroscopic methods, elemental analyses and physical measurements. Final compounds were tested for their antioxidant and anticancer effects (HeLa and CaSki cervical cancer cell lines). More specifically, *meta*-chloropiperazine was valuable for the radical scavenging effects, whereas *para*-methyl piperazine had applied better anticancer effects. All compounds exhibited low cytotoxic values (197.9–327.5 μg/mL) against normal cell lines. Overall, all compounds provided favorably improved antioxidant and anticancer effects than berberine and good to moderate potencies when compared to the control drugs. Compound **5a** with methyl functionality and **5c** with chloro functionality exhibited significant anticancer and antioxidant potential, respectively, and can be considered further in the ongoing drug discovery process. The molecular designs and rationalization presented with this can be a tool to process for further modification in the molecular systems to obtain an advanced level of therapeutic potencies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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