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ORIGINAL PAPER

Synthesis of piperazine based *N*-Mannich bases of berberine and their antioxidant and anticancer evaluations

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Abstract Isoquinoline alkaloids possess versatile biological activities. Hence, in the current research an effort has been made to improve structurally important part of isoquinoline alkaloid berberine because it is recognized as the marking compound for the crude drugs. Synthesis of rationalized berberine has been performed via substituting various piperazine moieties bearing disubstituted electron withdrawing and electron donating groups to the berberine core via Mannich reaction. Intended scaffolds were inspected for their in vitro antioxidant potential using different bioassays, FRAP, DPPH and ABTS as well as anticancer efficacies against cervical cancer cell lines HeLa, CaSki adapting SRB assay. Also, an inspection of the cytotoxic nature of titled analogues has been carried out towards Madin-Darby canine kidney (MDCK) cell lines. Radical scavenging potential of the final derivatives **4a**–**i** was found to be excellent with IC_{50s}, <20 and <12 μg/mL in DPPH and ABTS assay, respectively, whereas some dichlorophenyl piperazine analogues revealed important Fe^{3+} decreasing power with absorption at around 2 nm in FRAP assay. Moreover, compounds **4a**–**i** appeared with significant inhibitors of the cervical cancer cell lines HeLa and CaSki with IC_{50s} ranging 4.346–6.321 and 3.408–6.081 μg/mL, with low level of cytotoxic values and higher therapeutic indices ranging 20.42–42.45 and 21.23–43.25, respectively. Therefore, from the bioassay results it can be mentioned that these analogues are effective double agents as the scavengers of reactive oxygen species and inhibitors of the cancerous cells. The correct structure of the final compounds was adequately confirmed on the basis of FT-IR, ^{1}H NMR and mass spectroscopy data as well as elemental analyses.

Keywords Berberine · Mannich bases · Piperazine · Antioxidant · Cervical cancer

Introduction

Free radicals are atoms or compounds that hold an unpaired electron and are extremely reactive, able of eliciting a fast change response that destabilizes other elements and produces many more free radicals. Scientific evidence suggests that antioxidants prevent chronic diseases including cancer and cardiovascular disease [1–5]. Among the chronic diseases, cancer is the most common epidemic all over the world. Cancers figure among the major reasons of fatalities and death rate globally, with approximately 14 million new cases and 8.2 million cancer-related fatalities in 2012 and the number of new cases is predicted to increase by about 70 % over the next two decades [6, 7]. Natural products, like plant extract, either as pure compounds or as consistent extracts, provide unlimited possibilities for new drug findings due to the unrivaled chemical variety the can offer [8]. Various researches suggest that therapeutic plants contain substances like proteins, unsaturated long chain fatty acids, aldehydes, alkaloids, essential oils, phenols and water or soluble ethanol compounds. These compounds are significant in therapeutic application against human and animal pathogens, including bacteria, fungi and viruses [9, 10]. In the plant, alkaloids are available in the free state, as salt or as amine or alkaloid *N*-oxides [11]. They are identified based on the amino acids that provide their nitrogen atom

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and aspect of their skeleton and normally easily separable from the other metabolites due to their basicity [12].

Berberine is an isoquinoline alkaloid, with a bright yellow color, that is simply seen in most of the natural herb components, which contain any significant amount. Berberine is primary alkaloid from origins and stem-bark of Berberis varieties. It is produced mainly from roots of *B. aristata* (5 % in roots and 4.2 % in stem-bark), *B. Petiolaris* (0.43 %), *B.vulgaris*, *B. aquifolium*, *B. thunbergii* and *B. asiatica* [13–15], *C. teeta* (rhizome 8–9 %) and *Hydrastis Canadensis* [16]. Berberine has been associated with tremendous free-radical scavenging potential as an antioxidant agent as reported by numerous research studies [17–19]. Also, derivatization of berberine core structure has resulted in the development of molecules with positively enhanced biological effects [20–23]. Moreover, berberine and its derivatives are associated with excellent cancerous cell inhibitors effects [24–26]. In the design of new drugs, the improvement of several components using the mixture of various pharmacophores in one framework may cause substances with exciting healing properties. Researchers have recently reported the tremendous potential of piperazine derivatives associated with different biological efficacies [27]. Moreover, substitution of piperazine derivatives to the base nucleus bearing nitrogen ring has been found to exert significant anticancer effects [28]. Also, piperazine derivatization would lead to the development of scaffolds furnishing enormous antioxidant efficacies [29]. According to the extensive variety of biological activities related with berberine and the piperazine, the fusion of these two moieties in the same compound is an exciting challenge for the growth of new pharmacologically effective agents. Derivatization has been conducted via developing *N*-Mannich bases as the biological results of this scaffold is well known, particularly as anti-oxidant [30–32] and anticancer providers [33, 34]. *N*-Mannich bases prepared with piperazine linkage was recently tried and adapted in the existing research for the efficient synthesis of berberine-piperazine systems [35].

Experimental section

Materials and methods

Highest quality chemicals and reagents were used in this study without prior purification. Veego Open capillary electronic apparatus VMP-D was utilized to obtain melting points of the synthesized compounds that were uncorrected. Bruker FT-IR spectrophotometer (KBr pellets) and Varian 400 MHz model spectrometer ($CDCl_3$ and DMSO as a solvent and TMS as internal standard) were used to obtain FT-IR and ^{1}H NMR spectra of the title compounds. TLC was carried out using appropriate mobile phase systems and silica gel-G coated microscopic glass slides (2 × 7.5 cm), and TLC spots were observed in UV light chamber. FT-IR bands were presented in cm^{-1} as well as ^{1}H NMR spectral results were 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 performed using a Heraeus Carlo Erba 1180 CHN analyzer.

*General procedure for the synthesis of berberrubine (**2**)*

Berberine hydrochloride (10 g, 0.01 mol) was added to a 50 mL round bottom flask. The reaction system was kept under reduced pressure (20–30 mmHg) using an oil pump, and heated to 190 °C followed by reacting for 40 min. The vacuum pump was switched off after the temperature dropped to room temperature. The reaction product was purified through silica gel column chromatography ($CHCl_3$/CH_3OH:15:1 and 10:1, eluting until no compound was observed in the eluent) to obtain a brownish red amorphous powder compound 2 (6.6 g, 85 %).

*General procedure for the preparation of Mannich base derivatives (**4a–i**)*

Compound 2 (0.5 g, 0.01 mol) was dissolved in 25.0 ml of the anhydrous ethanol, various piperazine derivatives (0.015 mol) and aqueous formaldehyde solution (37 %, 0.05 mol) were added, stirred at room temperature or 80 °C for 24 h. Concentrated under reduced pressure to give the crude product, which was purified using flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give final compound **4a**.

12-(1-(2,3-Dichlorophenyl) piperazine-1-ylmethyl)-berberrubine (4a)

Light yellow solid, yield: 58 %. m.p. 241–243 °C. IR (KBr) cm^{-1}: 3620 (–OH), 3027 (C–H, Ar), 1622–1547 (C=C, Ar), 1261 (Ar–N), 768 (C–Cl). ^{1}H NMR (400 MHz, chloroform) δ 9.87 (s, 1H, –OH of berberine ring), 9.31 (s, 1H, H-8), 7.78 (s, 1H, H-13), 7.51 (s, 1H, H-1), 7.15 (s, 1H, H-4), 6.93–6.75 (m, 3H, Ar–H, piperazine H-22, H-23, H-24), 6.13 (s, 2H, $-OCH_2O$), 6.05 (s, 1H, H-11), 5.95 (t, J = 5.4 Hz, 2H, H-6), 3.99 (s, 2H, CH_2, H-14 Mannich base), 3.93 (s, 3H, OCH_3), 3.80 (br s, 4H, piperazine, H-18, H-20), 3.49 (br s, 4H, piperazine, H-15, H-17), 3.15 (t, J = 5.3 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.5, 152.2, 149.6, 147.4, 145.2, 142.3, 137.1, 134.3, 130.8, 128.1, 126.5, 124.7, 122.5, 121.4, 119.8, 118.6, 108.5, 105.7, 103.8, 101.2, 99.1, 80.8, 69.7, 55.9, 52.1, 50.6, 26.8. HRMS m/z $[M\text{-}Cl]^+$ calcd for $[C_{30}H_{28}Cl_2N_3O_4]^+$: 564.15; found: 564.20. Anal. calcd

for $C_{30}H_{28}Cl_3N_3O_4$: C, 59.96; H, 4.70; N, 6.99. Found: C, 59.84; H, 4.56; N, 6.87.

12-(1-(2,4-Difluorophenyl) piperazine-1-ylmethyl)-berberrubine (4b)

Light yellow solid, yield: 61 %. m.p. 229–231 °C. IR (KBr) cm^{-1}: 3632 (OH), 3041 (C–H, Ar), 1618–1558 (C=C, Ar), 1252 (Ar–N). ^{1}H NMR (400 MHz, chloroform) δ 9.78 (s, 1H, –OH of berberine ring), 9.28 (s, 1H, H-8), 7.71 (s, 1H, H-13), 7.47 (s, 1H, H-1), 7.13 (s, 1H, H-4), 6.88–6.72 (m, 3H, Ar–H, piperazine H-22, H-23, H-25), 6.17 (s, 2H, –OCH_2O), 6.08 (s, 1H, H-11), 5.91 (t, J = 5.5 Hz, 2H, H-6), 3.98 (s, 2H, CH_2, H-14 Mannich base), 3.94 (s, 3H, OCH_3), 3.83 (br s, 4H, piperazine, H-18, H-20), 3.44 (br s, 4H, piperazine, H-15, H-17), 3.18 (t, J = 5.5 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.7, 152.0, 149.1, 147.8, 145.7, 142.7, 137.9, 134.6, 130.0, 128.8, 126.9, 124.1, 122.8, 121.7, 119.2, 118.4, 108.9, 105.6, 103.2, 101.4, 99.0, 80.1, 69.1, 55.0, 52.2, 50.8, 26.3. HRMS m/z [M-Cl]$^+$ calcd for $[C_{30}H_{28}F_2N_3O_4]^+$: 532.20; found: 532.60. Anal. calcd for $C_{30}H_{28}ClF_2N_3O_4$: C, 63.44; H, 4.97; N, 7.40. Found: C, 63.59; H, 5.04; N, 7.56.

12-(1-(2,4-Dimethoxyphenyl) piperazine-1-ylmethyl)-berberrubine (4c)

Light yellow solid, yield: 48 %. m.p. 232–234 °C. IR (KBr) cm^{-1}: 3641(OH), 3034 (C–H, Ar), 1611–1560 (C=C, Ar), 1255 (Ar–N). ^{1}H NMR (400 MHz, chloroform) δ 9.91 (s, 1H, –OH of berberine ring), 9.21 (s, 1H, H-8), 7.82 (s, 1H, H-13), 7.44 (s, 1H, H-1), 7.22 (s, 1H, H-4), 6.83–6.69 (m, 3H, Ar–H, piperazine H H-22, H-23, H-25), 6.21 (s, 2H, –OCH_2O), 6.02 (s, 1H, H-11), 5.88 (t, J = 5.5 Hz, 2H, H-6), 4.05 (s, 2H, CH_2, H-14 Mannich base), 3.95 (s, 3H, OCH_3), 3.92 (s, 3H, Ar-OCH_3 of piperazine), 3.86 (s, 3H, Ar-OCH_3 of piperazine), 3.78 (br s, 4H, piperazine, H-18, H-20), 3.45 (br s, 4H, piperazine, H-15, H-17), 3.21 (t, J = 5.4 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.1, 152.7, 149.0, 147.1, 145.8, 142.8, 137.4, 134.7, 130.3, 128.7, 126.8, 124.5, 122.1, 121.2, 119.5, 118.1, 108.0, 105.1, 103.5, 101.5, 99.6, 80.2, 69.0, 55.5, 54.8, 52.7, 51.1, 50.3, 26.6. HRMS m/z [M-Cl]$^+$ calcd for $[C_{32}H_{34}N_3O_6]^+$: 556.24; found: 556.40. Anal. calcd for $C_{32}H_{34}ClN_3O_6$: C, 64.91; H, 5.79; N, 7.10. Found: C, 64.77; H, 5.91; N, 7.23.

12-(1-(2,4-Dimethylphenyl) piperazine-1-ylmethyl)-berberrubine (4d)

Light yellow solid, yield: 65 %. m.p. 259–261 °C. IR (KBr) cm^{-1}: 3628 (OH), 3021 (C–H, Ar), 1615–1555 (C=C, Ar), 1263 (Ar–N). ^{1}H NMR (400 MHz, chloroform) δ 9.84 (s, 1H, –OH of berberine ring), 9.33 (s, 1H, H-8), 7.85 (s, 1H, H-13), 7.56 (s, 1H, H-1), 7.09 (s, 1H, H-4), 6.98–6.78 (m, 3H, Ar–H, piperazine H-22, H-23, H-25), 6.09 (s, 2H, –OCH_2O), 6.01 (s, 1H, H-11), 5.93 (t, J = 5.4 Hz, 2H, H-6), 4.03 (s, 2H, CH_2, H-14 Mannich base), 3.95 (s, 3H, OCH_3), 3.86 (br s, 4H, piperazine, H-18, H-20), 3.48 (br s, 4H, piperazine, H-15, H-17), 3.24 (t, J = 5.4 Hz, 2H, H-5), 2.30 (s, 3H, Ar-CH_3 of piperazine), 2.04 (s, 3H, Ar-CH_3 of piperazine). ^{13}C NMR (DMSO, 400 MHz): δ 155.9, 152.4, 149.3, 147.6, 145.5, 142.5, 137.7, 134.2, 130.4, 128.6, 126.7, 124.8, 122.3, 121.6, 119.7, 118.7, 108.4, 105.0, 103.7, 101.3, 99.7, 80.4, 69.6, 55.8, 52.5, 50.1, 26.0, 22.4, 19.7. HRMS m/z [M-Cl]$^+$ calcd for $[C_{32}H_{34}N_3O_4]^+$: 524.25; found: 524.40. Anal. calcd for $C_{32}H_{34}ClN_3O_4$: C, 68.62; H, 6.12; N, 7.50. Found: C, 68.76; H, 6.26; N, 7.33.

12-(1-(2,3-Difluorophenyl) piperazine-1-ylmethyl)-berberrubine (4e)

Light yellow solid, yield: 63 %. m.p. 247–249 °C. IR (KBr) cm^{-1}: 3638 (OH), 3037 (C–H, Ar), 1620–1553 (C=C, Ar), 1254 (Ar–N). ^{1}H NMR (400 MHz, chloroform) δ 9.76 (s, 1H, –OH of berberine ring), 9.17 (s, 1H, H-8), 7.73 (s, 1H, H-13), 7.61 (s, 1H, H-1), 7.18 (s, 1H, H-4), 6.80–6.68 (m, 3H, Ar–H, piperazine H-22, H-23, H-24), 6.11 (s, 2H, –OCH_2O), 6.07 (s, 1H, H-11), 5.85 (t, J = 5.3 Hz, 2H, H-6), 3.97 (s, 2H, CH_2, H-14 Mannich base), 3.89 (s, 3H, OCH_3), 3.81 (br s, 4H, piperazine, H-18, H-20), 3.51 (br s, 4H, piperazine, H-15, H-17), 3.16 (t, J = 5.3 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.2, 152.9, 149.8, 147.9, 145.6, 142.2, 137.8, 134.5, 130.7, 128.5, 126.2, 124.3, 122.4, 121.1, 119.4, 118.2, 108.7, 105.4, 103.4, 101.6, 99.2, 80.7, 69.9, 55.2, 52.6, 50.2, 26.1. HRMS m/z [M-Cl]$^+$ calcd for $[C_{30}H_{28}F_2N_3O_4]^+$: 532.20; found: 532.30. Anal. calcd for $C_{30}H_{28}ClF_2N_3O_4$: C, 63.44; H, 4.97; N, 7.40. Found: C, 63.35; H, 5.07; N, 7.62.

12-(1-(2,4-Dichlorophenyl) piperazine-1-ylmethyl)-berberrubine (3f)

Light yellow solid, yield: 64 %. m.p. 217–219 °C. IR (KBr) cm^{-1}: 3625 (OH), 3022 (C–H, Ar), 1613–1549 (C=C, Ar), 1257 (Ar–N), 781 (C–Cl). ^{1}H NMR (400 MHz, chloroform) δ 9.90 (s, 1H, –OH of berberine ring), 9.29 (s, 1H, H-8), 7.69 (s, 1H, H-13), 7.43 (s, 1H, H-1), 7.24 (s, 1H, H-4), 6.85–6.73 (m, 3H, Ar–H, piperazine H-22, H-23, H-25), 6.18 (s, 2H, –OCH_2O), 6.11 (s, 1H, H-11), 5.83 (t, J = 5.3 Hz, 2H, H-6), 4.01 (s, 2H, CH_2, H-14 Mannich base), 3.90 (s, 3H, OCH_3), 3.79 (br s, 4H, piperazine, H-18, H-20), 3.39 (br s, 4H, piperazine, H-15, H-17), 3.22 (t, J = 5.5 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.4, 152.6, 149.5, 147.3, 145.3, 142.1, 137.3, 134.4, 130.6, 128.4, 126.3, 124.4, 122.7, 121.8,

119.3, 118.5, 108.6, 105.3, 103.1, 101.0, 99.5, 80.3, 69.8, 55.1, 52.3, 50.7, 26.2. HRMS m/z [M-Cl]$^+$ calcd for $[C_{30}H_{28}Cl_2N_3O_4]^+$: 564.15; found: 564.60. Anal. calcd for $C_{30}H_{28}Cl_3N_3O_4$: C, 59.96; H, 4.70; N, 6.99. Found: C, 60.05; H, 4.84; N, 7.08.

12-(1-(2,3-Dimethylphenyl) piperazine-1-ylmethyl)-berberrubine (4g)

Light yellow solid, yield: 46 %. m.p. 255–257 °C. IR (KBr) cm^{-1}: 3643 (OH), 3039 (C–H, Ar), 1608–1565 (C=C, Ar), 1265 (Ar–N). ^{1}H NMR (400 MHz, chloroform) δ 9.83 (s, 1H, –OH of berberine ring), 9.36 (s, 1H, H-8), 7.80 (s, 1H, H-13), 7.55 (s, 1H, H-1), 7.11 (s, 1H, H-4), 6.79–6.67 (m, 3H, Ar–H, piperazine H-22, H-23, H-24), 6.20 (s, 2H, –OCH_2O), 6.09 (s, 1H, H-11), 5.96 (t, J = 5.4 Hz, 2H, H-6), 4.08 (s, 2H, CH_2, H-14 Mannich base), 3.88 (s, 3H, OCH_3), 3.85 (br s, 4H, piperazine, H-18, H-20), 3.46 (br s, 4H, piperazine, H-15, H-17), 3.19 (t, J = 5.3 Hz, 2H, H-5), 2.21 (s, 3H, Ar-CH_3 of piperazine), 1.90 (s, 3H, Ar-CH_3 of piperazine). ^{13}C NMR (DMSO, 400 MHz): δ 155.6, 152.1, 149.2, 147.7, 145.4, 142.4, 137.6, 134.1, 130.9, 128.7, 126.4, 124.9, 122.6, 121.3, 119.6, 118.8, 108.3, 105.2, 103.6, 101.8, 99.4, 80.5, 69.3, 55.4, 52.4, 50.4, 26.5, 19.6, 17.7. HRMS m/z [M-Cl]$^+$ calcd for $[C_{32}H_{34}N_3O_4]^+$: 524.25; found: 524.50. Anal. calcd for $C_{32}H_{34}ClN_3O_4$: C, 68.62; H, 6.12; N, 7.50. Found: C, 68.45; H, 6.04; N, 7.38.

12-(1-(3,4-Dichlorophenyl) piperazine-1-ylmethyl)-berberrubine (4h)

Light yellow solid, yield: 53 %. m.p. 244–246 °C. IR (KBr) cm^{-1}: 3631 (OH), 3025 (C–H, Ar), 1610–1548 (C=C, Ar), 1249 (Ar–N), 753 (C–Cl). ^{1}H NMR (400 MHz, chloroform) δ 9.77 (s, 1H, –OH of berberine ring), 9.30 (s, 1H, H-8), 7.75 (s, 1H, H-13), 7.46 (s, 1H, H-1), 7.16 (s, 1H, H-4), 6.84-6.77 (m, 3H, Ar–H, piperazine H-22, H-23, H-26), 6.23 (s, 2H, –OCH_2O), 6.10 (s, 1H, H-11), 5.84 (t, J = 5.3 Hz, 2H, H-6), 4.04 (s, 2H, CH_2, H-14 Mannich base), 3.91 (s, 3H, OCH_3), 3.82 (br s, 4H, piperazine, H-18, H-20), 3.43 (br s, 4H, piperazine, H-15, H-17), 3.25 (t, J = 5.5 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.3, 152.8, 149.7, 147.2, 145.1, 142.9, 137.5, 134.8, 130.2, 128.2, 126.1, 124.6, 122.9, 121.0, 119.1, 118.3, 108.8, 105.5, 103.9, 101.1, 99.8, 80.6, 69.2, 55.3, 52.9, 50.9, 26.4. HRMS m/z [M-Cl]$^+$ calcd for $[C_{30}H_{28}Cl_2N_3O_4]^+$: 564.15; found: 564.40. Anal. calcd for $C_{30}H_{28}Cl_3N_3O_4$: C, 59.96; H, 4.70; N, 6.99. Found: C, 59.81; H, 4.59; N, 6.83.

12-(1-(3,5-Dichlorophenyl) piperazine-1-ylmethyl)-berberrubin (4i)

Light yellow solid, yield: 67 %. m.p. 253–255 °C. IR (KBr) cm^{-1}: 3622 (OH), 3033 (C–H, Ar), 1616–1543 (C=C, Ar), 1257 (Ar–N), 775 (C–Cl). ^{1}H NMR (400 MHz, chloroform) δ 9.94 (s, 1H, –OH of berberine ring), 9.35 (s, 1H, H-8), 7.83 (s, 1H, H-13), 7.42 (s, 1H, H-1), 7.08 (s, 1H, H-4), 6.78–6.65 (m, 3H, Ar–H, piperazine H-22, H-24, H-26), 6.16 (s, 2H, –OCH_2O), 6.07 (s, 1H, H-11), 5.92 (t, J = 5.5 Hz, 2H, H-6), 4.06 (s, 2H, CH_2, H-14 Mannich base), 3.96 (s, 3H, OCH_3), 3.77 (br s, 4H, piperazine, H-18, H-20), 3.40 (br s, 4H, piperazine, H-15, H-17), 3.20 (t, J = 5.4 Hz, 2H, H-5). ^{13}C NMR (DMSO, 400 MHz): δ 155.7, 152.3, 149.4, 147.5, 145.0, 142.6, 137.2, 134.9, 130.5, 128.9, 126.6, 124.2, 122.0, 121.5, 119.9, 118.1, 108.1, 105.8, 103.0, 101.7, 99.6, 80.9, 69.5, 55.6, 52.8, 50.0, 26.7. HRMS m/z [M-Cl]$^+$ calcd for $[C_{30}H_{28}Cl_2N_3O_4]^+$: 564.15; found: 564.30. Anal. calcd for $C_{30}H_{28}Cl_3N_3O_4$: C, 59.96; H, 4.70; N, 6.99. Found: C, 59.87; H, 4.83; N, 7.12.

Evaluation of antioxidant activity

Antioxidant capacity by DPPH assay

Radical scavenging potency of the compounds examined was evaluated in vitro by DPPH analysis. DPPH is one of the few constants and from the commercial perspective available organic nitrogen radicals. DPPH radicals are viewed as an associate method for the initial testing of compounds able to scavenge activated oxygen species. They are more constant and easier to deal with than oxygen free radicals and DPPH analysis has been mostly used to evaluate the antioxidant action of different phenolic compounds. The DPPH radical displays absorbance at 517 nm that reduces by the bleaching of a violet-coloured methanol solution upon decrease by an anti-oxidant or a radical via hydrogen atom or electron transfer to the odd electron in DPPH generating the light yellow non-radical form (DPPH-H). This reduces proportionately with the improvement of non-radical types of DPPH [36]. The radical scavenging activity of berberine derivatives and starting materials towards the radical DPPH was measured as described [37] with modifications to adapt the screen for 96-well plates. Berberine derivatives (20 µL) was included in 96-well microplate, and 180 µL of DPPH was included to the wells. Methanol was used as the blank sample. The mixtures were remaining for 30 min at room temperature and the absorbances then were measured at 517 nm. The control contains all reagents except the scavenger. The

DPPH radical scavenging activity of ascorbic acid was also assayed for comparison; all tests were performed in triplicate. The results of this bioassay, RSA % (the radical scavenging activity in percentage) was determined according to Mensor et al. [38] as described in below equation.

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

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

Antioxidant capacity by ABTS assay

The second analysis is by the capability of antiradical elements to satisfy the $ABTS^{\cdot+}$, a blue-green chromophore with attribute absorption at 734 nm. The addition of antioxidants to the performed radical cation reduces it to ABTS, determining a decolorization. In this technique, an antioxidant is included in a pre-formed ABTS radical solution, and after a set period, the staying $ABTS^{\cdot+}$ is quantified spectrophotometrically at 734 nm, according to ABTS method as previously described [39] with some modifications [37]. In brief, 20 L of sample was combined with 180 μL of ABTS radical solution followed by 10 min of incubation under dark conditions and the absorbance was measured at 734 nm whereas ascorbic acid was used as a reference drug. The UV absorption data represented the radical scavenging rates which give the corresponding IC_{50s} for the test compounds. All measurements were created in triplicate and for each analysis a fresh $ABTS^{\cdot+}$ stock solution was prepared.

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

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

FRAP assay

The FRAP assay of the compounds performed using modified method as described by Benzie and Strain [40] with some modifications [41]. FRAP analysis conducted the in vitro antioxidant action for all the newly synthesized compounds by the decrease of a colorless Fe^{3+} tripyridyl triazine complex into a blue-colored Fe^{2+}-tripyridyltriazine complex. The antioxidant potentials of the compounds were estimated as their power to reduce the TPTZ–Fe(III) complex to TPTZ–Fe(II) complex, which is simple, fast, and reproducible. The stock solutions contained 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM hydrochloric acid and 20 mM ferric chloride hexahydrate solution. The fresh working solution was prepared via mixing acetate buffer (25 mL), TPTZ (2.5 mL), and ferric chloride hexahydrate solution (2.5 mL). The temperature of the solution was raised to 37 °C before use and allowed to react with the FRAP solution (300 μL) in a volume ratio of 10:1:1, respectively. The 1 ml of methanol as blank, test samples dissolved in methanol and ascorbic acid as standard dissolved in water. The reaction was permitted to run for 30 min. The colored product (ferrous tripyridyl triazine complex) was monitored at a wavelength of 593 nm. The experiments were performed in triplicate, and their mean was calculated for each compound.

In vitro anticancer bioassay

Cell cultures

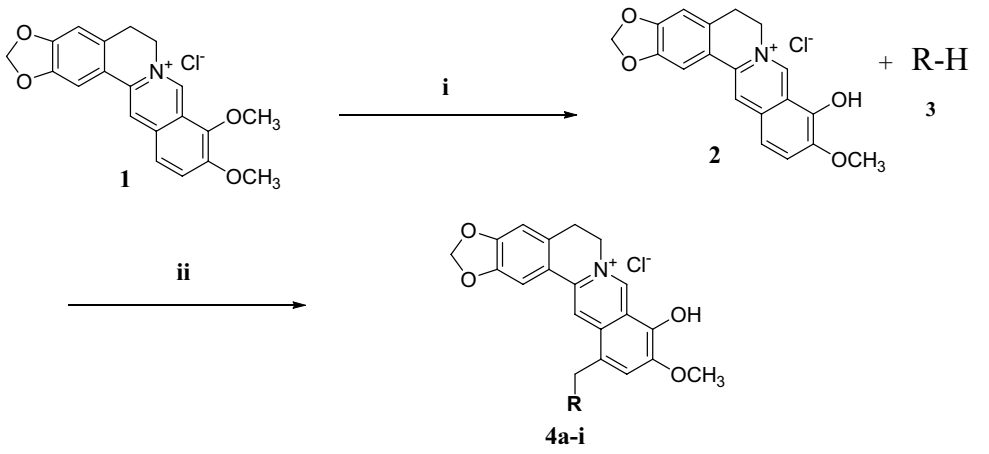
Human cervical cancer cell line (HeLa) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1 % of antibiotic–antimycotic solution (100×). CaSki and MDCK cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10 % FBS, and 1 % of antibiotic–antimycotic solution (100×). Both of the cells were cultured at 37 °C in a 5 % CO_2incubator.

The effects of Mannich base berberine derivatives on the different cell lines were determined by the SRB assay as described previously [37, 42]. Briefly, 5×10^3cells were incubated in triplicate on a 96-well plate under normal culture conditions overnight. HeLa, CaSki and MDCK were treated with different concentrations of Mannich base berberine derivatives (0.1, 1, 10 and 100 μL). Untreated cells served as control. After 48 h, cells were fixed with ice-cold 70 % (w/v) acetone (100 μL/well) for 1 h at 4 C. After incubation, the solvent was removed and plates were dried in an oven at 60 °C. Cells were then stained with SRB (0.4 % w/v in 1 % acetic acid, 100 μL/well) 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 carried out to determine the morphology of the cells. Bound dye was solubilized with 10 mM Tris base (100 μL/well) and absorbance was read at 540 nm to calculate the inhibition concentration of 50 % (IC_{50}), cytotoxic concentration of 50 % (CC_{50}) and therapeutic index (TI).

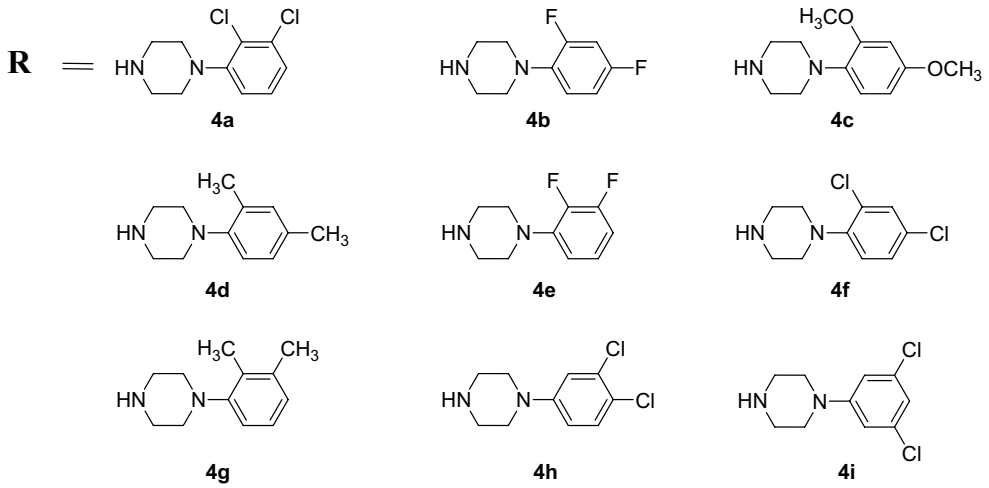
Results and discussion

Chemistry

The synthesis route of Mannich berberine derivatives was conveniently undertaken as outlined in Scheme 1. To begin



Reagents & conditions: i. 190°C, 20-30 mm Hg 40 min: **ii.** C_2H_5OH, HCHO, 80°C, 24 h



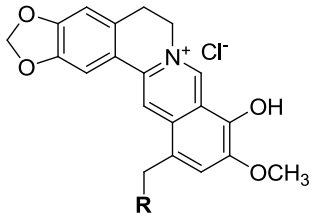
Scheme 1 Synthesis of piperazine linked berberine derivatives

with, berberrubine **2** was obtained in 85 % of yield through selective demethylation of berberine hydrochloride **1** at 190 °C temperature and 20–30 mmHg pressure [43] and then the appropriate piperazine derivatives with formaldehyde were added, stirred at 80 °C or room temperature to obtain the aminomethylation berberrubine derivatives **4a–i** [44].

The adequate production of titled analogues (**4a–i**) was confirmed adapting spectroscopic techniques (IR, ^{1}H NMR, and Mass spectrometry) and analyzing elemental quantification (CHN).

C–H Stretching and C=C of aromatics delivered their characteristic absorption bands at around 3043–3017 cm^{-1} and 1622–1546 cm^{-1}, respectively in FT-IR spectra of **4a–i**. The Ar–N bands were observed as a sharp peak nearby 1250–1270 cm^{-1} in Mannich bases along with sharp chlorine signal at 795–742 cm^{-1} while the broad band found at 3610–3650 cm^{-1} confirms the presence of –OH group. In the ^{1}HNMR spectra of Mannich bases **4a–i** show singlet peak at around 3.92–4.15 ppm (s, 2H), for the CH_2 group, which confirms the formation of Mannich bases. The ^{1}HNMR spectrum of berberine ring displayed singlet peaks at 9.31, 7.78, 7.51, 7.15, and 6.05 ppm for H-8, H-13, H-1, H-4, and H-11 protons, respectively, as well as singlet peak observe at 6.13 ppm presence of $–OCH_2O–$ of berberine ring. H-5 and H-6 proton atoms of berberine ring exerted a triplet peak at 3.15 and 5.95 ppm. The presence of methoxy group was confirmed while observing its characteristic peak as a singlet at around 3.92–4.17 ppm. The protons atom (H-18, H-20, H-15 and H-17) present on the piperazine moiety were resonated as a broad singlet at around 3.80 ppm as well as 3.49 ppm. Moreover, aromatic rings linked to the piperazine ring appeared to have a corresponding signal as multiples in the range 6.93–6.75 ppm. Mass spectrometric data were inaccurate as observed from the M^+ ion values for the final compounds **4a–i**. All of the novel compounds gave C, H and N analyses within 0.4 % points from the theoretical values, i.e. in an acceptable range.

Table 1 Screening results of DPPH, ABTS and FRAP radical scavenging activity of berberine derivatives **(4a–i)**



Compounds	[a]IC_{50} µM ± SD		FRAP
	DPPH	ABTS	Mean ± SD
4a	19.80 ± 0.94	11.64 ± 0.40	0.822 ± 0.07
4b	42.04 ± 2.56	9.052 ± 0.07	0.122 ± 0.01
4c	27.04 ± 0.61	18.84 ± 0.05	0.566 ± 0.03
4d	22.63 ± 1.63	11.52 ± 0.05	0.003 ± 0.02
4e	31.49 ± 1.74	15.24 ± 0.32	0.201 ± 0.06
4f	37.54 ± 2.34	12.24 ± 0.75	1.987 ± 0.15
4g	34.54 ± 1.26	16.52 ± 0.08	0.075 ± 0.21
4h	20.75 ± 0.56	7.774 ± 0.53	1.257 ± 0.31
4i	20.90 ± 0.37	8.189 ± 0.06	2.078 ± 0.27
Berberine	89.18 ± 1.60	230.1 ± 2.11	0.001 ± 0.66
Ascorbic acid	61.03 ± 0.66	27.02 ± 0.05	2.140 ± 0.78

[a] Antioxidant 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

DPPH 2,2-diphenyl-1-picrylhydrazyl, *ABTS* 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid, *FRAP* ferric reducing ability of plasma

Evaluation of biological activities

Antioxidant activities

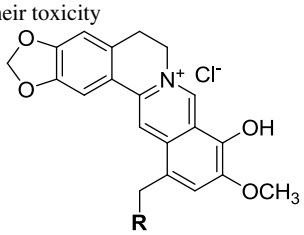
Examination of antioxidant potential of final berberine–piperazine adducts **4a–i** was carried out adapting three different antioxidant assays, DPPH, ABTS and FRAP shown in Table 1. From the results of these bioassays, it is worth mentioning that variation in functional groups presents on piperazine entity leads to the difference in antioxidant potential of the resultant molecules. Overall, the results suggested that the final compounds demonstrated significant antioxidant power as free-radical scavengers with IC_{50s} ranging 19.80 ± 0.94–42.04 ± 2.56 and 7.774 ± 0.53–18.84 ± 0.05 µM in DPPH and ABTS bioassay, respectively, when compared to that of berberine itself. Analogue **4a** with two electron withdrawing chlorine atoms on the piperazine entity attached to the berberine ring presented most active radical scavenging potential in DPPH assay with 19.80 ± 0.94 µM of IC_{50} level, comparable to that of control drug ascorbic acid at 61.03 ± 0.66 µM and far better than berberine at 89.18 ± 1.60 µM. Another two chlorine-based compounds with 3,4-dichlorophenyl piperazine (**4h**) and 3,5-dichlorophenyl piperazine (**4i**) moieties appeared with appreciable antioxidant effects in DPPH bioassay with IC_{50s}20.75 ± 0.56 and 20.90 ± 0.37 µM, respectively. Most importantly, these two derivatives established the most powerful antioxidant action in ABTS bioassay with 7.774 ± 0.53 and 8.189 ± 0.06 µM of IC_{50s}, respectively. In addition, a compound (**4d**) with electron releasing dimethyl functionality showed 22.63 ± 1.63 µM of IC_{50} in DPPH assay, whereas a compound (**4b**) with highly electronegative fluorine atoms exerted 9.052 ± 0.07 µM of IC_{50} in ABTS bioassay, hence these two compounds can be considered to have appreciable level of antioxidant power in the respective assay. The rest of the compounds showed better level of radical scavenging power when compared to the control ascorbic acid. Nevertheless, all compounds have better antioxidant efficacies than parent berberine as assayed by DPPH and ABTS tests.

Titled compounds **4a–i** were also evaluated for their antioxidant effects in FRAP assay. Fe^{3+} ion, biologically inactive form of iron, can be converted to Fe^{2+} ion via reduction depending on pH conditions and can be further oxidized through Fenton-type reaction producing hydroxyl radical via superoxide anions production. An antioxidant compound has reductive capabilities and is evaluated by Fe^{3+} to Fe^{2+} transformation upon releasing an electron in the presence of test compounds. So, the concentration of Fe^{2+} can be monitored by measuring the formation of Fe^{2+}-tripyridyltriazine complex with blue color. The higher the absorbance of the compounds, greater the reducing power and a compound **4i** with 3,5-dichlorophenyl piperazine entity reduced metal ion complexes to their lower oxidation state or takes part in electron transfer reaction very effectively with a absorption value of 2.078 nm as compared to that of the control ascorbic acid with absorption value of 2.140 nm at a concentration 10 µg/mL. This compound has significantly increased antioxidant potential in FRAP assay when compared to berberine with the absorption value of 0.001 nm. A compound **4f** with 2,4-dichlorophenyl piperazine moiety attached to the berberine ring showed tremendous ability of electron donor to scavenge free radicals in FRAP assay with the absorption value of 1.987 nm nearer to that of control ascorbic acid at a concentration 10 µg/mL. The rest of the compounds showed lower absorbance as compared to the standard but were of high efficacies when compared to berberine.

Anticancer activities

Results of sulforhodamine B (SRB) bioassay adapted to screen the cervical cancer cell line (HeLa and CaSki) potential of title compounds **4a–i** are furnished in Tables 2 and 3. Results of anticancer examination were worth stating

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

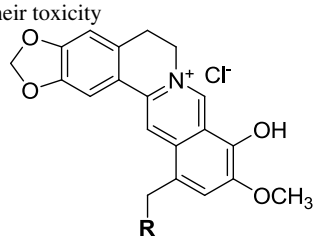


Compounds	[a]IC_{50} μM ± SD HeLa	CC_{50} μM ± SD MDCK	TI
4a	7.753 ± 0.06	312.6 ± 0.59	40.31
4b	11.12 ± 0.07	227.2 ± 1.07	20.43
4c	7.340 ± 0.04	248.9 ± 2.03	33.91
4d	8.257 ± 0.03	214.6 ± 0.46	25.99
4e	8.577 ± 0.05	238.7 ± 1.08	27.83
4f	7.896 ± 0.17	212.1 ± 0.75	26.86
4g	9.357 ± 0.32	279.0 ± 1.34	29.81
4h	7.526 ± 0.18	320.1 ± 0.08	42.53
4i	7.327 ± 0.08	310.6 ± 1.42	42.39
Berberine	13.42 ± 0.23	300.4 ± 0.47	22.38
Doxorubicin	0.486 ± 0.04	250.2 ± 0.57	514.8

[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

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

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



Compounds	[a]IC_{50} μM ± SD CaSki	CC_{50} μM ± SD MDCK	TI
4a	7.798 ± 0.25	312.6 ± 0.59	40.08
4b	10.70 ± 0.16	227.2 ± 1.07	21.23
4c	5.755 ± 0.17	248.9 ± 2.03	43.24
4d	7.856 ± 0.24	214.6 ± 0.46	27.31
4e	10.58 ± 0.56	238.7 ± 1.08	22.56
4f	8.932 ± 0.47	212.1 ± 0.75	23.74
4g	10.43 ± 1.05	279.0 ± 1.34	26.74
4h	8.090 ± 0.37	320.1 ± 0.08	39.56
4i	7.606 ± 0.08	310.6 ± 1.42	40.83
Berberine	15.17 ± 0.19	300.4 ± 0.47	19.80
Doxorubicin	1.076 ± 0.04	250.2 ± 0.57	232.5

[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

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

as overall all compounds exhibited a good level of cancerous cell inhibitory potential with IC_{50s} ranging 7.327–11.12 and 5.755–10.70 μM against HeLa and CaSki cell lines, respectively, when compared to that of berberine itself with IC_{50s} of 13.42 μM (HeLa) and 15.17 μM (CaSki). Moreover, similar to the antioxidant efficacies, it can be said that variation in the functionalities of groups attached to the piperazine ring led to the change in the anticancer efficacies of the resultant molecules. Secondly, final compounds **4a–i** exerted tolerable cytotoxic nature with cytotoxicity values ranging 212.1–320.1 μM against MDCK cell lines thereby exhibiting potent therapeutic index (TI) in the range 20.43–42.53 for HeLa and 21.23–43.24 for CaSki cell lines. From the TIs, it can be stated that the title compounds demonstrated better anticancer efficacies against HeLa cell line than CaSki cell lines of cervical cancer. Doxorubicin was used as a control [45, 46].

Two analogues bearing 3,4-dichlorophenyl piperazine (**4h**) and 3,5-dichlorophenyl piperazine (**4i**) moiety appeared with most potent anticancer action against HeLa cell lines with 7.526 ± 0.18 and 7.327 ± 0.08 μM of IC_{50s} and 42.53 and 42.39 of TIs, respectively. They had lower cytotoxic values of 320.1 ± 0.08 and 310.6 ± 1.42 μM, respectively. Moreover, an analogue with 2,3-dichlorophenyl piperazine entity showed a good level of HeLa cancer cell inhibitory effects with 7.753 ± 0.06 μM of IC_{50}, 312.6 ± 0.59 μM of cytotoxicity and 40.31 of the therapeutic index. All the remaining analogues except **4b** displayed TI level (25.99–33.91 μM) greater than berberine with 24.83. Finally, an analogue with electron donating alkoxy functional groups, **4c**, bearing 2,4-dimethoxyophenyl piperazine entity furnished a significant level of CaSki cell line inhibitory efficacy with 5.755 ± 0.17 μM of IC_{50} and 248.9 ± 2.03 μM of CC_{50} thereby releasing high therapeutic index of 43.24. Furthermore, compounds **4h** and **4i** had an appreciable level of CaSki cell line inhibitory potential with 8.090 ± 0.37 and 7.606 ± 0.08 μM of IC_{50s}, respectively again. These compounds have shown 320.1 ± 0.08 and 310.6 ± 1.42 μM of cytotoxicity levels, thereby introducing better TIs as 39.56 and 40.83, which were considered better than parent compound berberine with TI level of 19.80 against CaSki cell lines. A compound (**4a**) was also found to fall in a considerable range of anticancer effects against CaSki cell lines with 40.08 of TI. All remaining analogues showed TIs in the range 21.23–27.31 that was good when compared to that of berberine. Concerning the activity concerning the functional group attached to the piperazine ring, the order falls in the way chloro > methoxy > fluoro > methyl.

Conclusion

To sum up, Mannich bases introducing piperazine moieties enriched with different electron withdrawing and electron releasing functional groups were furnished on the berberine core to evaluate the antioxidant and anticancer potentials. Efficient synthesis of final analogues was confirmed using different spectroscopy methods and elemental analysis as well as physical tests. Compounds **4h** and **4i** with 3,4-dichloro and 3,5-dichloro functional groups performed well in all the bioassay tested and can be considered as a most potent antioxidant and anticancer agents for cervical cancer cell lines. All compounds exercised low cytotoxic values against normal cell lines. Final compounds exhibited better pharmacological action tested in this study than the parent berberine and this modification is likely to enhance their ability to bind to the target of drug action mainly through hydrophobic effect, conjugation effect and hydrogen bond on 9-hydroxyl. The presence of dichloro function was found beneficial for the antioxidant potencies as well as against HeLa cell lines, whereas dialkoxy substitution was crucial to enhancing the anticancer potential against CaSki cell lines. Thus, the rationalized molecular designs presented in this study would be of considerable interest for ongoing drug developing progress.

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