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ORIGINAL ARTICLE

Synthesis of *N*-Mannich bases of berberine linking piperazine moieties revealing anticancer and antioxidant effects

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Abstract A new Mannich base series of piperazine linked berberine analogues was furnished in this study to screen the antioxidant and anticancer potential of the resultant analogues. Alkoxy group at a C-9 position of berberine was converted to hydroxyl functionality to enhance the ability of final scaffolds binding to the target of drug action mainly through hydrophobic effect, conjugation effect, whereas Mannich base functionality was introduced on the C-12 position of berberine. Scaffolds were investigated for their free radical scavenging antioxidant potential in FRAP and DPPH assay, whereas tested to check their Fe^{+3} reducing power in ABTS assay. The radical scavenging potential of the final derivatives **4a**–**j** was found excellent with IC_{50}s, < 13 μg/mL and < 8 μg/mL in DPPH and ABTS assay, respectively, whereas some analogues showed significant Fe^{+3} reducing power with absorption at around 2 nm in the FRAP assay. Anticancer effects of titled compounds were inspected against cervical cancer cell line Hela and Caski adapting SRB assay, in which analogues **4a**–**j** presented < 6 μg/mL of IC_{50}s, and > 30 of therapeutic indices, thus exerting low cytotoxic values against Malin–Darby canine kidney (MDCK) cell lines at CC_{50}s > 125 μg/mL. Hence, from the bioassay outcomes it can be stated that these analogues are dual active agents as the scavengers of reactive oxygen species and inhibitors of the cancerous cells as compounds with halogen functional group have overall good pharmacological potential in assays studied in this research. Correct structure of the final compounds was adequately confirmed on the basis of FT-IR and ^{1}H NMR as well as elemental analyses.

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1. Introduction

Reactive oxygen species (ROS) carrying an unpaired electron in an atomic orbital are endogenous stimuli capable to interact with DNA which is the primary reason for the occurrence of cancer (Valko et al., 2004). One of the reasons for the

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generation of ROS responsible for extreme uncertainty and high reactivity is some kind of pathological states and physiochemical circumstances. These species can accept or donate an electron with adjunct molecules and can act as oxidants or reductants, important in the pathogenesis of many different diseases (Droge, 2002; Valko et al., 2006, 2007; Cheeseman and Slater, 1993). Interaction of ROS with DNA results into the damage of cell and homeostatic disruption which may outcomes into deleterious events such as cardiovascular disorders, diabetes, cancer, cirrhosis etc. (El-Gazzar et al., 2009; Kashif et al., 2008). "Oxidative stress" which is an imbalance toward the pro-oxidative state is the responsible factor to damage very important biological components like essential proteins, lipids and nucleic acids (Lobo et al., 2010) thereby furnishing excess quantities of reactive oxygen species. The process of cancer and its treatments create an discrepancy between anti-oxidant protection and toxic production.

Antioxidants can reduce oxidative stress caused carcinogenesis by an immediate scavenging of ROS and have an important role in the enhancement of these infected conditions. They are stable components and able to donate an electron to unstable ROS, and thus ROS can be neutralized and can no longer affect essential biological species (Mc Cord, 2000). The chain reactions often constructed by ROS can be broken by antioxidant molecules by the donation of an electron which stops catalysts of ROS which initiated chain reactions and as a result ROS can be quickly destroyed. By such means, antioxidant molecules works as an enzyme inhibitor, a radical scavenger, singlet oxygen quencher, hydrogen and electron donor, peroxide decomposer, synergist, and metal-chelating agents (Rice-Evans and Diplock, 1993; Krinsky, 1992). The aforementioned facts imply that scaffolds which hold dual antioxidant and anticancer activities are of tremendous significance in the current medication finding improvement. In fact, discovery of anticancer molecules from natural product leads is a major theme of the current decade worldwide with a reason that cancer is responsible for numbers of deaths in most developed, developing and underdeveloped countries.

Cancer is responsible for numbers of death each year worldwide, more specifically with the highest death rate in Asia and then Europe. As per the World Health Organization, death rate were 6.2 million in 1997 and 7.6 million in 2008, which means 13% of all fatalities were due to melanoma and that the international melanoma rate may improve by 50% to 15 million new cases by 2030. Among women, cervical cancer is the most typical which is responsible for an approximated 527,624 new cases and 265,653 fatalities in 2012. Cervical melanoma is the 2nd most typical females' melanoma in females older 15 to 44 years in Globe (Bray et al., 2013).

Despite the improvement in the area of melanoma research, both developing and developed countries are in the hold of this dangerous illness and still there is a need to discover and create therapeutic agents potentially effective against several types of cancer. It has identified that natural products signify the wealthiest source of high substance variety, offering the foundation for recognition of novel scaffolding components that provides starting points for rational drug design (Koehn and Carter, 2005). Natural products are small-molecule secondary metabolites that contribute to organism survival. This can be one of the factors that initiatives have been instructed to find appealing cancer therapeutic agents from natural resources. According to the latest evaluation, ~49% of cancer medication was either through natural products or their derivatives that are used as chemotherapeutic drugs (Newman and Cragg, 2012). The latest review states that there are 12 approved natural product anticancer agents (Basmadjian et al., 2014). Berberine, an isoquinoline plant alkaloid is acquired from different plant species including *Hydrastis canadensis* L., (Ranuncufaceae), *Berberineeris* species (Berberidaceae) and *Arcungelisia flaw* (Menispermaceae). Reports show that berberine has been observed to be efficient towards osteosarcoma, lung, liver, prostate and breast cancer (Wang et al., 2011; Patil et al., 2010) by using transcriptional control of some oncogene and carcinogenesis-related gene appearance and contacts with both DNA and RNA. It regulates ROS production and activates nuclear factor-B that make it responsible for the inhibitor of cancer cell growth because it affects N-acetyltransferase, cyclooxygenase-2, and topoisomerase activities and gene/protein expression. Berberin has efficacies as the most potent anticancer natural product alkaloid as it inhibits tumourigenic microorganisms, regulates oncogene and carcinogenesis-related gene expression, inhibits telomerase, inhibits cyclooxygenase-2, suppresses tumour cell proliferation, impacts cytochrome c discharge and caspase initial as well as enhances the possibilities of fighting against multidrug resistance problems (Sun et al., 2009). Naturally occurring berberine as well as their synthetic analogues have been shown to demonstrate interesting and different activities including antimicrobial, antileukemic, anti ulcerous, and enzyme-inhibiting, anti-inflammatory, anti-diarrhoea, glucose-lowering, cholesterol-lowering, neuroprotective, antidepressant, Alzheimers disease-ameliorating (Verpoorte, 1998; Bodiwala et al., 2011; Kuo et al., 2004; Yin et al., 2002; Leng et al., 2004; Peng et al., 2007; Cui et al., 2009; Kulkarni and Dhir, 2007; Asai et al., 2007) etc. It exhibits activity on LDLR (Yang et al., 2008) and is found to reveal cytotoxicity against HeLa, SVKO3, Hep-2 cancer cell lines (Orfila et al., 2000) and anti-leishmaniasis activity (Vennerstrom et al., 1990). Thus, in a view of the above-mentioned therapeutic role of berberine, their scaffolds have attracted significant interest in recent years.

Recent reports suggest that this piperazine derivative effectively prevents melanoma cell growth and causes caspase-dependent apoptosis via suppressing multiple signalling pathways implicated in cancer (Edward and Zhonglin, 2013). Moreover, alkaloids bearing piperazine residue are previously studied to deliver significant anticancer effects (Kohmoto et al., 1988). Some recent reports suggested that substituting different electron withdrawing and electron donating functional group bearing piperazine moieties to the heterocyclic or aromatic core results in analogues demonstrating appreciable anticancer efficacies (Patel and Park, 2013; Patel et al., 2011). According to the extensive variety of scientific actions associated with berberine and the piperazine, the mixture of these two moieties in the same compound is an exciting task for the growth of new pharmacologically effective agents. Derivatization has been conducted via developing *N*-Mannich bases as the scientific results of this scaffold is well known, particularly as antioxidant (Malhotra et al., 2012; Ma et al., 2013; Dong et al., 2012) and anticancer agents (Bala et al., 2014; Venkateshwarlu et al., 2014). *N*-Mannich bases equipped with piperazine linkage was recently attempted and adapted

in the present study for the effective synthesis of berberine-piperazine systems (Patel et al., 2012).

2. Experimental section

2.1. 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. Shimadzu 8400-S FT-IR spectrophotometer (KBr pellets) and Varian 500 MHz model spectrometer (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 n 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 done using a Heraeus Carlo Erba 1180 CHN analyzer.

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

Berberine hydrochloride (10 g, 0.01 mol) was included to a 50 mL round bottom flask. 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. The vacuum pump was turned off after the temperature 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 a brownish red amorphous powder compound **2** (6.6 g, 85%).

2.2.1. General procedure for preparation of compounds (**4a–j**)

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 formaldehyde aqueous solution (37%, 0.05 mol) were added, stirred at room temperature or at 80 °C for 24 h. It was then 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 obtain the final compound.

*2.2.1.1. 12-(1-(4-Methylphenyl)piperazine-1-ylmethyl)-berberrubine (**4a**).* Light yellow solid, Yield: 65%. m.p. 264–266 °C. IR (KBr) cm^{-1}: 3623 (OH), 3042 (C–H, Ar), 1630–1533 (C═C, Ar), 1242 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.79 (s, 1H, –OH of berberine ring), 9.42 (s, 1H, H-8), 7.89 (s, 1H, H-13), 7.44 (s, 1H, H-1), 7.05 (s, 1H, H-4), 6.90–6.71 (m, 4H, Ar–H, piperazine H-22, H-23, H-25, H-26), 6.22 (s, 2H, –OCH_2O), 6.03 (s, 1H, H-11), 5.78 (t, J = 5.5 Hz, 2H, H-6), 4.11 (s, 2H, CH_2, H-14 Mannich base), 3.97 (s, 3H, OCH3), 3.84 (br s, 4H, piperazine, H-18, H-20), 3.22 (br s, 4H, piperazine, H-15, H-17), 3.05 (t, J = 5.4 Hz, 2H, H-5), 1.95 (s, 3H, Ar–CH_3 of piperazine). Anal. Calcd. for $C_{31}H_{32}ClN_3O_4$: C, 68.19; H, 5.91; N, 7.70. Found: C, 68.25; H, 5.98; N, 7.81.

*2.2.1.2. 12-(1-(2-Chlorophenyl)piperazine-1-ylmethyl)-berberrubine (**4b**).* Light yellow solid, Yield: 70%. m.p. 271–272 °C. IR (KBr) cm^{-1}: 3642 (OH), 3049 (C–H, Ar), 1628–1545 (C═C, Ar), 1239 (Ar–N), 778 (C–Cl). ^{1}H NMR (500 MHz, Chloroform) δ 9.85 (s, 1H, –OH of berberine ring), 9.44 (s, 1H, H-8), 7.92 (s, 1H, H-13), 7.31 (s, 1H, H-1), 7.11 (s, 1H, H-4), 6.86–6.66 (m, 4H, Ar–H, piperazine H-22, H-23, H-24, H-25), 6.29 (s, 2H, –OCH_2O), 6.06 (s, 1H, H-11), 5.86 (t, J = 5.4 Hz, 2H, H-6), 4.09 (s, 2H, CH_2, H-14 Mannich base), 3.87 (s, 3H, OCH_3), 3.75 (br s, 4H, piperazine, H-18, H-20), 3.31 (br s, 4H, piperazine, H-15, H-17), 3.17 (t, J = 5.3 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}Cl_2N_3O_4$: C, 63.61; H, 5.16; N, 7.42. Found: C, 63.42; H, 5.24; N, 7.27.

*2.2.1.3. 12-(1-(3-Chlorophenyl)piperazine-1-ylmethyl)-berberrubine (**4c**).* Light yellow solid, Yield: 63%. m.p. 253–255 °C. IR (KBr) cm^{-1}: 3637 (OH), 3051 (C–H, Ar), 1631–1551 (C═C, Ar), 1244 (Ar–N), 757 (C–Cl). ^{1}H NMR (500 MHz, Chloroform) δ 9.92 (s, 1H, –OH of berberine ring), 9.49 (s, 1H, H-8), 7.86 (s, 1H, H-13), 7.35 (s, 1H, H-1), 7.10 (s, 1H, H-4), 6.77–6.54 (m, 4H, Ar–H, piperazine H-22, H-23, H-24, H-26), 6.31 (s, 2H, –OCH_2O), 6.04 (s, 1H, H-11), 5.80 (t, J = 5.5 Hz, 2H, H-6), 4.02 (s, 2H, CH_2, H-14 Mannich base), 3.85 (s, 3H, OCH_3), 3.71 (br s, 4H, piperazine, H-18, H-20), 3.25 (br s, 4H, piperazine, H-15, H-17), 3.07 (t, J = 5.5 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}Cl_2N_3O_4$: C, 63.61; H, 5.16; N, 7.42. Found: C, 63.78; H, 5.04; N, 7.68.

*2.2.1.4. 12-(1-(4-Chlorophenyl)piperazine-1-ylmethyl)-berberrubine (**4d**).* Light yellow solid, Yield: 72%. m.p. 259–261 °C. IR (KBr) cm^{-1}: 3648 (OH), 3043 (C–H, Ar), 1625–1543 (C═C, Ar), 1236 (Ar-N), 784 (C–Cl). ^{1}H NMR (500 MHz, Chloroform) δ 9.80 (s, 1H, –OH of berberine ring), 9.51 (s, 1H, H-8), 7.88 (s, 1H, H-13), 7.39 (s, 1H, H-1), 7.06 (s, 1H, H-4), 6.81–6.59 (m, 4H, Ar-H, piperazine H-22, H-23, H-25, H-26), 6.23 (s, 2H, –OCH_2O), 6.15 (s, 1H, H-11), 5.82 (t, J = 5.3 Hz, 2H, H-6), 3.96 (s, 2H, CH_2, H-14 Mannich base), 3.89 (s, 3H, OCH_3), 3.77 (br s, 4H, piperazine, H-18, H-20), 3.32 (br s, 4H, piperazine, H-15, H-17), 3.11 (t, J = 5.5 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}Cl_2N_3O_4$: C, 63.61; H, 5.16; N, 7.42. Found: C, 63.50; H, 5.17; N, 7.55.

*2.2.1.5. 12-(1-(2-Fluorophenyl)piperazine-1-ylmethyl)-berberrubine (**4e**).* Light yellow solid, Yield: 66%. m.p. 228–230 °C. IR (KBr) cm^{-1}: 3651 (OH), 3054 (C–H, Ar), 1627–1540 (C═C, Ar), 1249 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.88 (s, 1H, –OH of berberine ring), 9.45 (s, 1H, H-8), 7.80 (s, 1H, H-13), 7.37 (s, 1H, H-1), 7.09 (s, 1H, H-4), 6.87–6.61 (m, 4H, Ar–H, piperazine H-22, H-23, H-24, H-25), 6.28 (s, 2H, –OCH_2O), 6.17 (s, 1H, H-11), 5.77 (t, J = 5.4 Hz, 2H, H-6), 4.07 (s, 2H, CH_2, H-14 Mannich base), 3.95 (s, 3H, OCH_3), 3.83 (br s, 4H, piperazine, H-18, H-20), 3.37 (br s, 4H, piperazine, H-15, H-17), 3.20 (t, J = 5.3 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}ClFN_3O_4$: C, 65.51; H, 5.31; N, 7.64. Found: C, 65.34; H, 5.19; N, 7.53.

*2.2.1.6. 12-(1-(4-Fluorophenyl)piperazine-1-ylmethyl)-berberrubine (**4f**).* Light yellow solid, Yield: 69%. m.p. 239–241 °C. IR (KBr) cm^{-1}: 3644 (OH), 3045 (C–H, Ar), 1632–1539 (C═C, Ar), 1251 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.93 (s, 1H, –OH of berberine ring), 9.47 (s, 1H, H-8), 7.84 (s, 1H, H-13), 7.40 (s, 1H, H-1), 7.12 (s, 1H, H-4), 6.80–6.57

(m, 4H, Ar-H, piperazine H-22, H-23, H-25, H-26), 6.32 (s, 2H, –OCH_2O), 6.07 (s, 1H, H-11), 5.81 (t, J = 5.4 Hz, 2H, H-6), 3.95 (s, 2H, CH_2, H-14 Mannich base), 3.86 (s, 3H, OCH_3), 3.80 (br s, 4H, piperazine, H-18, H-20), 3.33 (br s, 4H, piperazine, H-15, H-17), 3.08 (t, J = 5.4 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}ClFN_3O_4$: C, 65.51; H, 5.31; N, 7.64. Found: C, 69.39; H, 5.42; N, 7.73.

*2.2.1.7. 12-(1-(4-Trifluoromethoxyphenyl)piperazine-1-ylmethyl)-berberrubine (**4g**).* Light yellow solid, Yield: 58%. m.p. 275–277 °C. IR (KBr) cm^{-1}: 3652 (OH), 3044 (C–H, Ar), 1626–1535 (C═C, Ar), 1238 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.95 (s, 1H, –OH of berberine ring), 9.43 (s, 1H, H-8), 7.90 (s, 1H, H-13), 7.42 (s, 1H, H-1), 7.07 (s, 1H, H-4), 6.92–6.68 (m, 4H, Ar-H, piperazine H-22, H-23, H-25, H-26), 6.26 (s, 2H, -OCH_2O), 6.12 (s, 1H, H-11), 5.92 (t, J = 5.5 Hz, 2H, H-6), 4.10 (s, 2H, CH_2, H-14 Mannich base), 3.96 (s, 3H, OCH_3), 3.76 (br s, 4H, piperazine, H-18, H-20), 3.29 (br s, 4H, piperazine, H-15, H-17), 3.06 (t, J = 5.4 Hz, 2H, H-5). Anal. Calcd. for $C_{31}H_{29}ClF_3N_3O_5$: C, 60.44; H, 4.74; N, 6.82. Found: C, 60.57; H, 4.86; N, 6.72.

*2.2.1.8. 12-(1-(4-Trifluoromethylphenyl)piperazine-1-ylmethyl)-berberrubine (**4h**).* Light yellow solid, Yield: 55%. m.p. 224–226 °C. IR (KBr) cm^{-1}: 3645 (OH), 3052 (C-H, Ar), 1629–1541 (C═C, Ar), 1240 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.83 (s, 1H, –OH of berberine ring), 9.46 (s, 1H, H-8), 7.78 (s, 1H, H-13), 7.33 (s, 1H, H-1), 7.13 (s, 1H, H-4), 6.91–6.60 (m, 4H, Ar–H, piperazine H-22, H-23, H-25, H-26), 6.30 (s, 2H, –OCH_2O), 6.14 (s, 1H, H-11), 5.77 (t, J = 5.5 Hz, 2H, H-6), 4.12 (s, 2H, CH_2, H-14 Mannich base), 3.90 (s, 3H, OCH_3), 3.78 (br s, 4H, piperazine, H-18, H-20), 3.26 (br s, 4H, piperazine, H-15, H-17), 3.09 (t, J = 5.3 Hz, 2H, H-5). Anal. Calcd. for $C_{31}H_{29}ClF_3N_3O_4$: C, 62.05; H, 4.87; N, 7.00. Found: C, 62.16; H, 4.96; N, 7.12.

*2.2.1.9. 12-(1-(2-Nitrophenyl)piperazine-1-ylmethyl)-berberrubine (**4i**).* Light yellow solid, Yield: 68%. m.p. 261–263 °C. IR (KBr) cm^{-1}: 3653 (OH), 3053 (C–H, Ar), 1619–1537 (C═C, Ar), 1563 (N–O stretch), 1332 (N–O stretch), 1237 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.90 (s, 1H, –OH of berberine ring), 9.53 (s, 1H, H-8), 7.74 (s, 1H, H-13), 7.47 (s, 1H, H-1), 7.04 (s, 1H, H-4), 6.88–6.58 (m, 4H, Ar–H, piperazine H-22, H-23, H-24, H-25), 6.27 (s, 2H, –OCH_2O), 6.08 (s, 1H, H-11), 5.87 (t, J = 5.3 Hz, 2H, H-6), 4.05 (s, 2H, CH_2, H-14 Mannich base), 3.88 (s, 3H, OCH_3), 3.72 (br s, 4H, piperazine, H-18, H-20), 3.27 (br s, 4H, piperazine, H-15, H-17), 3.13 (t, J = 5.4 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}ClN_4O_6$: C, 62.44; H, 5.07; N, 9.71. Found: C, 62.35; H, 5.18; N, 9.57.

*2.2.1.10. 12-(1-(4-Nitrophenyl)piperazine-1-ylmethyl)-berberrubine (**4j**).* Light yellow solid, Yield: 63%. m.p. 272–274 °C. IR (KBr) cm^{-1}: 3638 (OH), 3039 (C–H, Ar), 1615–1544 (C═C, Ar), 1526 (N–O stretch), 1341 (N–O stretch), 1235 (Ar–N). ^{1}H NMR (500 MHz, Chloroform) δ 9.87 (s, 1H, –OH of berberine ring), 9.50 (s, 1H, H-8), 7.82 (s, 1H, H-13), 7.49 (s, 1H, H-1), 7.14 (s, 1H, H-4), 6.84–6.55 (m, 4H, Ar-H, piperazine H-22, H-23, H-25, H-26), 6.25 (s, 2H, –OCH_2O), 6.13 (s, 1H, H-11), 5.94 (t, J = 5.3 Hz, 2H, H-6), 4.13 (s, 2H, CH_2, H-14 Mannich base), 3.92 (s, 3H, OCH_3), 3.79 (br s, 4H, piperazine, H-18, H-20), 3.24 (br s, 4H, piperazine, H-15, H-17), 3.08 (t, J = 5.5 Hz, 2H, H-5). Anal. Calcd. for $C_{30}H_{29}ClN_4O_6$: C, 62.44; H, 5.07; N, 9.71. Found: C, 62.53; H, 5.21; N, 9.83.

2.3. Antioxidant capacity by DPPH assay

Radical scavenging efficiency of the compounds examined was evaluated in vitro by the DPPH analysis. DPPH is among the few stable and commercially available organic nitrogen radicals. DPPH radicals are considered as an associate method for the preliminary screening of compounds capable of scavenging activated oxygen species, since they are more constant and easier to deal with than oxygen free radicals and DPPH assay continues to be commonly used to evaluate the antioxidant action of different phenolic substances. The DPPH solution contains a deep violet colour. Radical scavenging activity of antioxidant compounds can be calculated spectrophotometrically at 517 nm. The DPPH radical reveals absorbance at 517 nm, that reduces by the bleaching of a violet coloured methanol solution upon reduction by an antioxidant or a radical via hydrogen atom or electron move to the odd electron in DPPH generating the pale yellow non-radical form (DPPH-H). This decrease is proportional to the increase of non-radical forms of DPPH.

The DPPH radical solution was ready by dissolving an appropriate quantity of DPPH in methanol. The DPPH radical solution (180 μL) was added to a solution of the compound to be tested in methanol (20 μL) at various concentrations (0.1, 1, 10 and 100 μg/mL). The mixture was shaken extremely and incubated at room temperature in the dark for 30 min. The loss of the absorbance of the causing solution was then calculated spectrophotometrically at 517 nm. All measurements were developed in triplicate. Two controls were applied for this test: a negative control (blank) containing of methanol (20 μL) and the DPPH radical solution (180 μL) and a positive control including the referrals anti-oxidant (Ascorbic acid) in methanol and DPPH radical solution. The results of this bioassay, RSA% (the radical scavenging activity in percentage) was determined according to Mensor et al. (Mensor et al., 2001; Mistry et al., 2015) as described in the 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.

2.4. Antioxidant capacity by ABTS assay

The second is analysed by the capability of antiradical elements to satisfy the $ABTS^{\cdot+}$, a blue–green chromophore with attribute absorption at 734 nm. The inclusion of anti-oxidants to the conducted radical cation decreases it to ABTS, identifying a decolourization. In this technique, an antioxidant is included in a pre-formed ABTS radical solution, and after a set period, the staying $ABTS^{\bullet+}$ is quantified spectrophotometrically at 734 nm (Molyneux, 2004; Mistry et al., 2015). The $ABTS^{\cdot+}$ was created by responding 7 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), saved in the dark at room temperature for 12–16 h before use (the radical was constant for more than two days under these conditions).

The $ABTS^{\bullet+}$ solution was further diluted with methanol until finally the preliminary absorbance value of 0.70 ± 0.02 at 734 nm at 30 °C.

The 1000 µL stock solutions of titled compounds **4a**–**j** was established upon dissolving them in methanol and further dilutions furnishes 100 µL, 10 µL, 1 µL, and 0.1 µL of quantities of samples. An amount of 20 µL of each compound solution was added to 180 µL of diluted $ABTS^{\cdot+}$ solution were mixed in 96 well plates in a dark place which were then incubated for 10 min to measure UV absorption at 734 nm. A mixture of 180 µL ABTS and 20 µL mL methanol was applied as a control determination while ascorbic acid was applied as a reference drug. The UV absorption data represented the radical scavenging rates that give the corresponding IC_{50s} for the test compounds. All measurements were produced in triplicate and for every single analysis a fresh $ABTS^{\cdot+}$ stock solution was prepared.

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

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

2.5. FRAP assay

The FRAP assay of the compounds was performed using modified method as described by Benzie and Strain (Benzie and Strain, 1999). FRAP assay conducted the in vitro antioxidant action for all the newly synthesized compounds by the decrease of a colourless Fe^{3+}-tripyridyltriazine complex into a blue-coloured 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 solutions contained 300 mM acetate buffer (3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ 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 ready by 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 and the reaction was allowed to run for 30 min. The coloured product (ferrous tripyridyl triazine complex) was monitored at a wavelength of 593 nm. The experiments were conducted in triplicate, and their mean was measured for each compound.

2.6. In vitro anticancer bioassay

2.6.1. Cell cultures

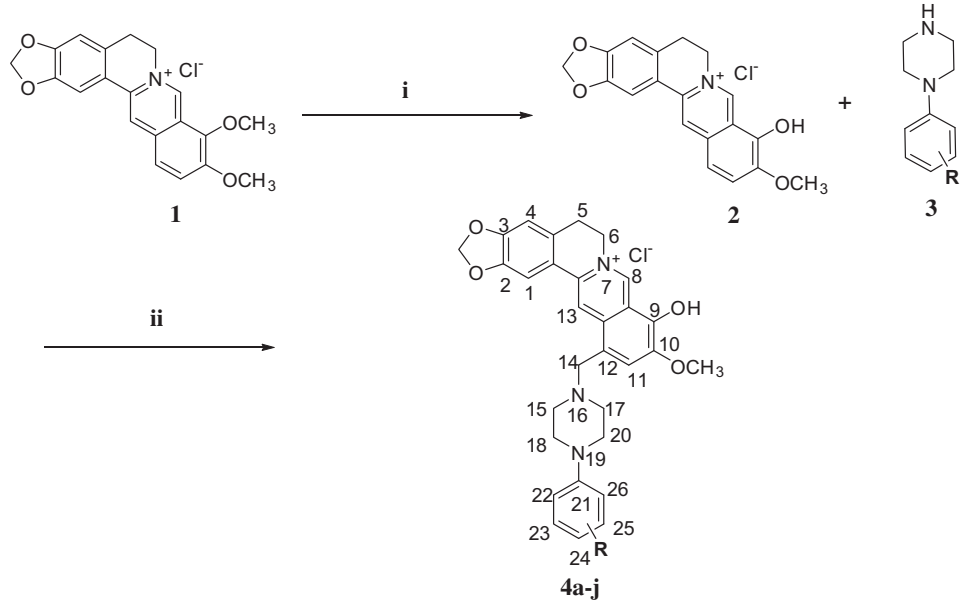
Human cervical cancer cell line (HeLa) was keep up in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% of Antibiotic–Antimycotic solution (100X). CaSki and MDCK cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% FBS, and 1% of Antibiotic–Antimycotic solution (100X). Both of the cells were cultured at 37 °C in a 5% CO_2 incubator.

The effects of Mannich base berberine derivatives on the different cell lines were determined by the SRB assay as described previously (Adaramoye et al., 2011; Mistry et al., 2015). Briefly, 5×10^3 cells 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 µL, 1 µL, 10 µL 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, solvent was removed and plates were dried in an oven at 60 °C temperature. Cells were then stained to the 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 on a plate reader. Spectrophotometric data were recorded at 510 nm to calculate the inhibition concentration of 50% (IC_{50}), cytotoxic concentration of 50% (CC_{50}) and therapeutic index (TI).

3. Results and discussion

3.1. Chemistry

The synthesis route of Mannich berberine derivatives was conveniently undertaken as outlined in Schemes 1. To begin 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 (Iwasa et al., 1996) and then the appropriate piperazine derivatives with formaldehyde were added, stirred at 80 °C or room temperature to obtain the aminomethylation berberrubine derivatives **4a**–**j** (Bekircan and Bektas, 2008). The correct formation of the structure of compounds **4a**–**j** was confirmed by recording the FT-IR, ^{1}H NMR, Mass spectrometry and elemental analysis (CHN). The FT-IR spectra of compound **4a**–**j** show the C–H stretching for aromatics, and C=C band of aromatics gave an absorption band at about 3041–3012 cm^{-1} and 1618–1541 cm^{-1}. A single sharp absorption band, attributed to Ar-N bands, was identified in the 1245–1275 cm^{-1} in Mannich bases along with sharp chlorine signal at 797–741 cm^{-1}. The presence of –OH group produced a broad absorption band at values 3615–3658 cm^{-1}. The corresponding peak at 1563 cm^{-1} and 1332 which suggests the presence of N–O stretch. In the ^{1}H NMR spectra of Mannich bases, **4a**–**j** the signals of the respectively prepared derivatives were verified on the basis of their chemical shifts, multiplicities, and coupling constants. Compounds **4a**–**j** show singlet peak at around 3.91–4.18 ppm (s, 2H), for the CH_2 group, which confirms the formation of Mannich bases. The ^{1}H NMR spectrum of berberine ring showed a singlet at 9.42, 7.89, 7.44, 7.05, and 6.03 ppm for H-8, H-13, H-1, H-4, and H-11 respectively. Compounds **4a**–**j** displayed characteristic >O–CH2–O< signals at around 6.22 ppm (s, 2H, $–OCH_2O$). Also, a triplet peak was shown at 3.05, and 5.78 ppm due to H-5 and H-6 proton atoms of berberine ring. The ^{1}H NMR spectrum of **4a** exhibited a sharp singlet at 1.95 ppm integrating for three protons of the methyl group



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

R = **4a** 4-CH_3
4b 2-Cl
4c 3-Cl
4d 4-Cl
4e 2-F
4f 4-F
4g 4-OCF_3
4h 4-CF_3
4i 2-NO_2
4j 4-NO_2

Scheme 1 Synthesis of piperazine linked berberine derivatives.

of piperazine moiety. The protons of (H-18, H-20, H-15 and H-17) piperazine nucleus were observed as two broad singlets at around 3.84 ppm as well as 3.22 ppm. Moreover, aromatic rings linked to the piperazine ring appeared to have a corresponding signal as multiples in the range 6.90–6.71 ppm. The signal at 9.79 ppm was due to the –OH group present in berberine ring. 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.

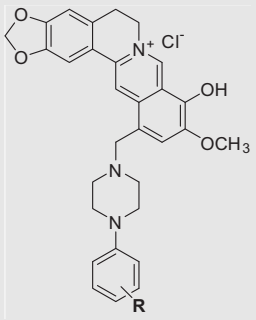
3.2. Evaluation of biological activities

3.2.1. Antioxidant activities

Titled analogues **4a**–**j** were studied to check their radical scavenging efficacies as antioxidants in DPPH and ABTS bioassay, and the results are summarized in Table 1. The presence of different functional groups as alkyl, trifluoroalkoxy, trifluoromethyl, halo (F, Cl) and nitro led to the variety of antioxidant activity patterns for the resultant molecules. In general, title analogues represented better activity with IC_{50s} ranging from 12.17 ± 0.37–23.86 ± 3.47 μg/mL and 4.644 ± 0.02–12.96 ± 0.30 μg/mL in DPPH and ABTS bioassay, respectively than parent berberine with 33.16 ± 1.60 μg/mL and 86.29 ± 2.11 μg/mL of IC_{50s} in respective bioassay. Analogue **4e** and **4f** with electron withdrawing and highly electronegative fluorine atom on the piperazine entity attached to the berberine ring presented most effective radical scavenging potential in DPPH assay with around 12.17 μg/mL of IC_{50s}, comparable to that of control drug ascorbic acid at 10.75 μg/mL and far better than berberine at 33.16 μg/mL. Also, compound **4f** also exhibited significant antioxidant power in ABTS assay with 4.644 μg/mL of $IC_{50,}$ which was further comparable to that of the ascorbic acid standard at 5.760 μg/mL. A compound (**4d**) with electron withdrawing chlorine atom presented an appreciable level of radical scavenging activity with IC_{50s}, 12.23 ± 0.03 μg/mL and 5.817 ± 0.04 μg/mL in DPPH and ABTS assay, respectively and were nearer to that of control ascorbic acid. All compounds exhibited a tremendous level of antioxidant power in ABTS assay when compared to results appeared in the DPPH assay in contrast with the antioxidant power of berberine. Hence, compounds with halogen functionality **4d**–**4f** can be considered to have an appreciable level of antioxidant power in the respective assay. Rest of the set of compounds showed good to moderate level of radical scavenging power when compared to control ascorbic acid. In terms of functional group presence and activity relationships, the antioxidant efficacies of the final analogues can be ordered as F > Cl > NO_2 > CH_3 > OCF_3 > CF_3.

FRAP assay was adapted to inspect the Fe^{+3} ion reducing capabilities of final berberine-piperazine Mannich bases. An

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



Compounds	R	IC_{50} µg/mL ± SD[a]		FRAP (10 µg/mL)[d]
		DPPH[b]	ABTS[c]	
4a	4-CH_3	19.33 ± 1.12	12.96 ± 0.30	2.221 ± 0.26
4b	2-Cl	16.89 ± 0.28	6.327 ± 0.16	1.907 ± 0.57
4c	3-Cl	21.30 ± 2.28	5.280 ± 0.50	0.781 ± 1.23
4d	4-Cl	12.23 ± 0.03	5.817 ± 0.04	1.743 ± 0.62
4e	2-F	12.17 ± 0.37	6.461 ± 0.08	1.399 ± 0.72
4f	4-F	12.17 ± 1.45	4.644 ± 0.02	0.002 ± 1.05
4g	4-OCF_3	21.13 ± 1.68	11.61 ± 0.52	0.005 ± 1.32
4h	4-CF_3	23.86 ± 3.47	8.067 ± 0.12	0.004 ± 0.56
4i	2-NO_2	16.95 ± 1.03	8.183 ± 0.05	0.004 ± 0.48
4j	4-NO_2	15.07 ± 1.38	8.795 ± 0.23	0.005 ± 0.73
Berberine	–	33.16 ± 1.60	86.29 ± 2.11	0.001 ± 0.66
Ascorbic acid	–	10.75 ± 0.66	4.760 ± 0.05	2.140 ± 0.78

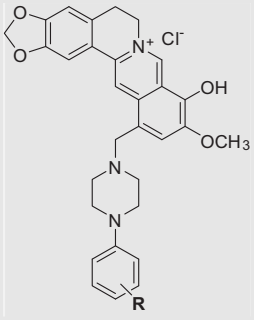
[a] Antioxidant activities are shown as IC_{50} values in µg/mL. All assays were carried out in triplicate, and the results expressed as an average ± standard deviation.
[b] DPPH = 2,2-diphenyl-1-picrylhydrazyl.
[c] ABTS = 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid.
[d] FRAP = ferric reducing ability of plasma.

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. Therefore, the concentration of Fe^{+2} can be monitored by measuring the formation of Fe^{+2}-tripyridyltriazine complex with blue colour. The higher the absorbance of the compounds indicated greater reducing power. Compounds **4a–j** exerted absorption value ranging from 0.002 ± 1.05 nm to 2.221 ± 0.26 nm. The scaffolds which reduced metal ion complexes to their lower oxidation state in FRAP assay were **4a** with electron releasing methyl functional group exhibiting 2.221 nm of absorption level as well as two another analogues (**4b** and **4d**) with electron withdrawing chlorine groups with 1.907 nm and 1.743 nm of absorption levels. The results of FRAP assay of these analogues are comparable to that of control compound ascorbic acid having absorption value of 2.140 nm in FRAP assay. Compounds **4f–4j** was found inactive in FRAP assay as compared to ascorbic acid and were of equal potency as parent berberine. It can be stated that in FRAP assay, an opposite trend of results appear when compared to other two antioxidant assays, as compound **4a** that was least active in FRAP and ABTS assay furnished potent activity in FRAP assay. The rest of the compounds showed lower absorbance as compared to the standard but were of high efficacies when compared to berberine.

3.2.2. *Anticancer activities*

Inhibitory efficacies of the cervical cancer cell line growth of compound **4a–j** were studied using sulforhodamine B (SRB) bioassay and the results are presented in Table 2 for Hela cell lines and Table 3 for Caski cell lines. In terms of therapeutic indices (TIs), it is worth mentioning that all compounds exhibited a reasonable level of anticancer potential against Hela and Caski cell lines when compared to the therapeutic index of parent compound berberine. Compounds **4a–j** furnished IC_{50}s in the range 3.983 ± 0.07 to 5.972 ± 0.05 µg/mL with a low level of cytotoxicity with CC_{50}s 118.1 ± 1.07 to 194.2 ± 1.92 µg/mL against Hela cell lines thereby establishing significant therapeutic indices better than berberine. A compound **4d** with electron withdrawing chlorine atom attached to the para position of the phenyl ring of piperazine moiety signifies highest anticancer effects against Hela cell lines with 4.243 ± 0.03 µg/mL of IC_{50}, 194.2 ± 1.92 µg/mL of CC_{50} with 45.77 of TI. In addition, an another analogue (**4c**) with 3-chlorophenyl piperazine ring attached via Mannich base

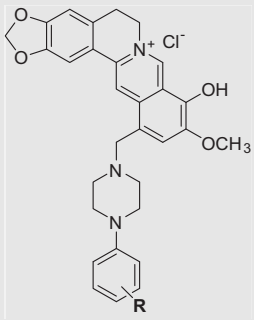
Table 2 *In vitro* anticancer activity of synthesized compounds against Hela cancer cell and their toxicity.



Compounds	R	IC_{50} µg/ml ± SD[a]	CC_{50} µg/ml ± SD[b]	TI[c]
		Hela	MDCK	
4a	4-CH_3	4.353 ± 0.12	142.4 ± 0.04	32.71
4b	2-Cl	5.412 ± 0.06	147.8 ± 1.53	27.31
4c	3-Cl	3.983 ± 0.07	144.7 ± 0.76	36.33
4d	4-Cl	4.243 ± 0.03	194.2 ± 1.92	45.77
4e	2-F	5.876 ± 0.11	126.1 ± 1.33	21.46
4f	4-F	4.420 ± 0.02	139.3 ± 0.73	31.52
4g	4-OCF_3	5.643 ± 0.05	159.2 ± 2.28	28.21
4h	4-CF_3	4.255 ± 0.05	118.1 ± 1.07	27.76
4i	2-NO_2	5.972 ± 0.05	129.1 ± 2.20	21.62
4j	4-NO_2	5.952 ± 0.16	140.9 ± 2.23	23.67
Berberine	–	4.499 ± 0.08	113.8 ± 0.51	25.29

[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.
[b] CC_{50}-cytotoxicity concentration of 50%.
[c] TI-therapeutic index.

Table 3 *In vitro* anticancer activity of synthesized compounds against Caski cancer cell and their toxicity.



Compounds	R	IC_{50} μg/ml ± SD[a] Caski	CC_{50} μg/ml ± SD[b] MDCK	TI[c]
4a	4-CH_3	5.003 ± 0.25	142.4 ± 0.04	28.46
4b	2-Cl	4.353 ± 0.10	147.8 ± 1.53	33.95
4c	3-Cl	4.920 ± 0.13	144.7 ± 0.76	29.41
4d	4-Cl	6.977 ± 0.31	194.2 ± 1.92	27.83
4e	2-F	4.623 ± 0.23	126.1 ± 1.33	27.28
4f	4-F	5.777 ± 0.19	139.3 ± 0.73	24.11
4g	4-OCF_3	4.979 ± 0.14	159.2 ± 2.28	31.97
4h	4-CF_3	4.821 ± 0.15	118.1 ± 1.07	24.50
4i	2-NO_2	4.670 ± 0.13	129.1 ± 2.20	27.64
4j	4-NO_2	4.560 ± 0.34	140.9 ± 2.23	30.90
Berberine	–	5.643 ± 0.11	113.8 ± 0.51	20.17

[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.
[b] CC_{50}-cytotoxicity concentration of 50%.
[c] TI-therapeutic index.

functionality to the berberine ring exerted the second highest level of Hela cell line inhibitory potential with 3.983 ± 0.07 μg/mL of IC_{50}, 144.7 ± 0.76 μg/mL of CC_{50} with 36.33 of TI. Compound **4a** with alkyl, **4b** with chlorine, **4f** with electronegative fluorine atom, **4g** and **4h** with trifluoro functionality expressed 32.71, 27.31, 31.52, 28.21 and 27.76 of therapeutic indices, better than that of berberine at 25.29 against Hela cervical cancer cell lines. A compound with a nitro functional group (**4i** and **4j**) appeared with diminished anticancer potential against Hela cell lines than that of berberine. An opposite trend of anticancer action was observed while studying cancerous cell inhibitory potential of compounds **4a**–**j** against cervical cancer cell line Caski. For example, compound **4j** with para-nitrophenyl piperazine moiety demonstrated high levels of anti Caski cell line potency with IC_{50} 4.560 ± 0.34 μg/mL and showed tolerable cytotoxicity of 140.9 ± 2.23 μg/mL thereby furnished 30.90 of TI, comparable to that of berberine at 20.17. However, the most potent compound (**4b**) against Caski cell line was with 2-chlorophenyl piperazine entity, having 4.353 ± 0.10 of IC_{50}, 147.8 ± 1.53 of CC_50 and 33.95 of the therapeutic index. Moreover, a compound with electronegative trifluoromethoxy functional group presented the second highest action against the Caski cell line with 4.979 ± 0.14 of IC50, 159.2 ± 2.28 of CC_{50} and 31.97 of the therapeutic index. All the final compounds exhibited better therapeutic indices against the Caski cell line than berberine itself.

4. Conclusion

A Mannich base series linking different EWD and ED functional groups bearing piperazine entities to the natural alkaloid substance berberine was constructed and examined for their in vitro antioxidant and anticancer potential. Overall, results of FRAP, DPPH and ABTS bioassay for the antioxidant activity and SRB bioassay against Hela and Caski cell lines of cervical cancer activity were quite fascinating when compared to results obtained for the parent berberine compound. Final Mannich bases with the chlorine and fluorine functional group presented significant radical scavenging effects in DPPH and FRAP assays, whereas those with alkyl functionality exercised a reasonable level of Fe^{+3} reducing power in the FRAP assay. Furthermore, the anticancer potential of chlorinated analogues was tremendous against Hela and Caski cell lines in addition to the reasonable level of activity observed for those analogues bearing nitro and trifluoromethoxy functional groups. Thus, results are worth stating that placement of different functional groups on the moiety attached to the berberine may result in positively enhanced anticancer and antioxidant effects. Overall, title Mannich bases appeared with better pharmacological efficacies in all the bioassays tested in this study when compared to the parent compound berberine and control drugs hence proving the rationalization of the work worth considering.

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