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RESEARCH ARTICLE

Synthesis and *In Vitro* Antioxidant Evaluation of Some Indole-2-carboxylic Acid-Aryl Amine Conjugates

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Abstract: A simple and efficient protocol for the synthesis of novel indole-2-carboxylic acid analogues having aryl amine moieties (**2a-k**) has been described. The synthesized compounds were characterized by spectroscopic techniques (IR, ^{1}H NMR, ^{13}C NMR and MS-EI) and further screened for their antioxidant activity by using various *in vitro* assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) scavenging assay, 2,2$^{'}$-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$**)** radical scavenging activity, ferric reducing antioxidant power, cupric ion reducing ability method and phosphomolybdate method. In all the antioxidant assays performed, compounds **2b**, **2d**, **2e** and **2f** showed promising antioxidant activity. Whereas, compound **2c** displayed potent antioxidant activity.

Keywords: Free radicals, Antioxidant activity, Indole-2-carboxylic acid, Aryl amines

Introduction

Reactive oxygen species (ROS) and free radicals have attracted increasing attention over the past decade. Free radicals are exacerbating factors in cellular injury and aging process[1-3]. Antioxidants, used to prevent or inhibit the natural phenomena of oxidation, have a broad application in diverse industrial fields as they have a huge importance either as industrial additives or health agents[4,5]. Research data have revealed that they could be suitable for preventive and therauptic purposes in several diseases related with oxidative stress (*e.g.* atherosclerosis, inflammatory injury, cancer and cardiovascular diseases)[6,7]. In the vast heterocyclic structural space, the indole nucleus occupies a position of major importance. Many indole derivatives including fused derivatives form the basis of a range of pharmaceuticals[8,9].

Indole moiety occurs widely in synthetic and natural products containing an important class of therapeutic agents[10,11]. In the last decade, antioxidant activity of synthetic indole

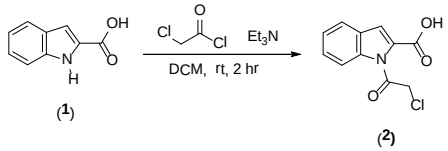
derivatives and their possible activity mechanisms have been widely studied[11]. Generally, pharmacophores containing typically the phenol moiety possess good antioxidant activity[12]. In addition to the traditional O–H bond type antioxidant, tricyclic amines having N–H bond functions as the antioxidant have attracted much research attention because aromatic amines (Ar_2NH) have always been the central structure in many currently used drugs[13]. It was envisaged, that the two pharmacophores if linked together would generate novel molecular templates, which are likely to exhibit interesting antioxidant properties. Owing to the importance and in continuation of our work on synthesis of novel biologically active heterocyclic compounds[14-17,] in the present investigation we have focused on the synthesis and evaluation of antioxidant activity of novel indole-2-carboxylic acid analogues. Our study reveals that assembling of indole-2-carboxylic acid with aryl amine moiety dispatch the generous support for the enhancement in the antioxidant activity.

Experimental

All the chemicals and reagents were purchased commercially from Merck, Himedia and SD Fine Chemicals and were used without further purification. Melting points of the synthesized compounds were determined in open capillaries and are uncorrected. Reactions are monitored by thin-layer chromatography (TLC) on silica gel 60 F_{254} aluminum sheets (Merck). IR spectra are recorded in KBr on FT-IR021 spectrophotometer (ν_{max} in cm^{-1}). ^1H NMR and ^{13}C NMR on Jeol GSX 400-MHz spectrometer (chemical shift in δ ppm downfield from TMS as an internal reference). The mass spectra were recorded on Waters-Q-TOF Ultima spectrometer. The elemental analysis was determined on FLASH EA-1112 series instrument. All the compounds gave C, H, O and N analysis within ±0.4% of the theoretical values.

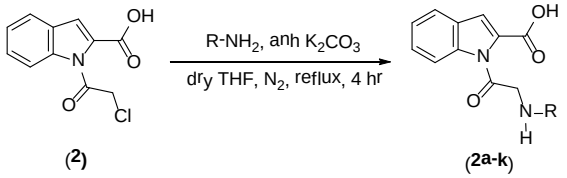
Chemistry

N-Acylation of indole-2-carboxylic acid with 2-chloroacetylchloride in the presence of triethylamine (TEA) as a base yields the key scaffold 1-(2-chloroacetyl)-1*H*-indole-2-carboxylic acid (**2**) Scheme 1. Further, coupling of various aryl amines to key scaffold (**2**) by base condensation reaction affords novel indole-2-carboxylic acid analogues (**2a-k**) in moderate to good yield, which were identified by spectroscopic techniques: NMR (^1H NMR, ^{13}C NMR), FT-IR and MS-EI. A typical synthetic strategy employed to obtain the title compound (**2a-k**) is depicted in Scheme 2.



(**1**) (**2**)

Scheme 1. Reaction for the synthesis of indole-2-carboxylic acid-aryl amine conjugates **(2a-k)**



(**2**) (**2a-k**)

Scheme 2. Pathway for the synthesis of indole-2-carboxylic acid-aryl amine conjugates (**2a-k)**

*Procedure for the synthesis of 1-(2-chloroacetyl)-1H-indole-2-carboxylic acid (compound **2**)*

To a well stirred solution of indole-2-carboxylic acid (1 mM) and triethylamine (1.2 mM) in 10 mL dichloromethane, 2-chloroacetylchloride (1.2 mM) in 5 ml was added drop by drop for 10 min, then the reaction mixture was stirred at room temperature for about 2 h. Progress of the reaction was monitored by TLC using hexane:ethyl acetate (6:4) mixture as mobile phase. After the completion of reaction, the reaction mass was quenched in ice cold water and the product was extracted with diethyl ether. The organic layer was washed with brine solution followed by distilled water and dried briefly (Na_2SO_4) and the volatiles were removed *in vacuo* at <30 ^oC, the resulting brownish solid was identified.

Brownish solid, yield (85%), m.p. 185-188 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3349.75 (COOH), 2926.60 (Ar-H), 1603.2 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.0 (s, 1H, COOH), 7.33 (s, 1H, CH of indole), 6.87-8.11 (m, 4H, Ar-H), 4.49 (s, 2H, CH_2Cl); Mass (*m/z*): M^+ 238.02; Anal. Calcd. for $C_{11}H_8ClNO_3$: C, 55.60; H, 3.39; N, 5.89; O, 20.20%; Found; C, 55.57; H, 3.43; N, 5.85; O, 20.24%.

General procedure for the synthesis for the indole-2-carboxylic acid conjugated with aryl amine analogues (**2a-k**)

A mixture of aryl amines (1.2 mM) and K_2CO_3 (1 mM) in dry tetrahydrofuran (dry THF, 10 mL) were stirred under N_2 atmosphere for 30 min at room temperature. The mixture was treated drop wise with 1-(2-chloroacetyl)-1*H*-indole-2-carboxylic acid (1 mM) in dry THF (10 mL) and refluxed for 4 h. The progress of the reaction was monitored by TLC. The reaction mixture was then desolventized in rotary evaporator and the product was extracted in ethyl acetate and washed with water and dried over anhydrous Na_2SO_4. The products were separated and purified by column chromatography, using hexane:ethyl acetate=60:40.

*1-(2-(Phenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2a**)*

Brown solid, m.p. 137-139 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3623.32 (COOH), 3330.46 (N-H), 2854.17-2938.52 (Ar-H),1653.66 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) (δ ppm): 11.0 (s, 1H, COOH), 7.1-8.11 (m, 9H, Ar-H), 7.33 (s, 1H, CH of indole), 4.17 (s, 2H, CH_2N-H), 4.0 (s, 1H, NH); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.2, 160.1, 147.5, 141.1, 129.5, 127.4, 124.4, 123.5, 120.8, 115.6, 53.1; Mass (*m/z*): M^+ 295.3; Anal. Calcd. for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52; O, 16.31%; Found; C, 69.40; H, 4.81; N, 9.53; O, 16.30%.

*1-(2-(2-Hydroxyphenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2b**)*

Light yellow solid, m.p. 157-159 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3344.16 (COOH), 3316.12 (N-H), 2853.51-2930.85 (Ar-H), 1605.40 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) (δ ppm): 11.2 (s, 1H, COOH), 7.23 (s, 1H, CH of indole), 6.83-8.20 (m, 8H, Ar-H), 5.35 (s, 1H, OH of Phenol), 4.3 (s, 1H, NH), 4.15 (s, 2H, CH_2N-H); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.3, 160.1, 141.7, 140.9, 139.8, 127.3, 124.4, 123.2, 120.0, 118.6, 114.8, 53.3; Mass (*m/z*): M^+ 311.50; Anal. Calcd. for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03; O, 20.62%; Found; C, 65.83; H, 4.52; N, 9.07; O, 20.60%.

*1-(2-(4-Hydroxyphenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2c**)*

Yellow solid, m.p. 153-155 ^oC,IR (KBr) ν_{max} (cm^{-1}): 3622.63 (COOH), 3306.46 (N-H), 2853.58-2923.56 (Ar-H), 1689.98 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) (δ ppm: 11.0 (s, 1H, COOH), 7.4 (s, 1H, CH of indole), 6.92-8.11 (m, 8H, Ar-H), 5.42 (s, 1H, OH), 4.17 (s, 2H, CH_2N-H),), 4.0 (s, 1H, NH); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.4, 160.2, 146.8, 141.1,

127.5, 126.6, 124.3, 119.5, 116.8, 115.8, 53.2; Mass (*m/z*): M^+ 311.13; Anal. Calcd. for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03; O, 20.62%; Found; C, 65.83; H, 4.52; N, 9.06; O, 20.60%.

*1-(2-(2-Methoxyphenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2d**)*

Pale brown solid, m.p. 160-162 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3394.32 (COOH), 3334.32 (N-H), 2862.17-2938.52 (Ar-H), 1615.52 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) (δ ppm): 11.3 (s, 1H, COOH), 7.35 (s, 1H, CH of indole), 6.86-8.21 (m, 8H, Ar-H), 4.19 (s, 2H, CH_2N-H), 4.0 (s, 1H, NH), 3.83 (s, 3H, OCH_3); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.3, 160.1, 144.6, 141.1, 128.3, 127.4, 124.4, 123.6, 121.8, 119.6, 55.9, 53.1; Mass (*m/z*): M^+ 325.01; Anal. Calcd. for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.97; N, 8.64; O, 19.73%; Found; C, 66.68; H, 4.96; N, 8.61; O, 19.70%.

*1-(2-(4-Methoxyphenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2e**)*

Pale brown semisolid, IR (KBr) ν_{max} (cm^{-1}): 3589.51 (COOH), 3316.16 (N-H), 2852.54-2924.52 (Ar-H), 1630.88 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.0 (s, 1H, COOH), 7.42 (s, 1H, CH of indole), 6.87-8.11(m, 8H, Ar-H), 4.27 (s, 2H, CH_2N-H), 4.1 (s, 1H, NH), 3.86 (s, 3H, OCH_3); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.2, 160.3, 151.6, 141.2, 139.6, 127.4, 124.2, 123.5, 119.9, 55.9, 53.1; Mass (*m/z*): M^+ 325.13; Anal. Calcd. for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.97; N, 8.64; O, 19.73%; Found; C, 66.65; H, 4.99; N, 8.62; O, 19.71%.

*1-(2-(2,4-Dimethoxyphenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2f**)*

Yellow semisolid, IR (KBr) ν_{max} (cm^{-1}): 3626.48 (COOH), 3334.46 (N-H), 2853.17-2924.52 (Ar-H), 1687.66 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.0 (s, 1H, COOH), 7.85-8.11 (m, 8H, Ar-H), 7.33 (s, 1H, CH of indole), 4.19 (s, 1H, NH), 4.12 (s, 2H, CH_2N-H), 3.87 (s, 6H, OCH_3); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.4, 162.3, 160.3, 145.6, 141.1, 130.7, 126.4, 124.1, 119.8, 101.3, 55.8, 53.2; Mass (*m/z*): M^+ 355.26; Anal. Calcd. for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91; O, 22.58%; Found; C, 64.43; H, 5.13; N, 7.95; O, 22.54%.

*1-(2-(p-Tolylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2g**)*

White solid, m.p. 167-169 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3621.66 (COOH), 3340.16 (N-H), 2854.13-2954.82 (Ar-H), 1618.95 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.6 (s, 1H, COOH), 7.13 (s, 1H, CH of indole), 7.0-8.11(m, 8H, Ar-H), 4.27 (s, 2H, CH_2N-H), 4.1 (s, 1H, NH), 2.32 (s, 3H, CH_3); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.3, 160.2, 144.5, 141.2, 130.7, 126.8, 124.3, 119.8, 113.2, 53.6, 21.4; Mass (*m/z*): M^+ 309.06; Anal. Calcd. for $C_{18}H_{16}N_2O_3$: C, 70.12; H, 5.23; N, 9.09; O, 15.57%; Found; C, 70.15; H, 5.26; N, 9.07; O, 15.55%.

*1-(2-(4-Chlorophenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2h**)*

Light brown solid, m.p. 143-145 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3538.31 (COOH), 3309.16 (N-H), 2853.17-2935.52 (Ar-H),1620.88 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.1 (s, 1H, COOH), 6.87-8.11 (m, 8H, Ar-H), 7.33 (s, 1H, CH of indole), 4.18 (s, 2H, CH_2N-H), 4.1 (s, 1H, NH); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.3, 145.7, 141.6, 129.6, 126.0, 124.2, 123.4, 119.7, 114.8, 53.2; Mass (*m/z*): M^+ 330.08; Anal. Calcd. for $C_{17}H_{13}ClN_2O_3$: C, 62.11; H, 3.99N, 8.52; O, 14.60%; Found; C, 62.13; H, 3.97; N, 8.56; O, 14.61%.

*1-(2-(4-Bromophenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2i**)*

Brown solid, m.p. 138-140 ^oC, IR (KBr) ν_{max} (cm^{-1}): 3438.31 (COOH), 3312.96 (N-H), 2848.17-2924.52 (Ar-H), 1618.95 (C=O); ^1H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.2 (s, 1H,

COOH), 6.87-8.11 (m, 8H, Ar-H), 7.31 (s, 1H, CH of indole), 4.18 (s, 2H, CH_2N-H), 4.0 (s, 1H, NH); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.2, 160.2, 146.5, 132.3, 126.4, 124.3, 119.7, 115.0, 114.2, 53.1; Mass (*m/z*): M^+ 373.06; Anal. Calcd. for $C_{17}H_{13}BrN_2O_3$: C, 54.71; H, 3.51; N, 7.51; O, 12.86%; Found; C, 54.73; H, 3.54; N, 7.48; O, 12.87%.

*1-(2-(2-Nhitrophenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2j**)*

Pale yellow solid, m.p. 118-120 ^{o}C, IR (KB) ν_{max} (cm^{-1}): 3621.66 (COOH), 3353.60 (N-H), 2854.13-2923.56 (Ar-H), 1625.70 (C=O); ^{1}H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.6 (s, 1H, COOH), 6.87-8.11 (m, 8H, Ar-H), 7.31 (s, 1H, CH of indole), 4.17 (s, 2H, CH_2N-H), 4.0 (s, 1H, NH); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.2, 160.1, 145.4, 131.7, 135.4, 125.6, 124.2, 123.5, 119.8, 108.6, 52.2; Mass (*m/z*): M^+ 340.05; Anal. Calcd. for $C_{17}H_{13}N_3O_5$: C, 60.18; H, 3.86; N, 12.38; O, 23.58%; Found; C, 60.16; H, 3.85; N, 12.41; O, 23.59%.

*1-(2-(4-Nitrophenylamino)acetyl)-1H-indole-2-carboxylic acid (compound **2k**)*

Pale yellow solid, m.p. 125-128 ^{o}C, IR (KBr) ν_{max} (cm^{-1}): 3478.95 (COOH), 3358.43 (N-H), 2853.17-2924.52 (Ar-H), 1630.52 (C=O); ^{1}H NMR (400 MHz) ($CDCl_3$) δ ppm): 11.0 (s, 1H, COOH), 6.87-8.20 (m, 8H, Ar-H),), 7.30 (s, 1H, CH of indole), 4.17 (s, 2H, CH_2N-H 4.0 (s, 1H, NH); ^{13}C HMR ($CDCl_3$) (δ ppm): 168.4, 160.3, 153.8, 141.2, 136.2, 127.7, 126.5, 123.5, 119.8, 114.3, 108.4, 53.1; Mass (*m/z*): M^+ 341.20; Anal. Calcd. for $C_{17}H_{13}N_3O_5$: C, 60.18; H, 3.86; N, 12.38; O, 23.58%; Found; C, 60.19; H, 3.84; N, 12.36; O, 23.59%.

Results and Discussion

A series of novel indole-2-carboxylic acid derivatives (**2a-k**) were synthesized. Scheme 1 and Scheme 2 illustrates the way used for the preparation of target molecules. The starting material, indole-2-carboxylic acid (**1**) reacts with chloroacetyl chloride in the presence of triethylamine as a base to yield 1-(2-chloroacetyl)-1*H*-indole-2-carboxylic acid (**2**), a key scaffold Scheme 1. Further, in order to assemble the key scaffold (**2**) and different aryl amines and to study the effect on antioxidant properties, the key scaffold (**2**) was conjugated with different aryl amines in the presence of potassium carbonate as a base to afford desired products (**2a-k**) Scheme 2. Structural conformation was done using IR, ^{1}H NMR, ^{13}C NMR, mass spectra and elemental analysis. In the IR spectrum of 1-(2-chloroacetyl)-1*H*-indole-2-carboxylic acid (**2**) the C=O stretching band was observed at 1603.2 cm^{-1} and absence of N-H band at 3310 cm^{-1} confirms the *N*-acylation reaction. The structure of the compound (**2)** was further confirmed by ^{1}H NMR spectrum where the absence of the signal at δ 11.1 ppm which corresponds to N-H proton of indole ring. All the respective aromatic protons were signaled at δ 6.87–8.11 ppm. ^{1}H NMR spectra of all indole-2-carboxylic acid aryl-amine conjugates (**2a-k**) showed multiplet for Ar–H proton at δ 6.76–8.11 ppm and singlet for N-H protons at δ 4.0-4.1 ppm. All the analogues showed mass according to their M^+ ions.

Antioxidant activities

All the newly synthesized series of indole-2-carboxylic acid conjugated aryl-amine derivatives (**2a-k**) were screened for their antioxidant potential by employing different *in vitro* assays such as, DPPH free radical scavenging assay and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$**)** radical scavenging activity, ferric reducing antioxidant power, cupric ion reducing ability method and phosphomolybdate method. All the *in vitro* assay measurements were performed in triplicates.

DPPH free radical scavenging activity

The newly synthesized compounds were screened for free radical scavenging activity by DPPH method[18]. DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. The samples and the standards like butylated hydroxy anisole (BHA) and ascorbic acid were prepared at concentrations of 10, 25, 50, 100, 200 and 500 µM. Among the tested indole-2-carboxylic acid conjugated with aryl amine analogues (**2a-k**) displayed certain degree of activity. The results of the analogues appeared to be related to the nature of the substituent group on the substituted phenyl ring of aryl amines. Among the synthesized analogues, compound **2c** exhibited better scavenging activity (Table 2), this may be due to the presence of hydroxyl group on the *para* substituted phenyl ring. Chloro, bromo and nitro substituted indole-2-carboxylic acid derivatives (**2h-k**) showed least scavenging activities compared to standard.

Table 1. Chemical structures and yields of indole-2-carboxylic acid aryl-amine conjugates

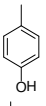
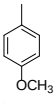
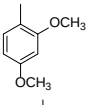
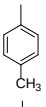
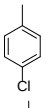
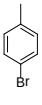
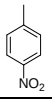
Compound	R	Yield, %
2a		83
2b		82
2c		84
2d		81
2e		78
2f		71
2g		75
2h		87
2i		77
2j		75
2k		84

Table 2. Concentration required for 50% scavenging (IC_{50}) of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activities of the compounds (**2a-k**) and the standard antioxidant compounds BHA and Ascorbic acid. Each value represents mean ± SD (n=3)

Tested compounds	Scavenging activity (IC_{50})[a]	
	$DPPH^{\bullet}$	$ABTS^{\bullet+}$
BHA	12.2±0.12	20.9±0.12
Ascorbic acid	11.1±0.11	18.3±0.14
01	124±0.34	88.4±0.16
2a	156±0.21	132.1±0.35
2b	18±0.25	36.6±0.24
2c	15±0.63	29.3±0.45
2d	81±0.62	153.16±0.81
2e	61±0.54	132.62±0.35
2f	26±0.20	32.7±0.12
2g	132±0.51	189.03±0.33
2h	189±0.11	173.12±0.10
2i	301±0.72	350.85±0.53
2j	234±0.45	203.34±0.60
2k	261±0.16	293.32±0.27

[a]*The values are expressed as µM concentration. Lower IC_{50} values indicate higher radical scavenging activity*

$ABTS^{\bullet+}$ radical scavenging activity

The synthesized indole-2-carboxylic acid aryl-amine conjugates were subjected to $ABTS^{\bullet+}$ radical scavenging activity[19]. This assay is based on a decolorization technique in which the radical is generated directly in a stable form prior to reaction with putative antioxidants. In this assay, the technique is based on the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. Amongst the synthesized analogues, compounds **2c, 2b** and **2f** showed promising $ABTS^{\bullet+}$ scavenging activity and compounds **2g**, **2e** and **2d** displayed moderate scavenging activity. Whereas, compounds **2h**, **2i**, **2j** and **2k** possessing chloro and nitro substituents showed very least $ABTS^{\bullet+}$ scavenging activity compared to standard BHA and ascorbic acid (Table 2).

Ferric reducing antioxidant power (FRAP) assay

All the synthesized indole-2-carboxylic acid aryl-amine conjugates were screened for ferric reducing antioxidant power[20]. The FRAP method is based on the reduction of Fe^{3+}-Fe^{2+}. The presence of antioxidants in the samples would result in the reducing Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can be then monitored by measuring the formation of Prussian blue at 700 nm. The reducing ability of the compound may serve as a significant indicator of its potential antioxidant activity. Compounds **2b**, **2f**, **2d** and **2e** displayed good ferric ion reducing ability compared to other scaffolds. Compound **2c** has the most powerful ferric ion reducing ability, this may be due to the electron donating power of hydroxy group present in the substituted phenyl ring. Also the results from the (Table 3) reveals that the presence of electron withdrawing groups on the substituted phenyl ring did not favors in the ferric ion reducing ability.

Table 3. Comparison of ferric ions (Fe^{3+}) reducing ability by Fe^{3+}-Fe^{2+} transformation methods, Cu^{2+}-Cu^{+} reducing ability and phosphomolybdenum method of the compounds (**2a-k**) to the standard antioxidant compounds BHA and Ascorbic acid at 10 µM

Tested compounds	Fe^{3+}-Fe^{2+} reducing ability[a]	Cu^{2+}-Cu^{+} reducing ability[a]	Phosphomolybdenum assay[b]
BHA	0.3490	0.3130	372.71
Ascorbic acid	0.4013	0.3612	388.12
01	0.1272	0.1420	93.48
2a	0.1272	0.1063	111.27
2b	0.3079	0.2897	282.16
2c	0.3324	0.3006	348.67
2d	0.2709	0.2513	276.17
2e	0.2681	0.2486	307.22
2f	0.2953	0.1953	294.22
2g	0.1536	0.1411	101.14
2h	0.1163	0.0931	83.87
2i	0.0615	0.0791	78.24
2j	0.0961	0.0061	70.97
2k	0.0817	0.1040	101.90

[a]*The values are expressed as absorbance. High absorbance indicates high reducing power.* [b]*The values are expressed as equivalent of Ascorbic acid (µM/mg)*

Cupric ion reducing ability

All the synthesized compounds were performed cupric ion reducing ability assay[21]. This method utilizes the copper(II)-neocuproine reagent as the chromogenic oxidizing agent. This reagent was useful at pH 7 and the absorbance of the Cu(I)-chelate formed as a result of redox reaction with the samples was measured at 450 nm. Among the synthesized derivatives, compounds **2c, 2b**, **2e** and **2d** exhibited the marked cupric ion reducing ability. All other indole-2-carboxylic acid aryl-amine conjugates such as **2f** and **2a** showed moderate cupric reducing activity. The results are showed in (Table 3).

Phosphomolybdenum method

The antioxidant activity for the synthesized compounds (**2a-k**) was evaluated by using phosphomolybdate method[22]. It determines the total antioxidant capacity. This assay is based on the reduction of Mo(VI) to Mo(V) in presence of the antioxidant compounds and the subsequent formation of a green phosphate Mo(V) complex at acidic pH, which is measured at 695 nm. The antioxidant capacity of the compounds was determined for 10 µM concentration. Among the synthesized analogues compound **2c**, **2e**, **2f**, **2b** and **2d** displayed good reducing ability of Mo(VI) to Mo(V) in better way due to the presence of electron donating groups such as hydroxy and methoxy in the substituted phenyl ring.

Antioxidant activities

DPPH Free radical scavenging activity

The newly synthesized compounds were screened for free radical scavenging activity by DPPH method[18]. Compounds of different concentrations were prepared in distilled ethanol, 1 mL of each compound solutions (**2a-k**) having different concentrations (10, 25, 50, 100, 200 and 500 µM) were taken in different test tubes, 4 mL of 0.1 mM ethanol solution of DPPH was added and shaken vigorously. The test tubes were then incubated in the dark room at r.t.

for 20 min. A DPPH blank was prepared without the compound and ethanol was used for the baseline correction. Changes (decrease) in the absorbance at 517 nm were measured using a UV-visible spectrometer (Shimadzu 160 A). The radical scavenging activities were expressed as the inhibition percentage and were calculated using the formula:

$$\text{Radical scavenging activity (\%)} = [(A_c\text{-}A_s)/A_c)\text{x}100]$$

Where A_c is absorbance of the control (without compound) and A_s is absorbance of the compounds (**2a-k**). The radical scavenging activity of BHA and ascorbic acid was also measured and compared with that of the different synthesized compound. The compound concentration providing 50% inhibition (IC_{50}) was calculated from the graph of RSA percentage against compound concentrations.

$ABTS^{\bullet+}$ radical scavenging activity

The analysis of $ABTS^{\bullet+}$ radical scavenging activity was determined according to Re method[19]. The $ABTS^{\bullet+}$ cation was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before the usage, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.700±0.025 at 734 nm with phosphate buffer (0.1M, pH 7.4). Then, 1 mL of $ABTS^{\bullet+}$ solution was added to the 1.5 mL compounds (**2a-k**) solution in ethanol at different concentrations (10, 25, 50, 100, 200 and 500 µM/mL). After 30 min, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (ethanol).

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

$$ABTS^{\bullet+}\text{ scavenging effect (\%)} = [(A_c\text{-}A_s) / A_c] \text{ x } 100$$

Where, $A_{control}$ is the initial concentration of the $ABTS^{\bullet+}$ and A_{sample} is the absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of the compounds (**2a-k**).

Ferric ion (Fe^{3+}) reducing antioxidant power assay

The reducing antioxidant power assay for the newly synthesized compounds was determined according to Oyaizu method[20]. 2 mL of the compounds (**2a-k**) having concentration 10 µM were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferric cyanide (2.5 mL, 1%). The mixture was incubated at 50 ^{o}C for 20 min. Later, the reaction mixture was acidified with trichloroacetic acid (2.5 mL, 10%). After $FeCl_3$ (0.5 mL, 0.1%) was added to this solution, the absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicates an increased reducing power.

Cupric ion (Cu^{2+}) reducing ability:

The cupric reducing ability antioxidant assay was determined according to the literature method[21]. Briefly, a mixture of $CuCl_2$ (1 mL, 0.01 M) solution, ethanolic neocuproine (Nc) (1 mL, $7.5\text{x}10^{-3}$ M) solution and Ammonium acetate (1 mL, 1 M) in a test tube were added to a solution of compounds (**2a-k**) having 10 µM concentration along with 0.1 mL distilled water. The mixture was incubated for 30 min. Then the absorbance was measured at 450 nm against reagent blank.

Evaluation of antioxidant capacity by Phosphomolybdenum method

The total antioxidant capacity was evaluated by the reported method[22]. An aliquot of 0.1 mL of compound solutions (10 µM) was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank 0.1 mL of methanol was used in place of compound. The tubes were capped and incubated in a

boiling water bath at 95 ^{o}C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank of spectrophotometer. The antioxidant capacities of the synthesized indole-2-carboxylic acid analogues (**2a-k**) were expressed as equivalent of ascorbic acid (μM/mg of compound).

Conclusion

In conclusion, we have synthesized a new series of indole-2-carboxylic acid conjugated with aryl amines (**2a-k**) and evaluated for their antioxidant power by various *in vitro* methods. Most of them demonstrated a broad spectrum of antioxidant activities. The simple indole-2-carboxylic acid derivatives **2b**, **2d**, **2e** and **2f** were concluded as good antioxidant activity, whereas **2c** displayed more potent derivative in all the *in vitro* antioxidant assays. It clearly demonstrated that the assembly of appropriate aryl-amine substituents on the *N*-acylated indole-2-carboxylic acid moiety would lead to the enhancement for potent antioxidant derivatives. It implied that, compounds **2c**, **2b**, **2d**, **2e** and **2f** may be considered as new promising lead candidates for further design and synthesis of this class of antioxidants.

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