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In vitro Antioxidant and Cytotoxic Studies of Natural Naphthoquinones and its Synthetic Naphthofuran Derivatives

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ABSTRACT

Antioxidants are emerging as potential prophylactic and therapeutic agents which scavenge free radicals and prevent the damage caused by them. The quinones and their derivatives containing hydroxyl group exhibits wide range of pharmacological activities such as antioxidant, antibacterial, antiviral, anticancer, antimalarial and antifungal activities. In particular the antioxidant and anticancer behaviors of these compounds continue to draw attention of researchers. In the present communication, three natural naphthoquinones 1-3 isolated from nutshells of Juglans regia Linn., and five synthetic naphthofuran derivatives 4-8 obtained by structural modification of natural naphthoquinones. The structures of these compounds were characterized by ^{1}HNMR, ^{13}CNMR, IR and Mass spectral studies. In vitro cytotoxicity using human hepatocellular liver carcinoma cell line (HepG2) and antioxidant study using ABTS and DPPH were carried out for these compounds. The naphthofurans 6, 7 and 8 displayed almost equivalent scavenging activity on DPPH assay and higher activity on ABTS assay relative to ascorbic acid. On the other hand naphthoquinones 1 and 3 showed lesser antioxidant activity but higher cytotoxic activity than naphthofurans except 5 which showed excellent cytotoxic activity.

Keywords: Antioxidant; Cytotoxic; Naphthoquinone; Naphthofuran

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INTRODUCTION

The antioxidant compounds are the most important species which can inhibit the oxidative stress in biological system and prevent any free radicals damage. The phenolic compounds are one of the significant antioxidants and also having anticancer activities [1]. The reactive oxygen species (ROS) and other related free radical species are witty to react either directly or indirectly, to damage all biomolecules. This damage can cause many diseases [2] including cancer [3]. Generally, antioxidant compounds donate protons to become more stable free radicals. This stability increases with the extent of delocalization and enhances the antioxidant ability [4,5]. As such, many synthesized compounds containing long chain resonance exhibited significant antioxidant activity. Furthermore, the compounds which can be considered a strong antioxidant usually possess common structural features. They often own multiple phenolic hydroxyl groups or exhibited substituted groups might influence on the scavenging ability. This indicates the existence of a close relationship between the chemical structure and the ability to scavenge free radicals. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) may be added to food products to retard oxidation reactions [6,7]. These synthetic antioxidants show stronger antioxidant activities than those of natural antioxidants, such as α-tocopherol and ascorbic acid.

The chemistry of quinones is largely dependent on the substituents being either on the quinonic or on adjacent rings. The quinone derivatives containing hydroxyl group exhibits wide range of pharmacological activities. Quinones that

have one or more hydroxyl groups attached directly to the quinone moiety are found in nature to great extent. In recent times, quinone derivatives including vitamin K, plumbagin, juglone, lawsone and shikonin attracted the attention of researchers all over the world due to its widespread occurrence, structural diversity and wide range of potent therapeutic activities [8]. The pharmacological properties such as antibacterial, antiviral, anticancer, antimalarial and antifungal are due to the interference of quinones in the electron transport chain by electron reduction processes, generating semiquinone radical ($Q^{\bullet -}$) and hydroquinone anion ($Q2^{-}$) [9-12]. Among the quinones, 1,2-naphthoquinone and 1,4-naphthoquinone nuclei are commonly encountered in natural products and their derivatives juglone, lawsone and plumbagin are well explored in pharmacologically as well as phytochemically. 1,4-Naphthoquinones have been found in plants such as Juglandaceae, Plumbaginaceae, Ebenceae, Lythraceae etc. [13-16]. Naphthofurans synthesized from naphthoquinones have attracted the attention owing to their well pronounced activities such as antifungal and cytotoxic [17]. *In vitro* cytotoxic activity of some naphthofuran derivatives using MTT assay against six human cancer cell lines (PA1, KB403, WRL68, COLO320DM, $CaCO_2$ and MCF7) has been studied [18]. From the literature survey, it could be ascertain that only a lesser number of naphthofuran derivatives were attempted to get bioactive targets.

Considering the importance of naphthoquinone and naphthofuran derivatives, the present study was designed i) to isolate Juglone (1), Lawsone (2) and Plumbagin (3) from nut shells of *Juglans regia* ii) to synthesize Ethyl-5-hydroxynaphtho[1,2-b]furan-3-carboxylate (4) and Diethyl naphtho[1,2-b:4,3-b′]difuran-3,4-dicarboxylate (5) from 1,4-naphthoquinone iii) to synthesize Diethyl-7-hydroxynaphtho [1,2-*b*:4,3-*b′*]difuran-3,4-dicarboxylate (6), 4-Ethoxycarbonyl-7-hydroxynaphtho[1,2-*b*:4,3-*b′*]difuran-3-carboxylic acid (7) and 7-Hydroxynaphtho[1,2-*b*:4,3-*b′*]difuran-3,4-dicarboxylic acid (8) from juglone and iv) to study *in vitro* antioxidant and cytotoxic activities. To the best of our knowledge, the compounds 1, 2, and 3 are isolated from the nut shells of *Juglans regia* for the first time. Particularly, isolation of 2 has not reported from this plant so far. This work has been planned to compare anticancer and antioxidant activities of the natural against synthetic derivatives. Our aim is to get more potent antioxidant and anticancer compounds by converting natural naphthoquinones into naphthofurans by structural modification. Recently we have reported the synthesis of compounds 4-8 and their antibacterial and cytotoxic studies using human cervical cancer cell line HeLa [19], in the present communication reporting the antioxidant and cytotoxic activity of these compounds using human hepatocellular liver carcinoma cell line (HepG2).

EXPERIMENTAL SECTION

Materials and Methods

1,4-Naphthoquinone, Ethyl-N,N-dimethyl aminoacrylate and DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) were purchased from Alfa aesar, India. ABTS [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonate)] was purchased from Sigma Aldrich, Bangalore, India. Fetal Bovine Serum (FBS) was purchased from Cistron laboratories. Trypsin, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and DMSO were purchased from Sisco research laboratory chemicals, Mumbai. Pre-coated silica gel 60 F_{254} plates (E. Merck, Germany) were used for TLC and visualized on UV (365 nm and 254 nm) and Iodine chamber. Silica gel (100-200 mesh) used as adsorbent for column chromatography was purchased from Acme, India Ltd. All the other chemicals and reagents were obtained from Sigma Aldrich, Mumbai/S.D. Fine Chemicals (India) Ltd and used without purification.

The melting points were determined using Buchi-540 melting point apparatus by open capillary method and are uncorrected. IR spectra were recorded as KBr pellet on Perkin-Elmer Paragon-1000; ^{1}H NMR spectra were recorded on Bruker-300 FT NMR in $CDCl_3$/DMSO-d_6 solvent using TMS as internal standard and ^{13}C NMR spectra were recorded on Bruker-500 FT NMR in $CDCl_3$ solvent using TMS as internal standard. Mass spectra were recorded on Perkin Elmer LCMS, which utilized electron spray ionization. UV was recorded on Beckman DU-40 spectrophotometer.

Isolation of Compounds

Hydroquinones are generally soluble in Chloroform/DCM. Hence, kernel removed *Juglans regia* (walnut) shells (2.4 kg) were ground to coarse powder; extracted with hexane for 12 h at room temperature and decanted; then extracted with chloroform thrice till the color of the extract become pale. The combined chloroform extract was filtered using Whatman filter paper (No.1) and concentrated at 40-45°C under reduced pressure in a rotary evaporator. The crude (24.5 g) obtained was dissolved in 200 mL of DCM and adsorbed on 75 g of silica gel (100-200 mesh), by evaporating DCM under reduced pressure using rotary evaporator. The silica gel adsorbed crude was loaded onto a column (8 cm × 120 cm) containing silica gel (100-200 mesh) up to 80 cm. It was successively eluted with hexane,

hexane/ethyl acetate, ethyl acetate and ethyl acetate/methanol. 100 mL fractions were collected at a time and checked using TLC and fractions containing similar spots by TLC were pooled together and concentrated.
The fraction collected in hexane/ethyl acetate (80:20) upon evaporation gave 490 mg of compound 1 as brown needles, R_f: 0.63 in hexane/ethyl acetate (9:1), that crystallized from ethyl acetate and hexane; mp: 162-165°C (lit 164-166°C). ^{1}H NMR (300 MHz, DMSO-d_6): 7.02-7.10 (2H, m, olefinic protons), 7.33-7.35 (1H, d, *J* = 6 Hz, Ar-H), 7.48-7.50 (1H, d, *J* = 6 Hz, Ar-H), 7.72-7.77 (1H, t, *J* = 15 Hz, Ar-H), 11.75 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (500 MHz, $CDCl_3$): 190.3, 184.2, 161.4, 139.6, 138.6, 136.5, 131.7, 124.5, 119.1, and 114.9; IR (KBr, cm^{-1}): 3066 (b), 1642 (s), 1597 (m), 1453 (m), 1363 (m), 1290 (s) and 1225 (s); MS (70 eV), m/z: 174 (M^+ ion and base peak), 146, 118, 92, 74, 63 and 53.

The fraction collected using ethyl acetate/methanol (80:20) upon evaporation gave yellow solid, which on crystallization using ethyl acetate/hexane yielded 280 mg of compound 2 as pale yellow needles, R_f: 0.54 in $CHCl_3$/MeOH (9:1); mp: 193-195°C (lit 194-197°C); ^{1}H NMR (300 MHz, $CDCl_3$): 6.370 (1H, s, olefinic proton), 7.363 (1H, s, OH, D_2O exchangeable), 7.701-7.751 (1H, t, *J* = 15 Hz, 1xAr-H), 7.780-7.829 (1H, t, *J* = 14.7 Hz, 1xAr-H), 8.112-8.138 (2H, d, *J* = 7.8 Hz and 7.8 Hz, 2xAr-H); ^{13}C NMR (500 MHz, $CDCl_3$): 184.9, 181.9, 156.3, 135.3, 133.1, 132.9, 129.4, 126.7, 126.5, and 110.7; IR (KBr, cm^{-1}): 3174 (b), 1679 (m), 1641 (s), 1583 (m), 1386 (s), 1345 (s), 1285 (m) and 1222 (s); MS (70 eV), m/z: 174 (M^+ ion peak), 146, 129, 118, 105 (base peak), 89, 77, 69, 50 and 41. The fraction collected in hexane/ethyl acetate (90:10) after evaporation afforded 120 mg of less polar compound 3 as orange needles, R_f: 0.53 in hexane/chloroform (1:1) that crystallized from petroleum-ether; mp: 75-78°C (lit 78-79°C); ^{1}H NMR (300 MHz, $CDCl_3$): 2.19 (3H, s, 1xCH_3), 6.803-6.808 (1H, d, *J* = 1.5 Hz olefinic proton), 7.233-7.264 (1H, m, *J* = 9.3 Hz, 1xAr-H), 7.597-7.627 (2H, m, *J* = 9Hz & 9 Hz, 2xAr-H), 11.969 (1H, s, OH, D_2O exchangeable); ^{13}C NMR (500 MHz, $CDCl_3$): 190.21, 184.69, 161.13, 149.57, 136.05, 135.40, 132.02, 124.11, 119.23, 115.08, and 16.49; IR (KBr, cm^{-1}): 1644 (s), 1607 (m), 1454 (m), 1363 (s), 1258 (s), 1231 (s) and 754 (s); MS (70 eV) m/z: 188 (M^+ ion and base peak), 173, 160, 145, 131, 120, 103, 92, 77 and 51.

Synthesis of Compounds 4-8
The compounds 4-8 were synthesized as per the reported procedure [19] and the structures of all the compounds were characterized using IR, Mass, ^{1}H and ^{13}C NMR spectral studies.

Biological Activity
***In vitro* antioxidant activity:**
To determine whether compounds 1,4-naphthoquinones and naphthofurans could exert significant antioxidant activity, compounds 1-8 were evaluated using DPPH and ABTS assay.

DPPH radical scavenging assay:
DPPH radical scavenging assay has been widely used to evaluate the antioxidant capacity, which is stable due to its resonance stability and special blockade of benzene rings [20]. The purple chromogen radical DPPH is reduced by antioxidant compounds to the corresponding pale yellow hydrazine. Various concentrations (5, 10, 20, 50, 100, and 200 μg/mL) of compound (0.3 mL) were mixed with 2.7 mL of 0.1 mM solution of DPPH in methanol. After shaken for 60 seconds in microplate reader, it was left in the dark at 37°C for 30 min. The absorbance was then measured at 515 nm using a spectrophotometer [21]. Methanol was used as the blank control and ascorbic acid served as positive control. The DPPH radical scavenging activities were calculated according to the following formula.

$$\% \text{ DPPH scavenging activity} = (A_{blank} - A_{sample})/A_{blank} \times 100$$

All experiments were carried out in triplicate and the results were expressed as mean values ± standard deviations. The compound concentration providing 50% inhibition (IC_{50}) was calculated from the values and graphs of percentage scavenging activity against concentration of sample/compound were plotted.

ABTS radical scavenging assay:
The ABTS test is the most popular among other indirect assays for water-soluble phenolics [22]. The idea of the method is to monitor the decay of the radical cation ABTS•+ produced by the oxidation of ABTS caused by the addition of a phenolic containing sample. In the absence of phenolics, ABTS•+ is rather stable, but it reacts energetically with H-atom donor, such as phenolics, being converted into a colorless form of ABTS. ABTS•+ has high molar absorptivity at 734 nm and can be easily determined spectrophotometrically. The advantage of ABTS derived free radical scavenging method over other antioxidant screening methods is that, the produced color remains

stable for more than 1 h and the reaction is stoichiometric. ABTS•+ was generated by the interaction of 5.0 ml ABTS solution (1.8 mM) mixed with 1.25 mL potassium persulfate (2.0 mM) and kept in dark at room temperature for 2 h, then diluted five times with phosphate buffer pH 7.0 (0.02 mM). Then 0.4 mL of various concentrations (5, 10, 20, 50, 100, and 200 μg/mL) of samples in methanol were taken, mixed with 3.6 mL of ABTS solution, and kept in the dark for 10 min. The absorbance was measured at 734 nm [23]. ABTS solution was used as the blank control and ascorbic acid served as positive control. The ABTS radical scavenging activities were calculated according to the following formula.

$$\% \text{ ABTS scavenging activity} = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

All experiments were carried out in triplicate and the results were expressed as mean values ± standard deviations. The compound concentration providing 50% inhibition (IC_{50}) was calculated from the values and graphs of percentage scavenging activity against concentration of sample/compound were plotted.

***In vitro* cytotoxic activity:**

Compounds 1-8 were tested for their *in vitro* cytotoxic activities using human hepatocellular carcinoma (HepG2) cell line [24]. Cell viability in the presence or absence of tested compounds was determined using the MTT method. The HepG2 cell line was procured from National Centre for Cell Sciences, Pune, India and maintained in standard MEM with FBS (10%) supplement, penicillin (100 U/mL) and streptomycin (100 μg/mL) solution. For cytotoxicity evaluation, exponentially growing cells were harvested and plated in 96 wells plate (2×10^4 /well) in MEM at 37°C under an atmosphere of humidified CO_2 (5%) and air (95%) for 24 h. After the cells had been washed with FBS, the medium was changed to serially diluted test samples in MEM. After 48 h of incubation, the cells were washed thrice with PBS, and MTT solution was added and incubated for 4 h at 37°C. Then, medium was removed. DMSO (100 μL) was added and absorbance at 570 nm was determined by microplate reader. IC_{50} was calculated using regression analysis in MS excel. Morphological figure given for cyclophosphamide standard [25] was used for comparison. The % cell viability was calculated using the following formula:

$$\% \text{ Cell viability} = \frac{\text{Mean OD of test sample}}{\text{Mean OD of negative control}} \times 100$$

The % cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = 100 - \% \text{ Cell viability.}$$

Data are presented with descriptive analysis (mean ± SD for 3 (n=3) independent experiments). Probability $p \leq 0.05$ was accepted as the significance level.

RESULTS AND DISCUSSION

Chemistry

Isolation of compounds 1-3:

In ^{1}H NMR spectrum of the Compound 1, the signal of H-2 and H-3 protons (i.e., olefinic protons) appears at 7.02-7.10 ppm. The -OH group signal appears at 11.75 ppm in DMSO-d_6, which is further confirmed by D_2O exchange. H-6 and H-8 protons appear as doublet at 7.33-7.35 and 7.48-7.50 ppm respectively. The remaining aromatic proton at H-7 appears as triplet at 7.72-7.77 ppm. The ^{13}C NMR spectrum of compound 1 show the presence of ten carbon signals, of which five are methine signals and the rest are quaternary carbon signals. The downfield signals at 184.2 and 190.3 ppm clearly belong to the carbonyl carbon atoms C-1 and C-4. Among the two carbonyl carbon atoms, the one at more deshielded (190.3 ppm) is assigned to C-4. The signal at 161.4 ppm is assigned to C-5 carbon. The two remaining quaternary carbon signals at 131.7 and 114.9 ppm are obviously due to C-9 and C-10. The three methine carbons of the naphthalene ring are appear at 114.9, 136.5 and 124.5 ppm but difficult to assign individually. The remaining olefinic methine signals at 138.6 and 139.6 could be assigned to C-2 and C-3. IR spectrum shows the presence of broad-low intensity band at 3066 cm^{-1}, which belongs to the presence of a hydroxyl group. The two *α,* β-unsaturated carbonyl bands at 1642 and 1596 cm^{-1} assigned to free carbonyl and carbonyl group chelated with *α*-hydroxyl respectively. The band at 1225 cm^{-1} is assigned to the C-O stretching vibration. Mass spectrum shows the parent ion peak (M^+) at 174. The melting point was found to be 162-165°C. The structural assignments were correlated with the published data [26] and confirmed that the compound 1 is 5-hydroxy-1,4-naphthoquinone known

as Juglone. In ^{1}H NMR spectrum of Compound 2, the signal of H-3 proton (i.e., olefinic proton) appears as a sharp singlet at 6.370 ppm. The -OH group signal appears at 7.363 ppm in $CDCl_3$, which is further confirmed by D_2O exchange. Doublet type of signals at 8.112-8.138 belongs to H-5 and H-8 and the triplet type signals at 7.701-7.751 and 7.780-7.829 belong to H-6 and H-7 protons respectively. The ^{13}C NMR spectrum shows the presence of ten carbon signals, of which five are methine signals and the rest are quaternary carbon signals. The downfield signals at 184.9 and 181.9 ppm belong to the carbonyl carbon atoms C-1 and C-4. Among the two carbonyl carbon atoms, the more deshielded signal (184.9 ppm) is assigned to C-4, whereas the signal at 156.3 ppm is assigned to C-2. The two remaining quaternary carbon atoms C-9 and C-10 resonate very closely together at 129.4 and 132.9 ppm. The four methine carbons of the naphthalene ring are difficult to assign individually. The remaining olefinic methine signal at 110.7 ppm could be assigned to C-3, due to its β-position with respect to the hydroxyl group. IR spectrum shows the presence of broad-medium intensity band at 3174 cm^{-1}, which belongs to the presence of a hydroxyl group. The two *α,* β-unsaturated carbonyl bands at 1679 and 1641 cm^{-1} assigned to free carbonyl and carbonyl group chelated with *α*-hydroxyl respectively. The band at 1222 cm^{-1} assigned to the C-O stretching vibration. Mass spectrum shows the parent ion peak (M^+) at 174. The melting point was found to be 193-195°C. The structural assignments were in agreement with the published data [27] and confirmed that the compound **2** is 2-hydroxy-1,4-naphthoquinone known as Lawsone. For the first time, the lawsone (isomer of Juglone) has been isolated from *Juglans regia* as a minor constituent. In ^{1}H NMR spectrum of Compound 3, the methyl signal appears at 2.19 ppm. H-3 proton (i.e., olefinic proton) appears at 6.803-6.808 ppm. The signal for -OH group appears at 11.969 ppm in $CDCl_3$, which is further confirmed by D_2O exchange. H-7 proton signals appeared as double doublet at 7.233-7.264 ppm. The multiplet appeared at 7.597-7.627 assigned to H-6 and H-8 protons. The ^{13}C NMR spectrum shows the presence of eleven carbon signals, of which six are methine signals, four are quaternary carbon signals and rest is methyl signal. The two downfield signals at 190.2 and 184.7 ppm are clearly belong to carbonyl atoms at C-4 and C-1, respectively. The signal at 149.6 is assigned to C-2 carbon whereas the signal at 161.1 ppm is assigned to C-5 carbon. The two remaining quaternary carbon atoms C-9 and C-10 resonate at 132.0 and 115.1 ppm, respectively. The upfield signal at 16.5 ppm is assigned to methyl group at C-2. The three methine carbons of the naphthalene ring are difficult to assign individually. The remaining olefinic methine signal at 136.0 ppm could be assigned to C-3. IR spectrum shows the presence of two carbonyl groups, with the hydrogen bonded one is appearing at 1607 and the other at 1644 cm^{-1}. The band at 1258 cm^{-1} assigned to the C-O stretching vibration. Mass spectrum shows the parent ion peak (M^+) at 188. The melting point was found to be 75-78°C. The structural assignments proved unequivocally that the compound 3 is 5-hydroxy-2-methyl-1,4-naphthoquinone known as Plumbagin and also in agreement with the published data [28]. Structures of compounds 1-3 are given in Figure 1 and spectral details are provided as the supplementary document.

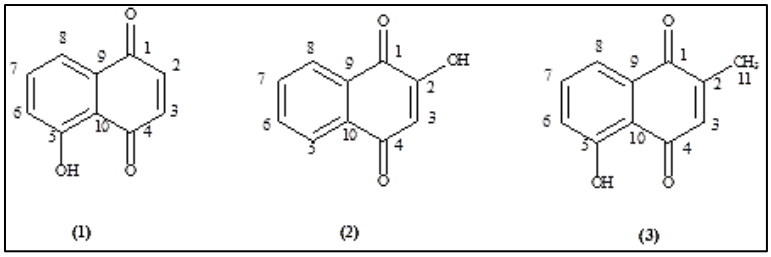
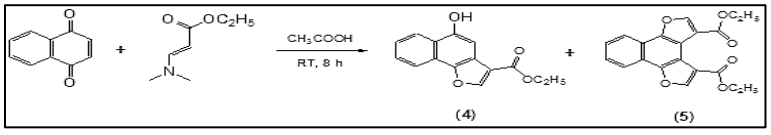


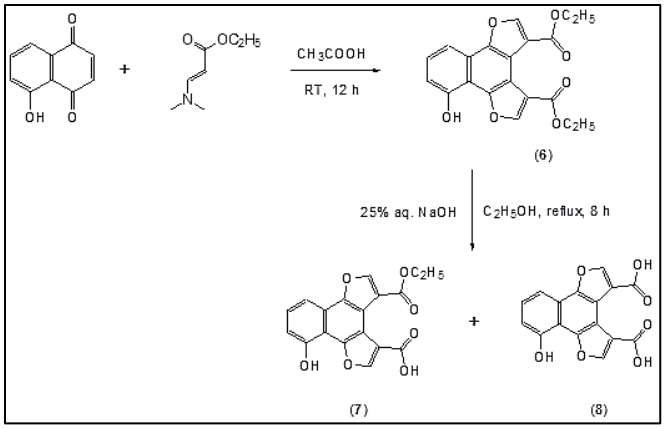
Figure 1: Structures of isolated compounds 1-3

Synthesis of Compounds 4-8

The compounds 4-8 were synthesized and characterized as per the procedure reported in our previous paper [19]. Scheme 1 reaction favours to two products [monofuran derivative (4) and difuran derivative (5)], whereas in scheme 2, we got only one compound [diester derivative (6)]; varying molar equivalents of N,N-Dimethyl aminoacrylic acid ethyl ester also did not yield monofuran derivative; hydrolysis of 6 with different molar eq of NaOH afforded mono acid (7) and diacid (8). However, mechanism for the formation of two products in scheme 1 and one product in Scheme 2 are unknown. Spectral details for compounds 4-8 are provided as the supplementary document.



Scheme 1: Synthetic route for compounds 4-5



Scheme 2: Synthetic route for compounds 6-8

Biological Activity

Antioxidant studies:

DPPH assay provides information on the reactivity of the samples with a stable free radical. Because of the odd electron, it shows a strong absorption band at 515 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolourization is stoichiometric with respect to the number of electrons taken up. ABTS is also a relatively stable free radical. The ABTS•+ model can be assessed the scavenging activity for both polar and non-polar samples and the spectral interference is lessened as the absorption maximum often used is a wavelength not normally encountered by natural products. Aromatic phenolic compounds have the ability to donate hydrogen and electrons, and can thus be detected by antioxidant assay methods. Hence, the antioxidant activity was measured by DPPH and ABTS radicals scavenging method for the compounds 1-8 and the results are shown in Figures 2 and 3.

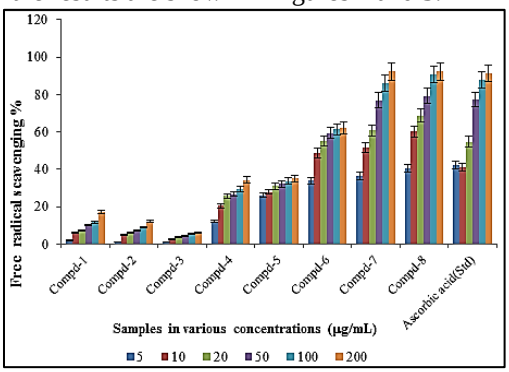


Figure 2: DPPH free radical scavenging activities of compounds (1-8)

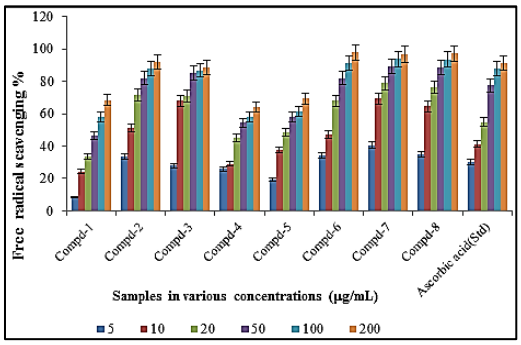


Figure 3: ABTS free radical scavenging activities of compounds (1-8)

Antioxidant assay of any organic compound is mainly depends on its structure. For a compound showing antioxidant activity, presence of phenolic -OH group is very essential; but not important [29]. Among the isolated compounds, phenolic -OH group is present in 1 and 3, which is absent in 2. But in 1 and 3, hydrogen atoms of hydroxyl groups

are not available for interaction, as they are already involved in a strong intra-molecular hydrogen bonding (between the hydrogen atom of -OH group and oxygen atom of adjacent ketone group). In addition, intermolecular hydrogen bonding is also present in these two compounds. Due to the presence of these hydrogen bonding, the -OH group present in these compounds are not behaving as phenolic -OH group. Compound 2 contain only intermolecular hydrogen bonding. Generally, introduction of electron donating groups (such as hydroxyl) to a molecule increases the antioxidant activity. The compounds 6, 7 and 8 displayed almost equivalent scavenging activity on DPPH• radical (IC_{50} = 10.27, 9.72, and 8.31 µg/mL respectively) relative to ascorbic acid (IC_{50} = 5.88 µg/mL). This may be due to the presence of phenolic -OH group in all these three compounds. Though the structures of 5 and 6 are similar, phenolic -OH is absent in 5. Hence, antioxidant activity is absent in 5. Among the isolated compounds 1-3 isolated from chloroform extract, the ABTS•+ scavenging ability of the 2 and 3 was higher than that of ascorbic acid, but 1 showed lesser activity than ascorbic acid. Among the compounds 4-8, the ABTS•+ scavenging ability of 6, 7 and 8 was higher than that of ascorbic acid (i.e., IC_{50} = 10.60, 6.14, 7.75 µg/mL respectively); 5 showed almost equivalent activity (IC_{50} = 20.67 µg/mL) with ascorbic acid and 4 had lesser activity (IC_{50} = 45.97 µg/mL) than ascorbic acid. While comparing the antioxidant activities of DPPH and ABTS, the antioxidant activity (IC_{50}) of 6, 7 and 8 by DPPH were in accordance with the results obtained by ABTS method. The results of rest of the compounds were not comparable with each other. DPPH assay was carried out in methanol solvent, whereas water was used as a solvent in ABTS method. The difference in solvating power of these two solvents towards the samples could be the reason for such a difference in antioxidant activities.

Cytotoxic studies:

The predictive value of *in vitro* cytotoxicity test is based on the idea of 'basal' cytotoxicity i.e. toxic chemicals affect basic functions of cells which are common to all cells and that the toxicity can be measured by assessing cellular damage. The development of *in vitro* cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible and to carry out tests with small quantities of compound [30]. In the previous paper we have reported the cytotoxicity of the compounds 1-8 in HeLa cell line [19]. Even though once the compound is toxic to cells, it will exhibit cytotoxic effects to most cancer cell lines. However, it was observed that some compounds are toxic in one cell line but not in other cell line [31]. For example, the anti-cancer activities of berberine (BBR) have been reported extensively in various cancer cell lines but, the minimal inhibitory concentrations of BBR varied greatly among different cell lines and very few studies have been devoted to elucidate this aspect. Hence we have made an attempt to check the toxicity of synthesized and isolated compounds in HepG2 cell line. The cytotoxic effects of the compounds 1-8 at various concentrations were evaluated against HepG2 cell lines and % cell viability was measured by MTT assay. The amount of formazan crystals produced by MTT is directly proportional to the number of viable cells. The compounds are usually regarded as interesting for *in vitro* cytotoxic activity if IC_{50} <100 µg/ml [32]. Among the tested compounds, the compounds 1, 3 and 5 showed promising anti-proliferative activity against HepG2 cell line with IC_{50} 48.61 µg/mL, 58.63 µg/mL and 26.05 µg/mL respectively along with standard (Cyclophosphamide), which showed 22.15 µg/mL. Rest of the compounds did not show any activities at all concentrations tested. The % cell viability and % cytotoxicity of compounds 1, 3, and 5 are shown in Figures 4 and 5 respectively. Microscopic images of control cancer cell and apoptotic morphological changes in HepG2 cell line treated with compounds 1, 3 and 5 are shown in Figure 6. From the results the Compound **5** without any hydroxyl functionality exhibited significant cytotoxicity. However, the same compound did not exhibit cytotoxic effect against HeLa cell growth in our previous work [19]. Similarly, compound **7** shares the structural similarity with Juglone, Plumbagin, and compound **5** respectively but did not show cytotoxicity against HepG2 cells. The anomaly may be due to penetration/ concentration difference of compounds varies with different cell lines. Comparison of transportation and distribution of BBR among three cancer cell lines, HepG2, HeLa and SY5Y have been studied and the HPLC results showed that BBR was capable of penetrating all the cell lines whereas the cumulative concentrations were significantly different. HepG2 cells accumulated higher level of BBR for longer duration than the other two cell lines [31]. The biological effects of quinones are largely mediated by the formation of reactive oxygen species through redox activation and the covalent modification. Juglone is one of the important derivatives of quinone and it is a strong cytotoxic agent. Its cytotoxicity is based on its high reactivity with oxygen, and it is frequently applied as a free radical enhancer [33]. Juglone is reported to occur in various parts of walnut plants including fresh walnut leaves and nut hulls [34,35]. Although, the mode of action of Juglone is really unknown, there has been some indication of cytotoxicity [36].

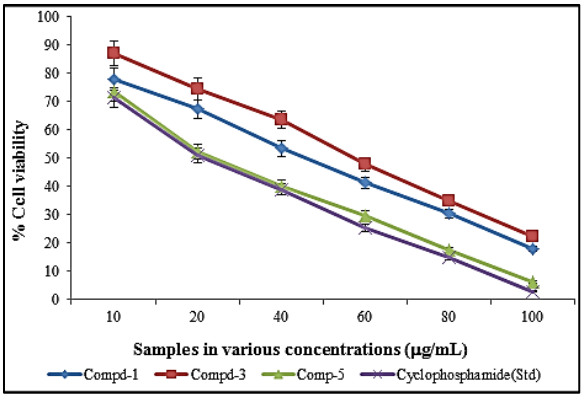


Figure 4: *In vitro* cell viability of compounds-1, 3 and 5 (MTT assay method)

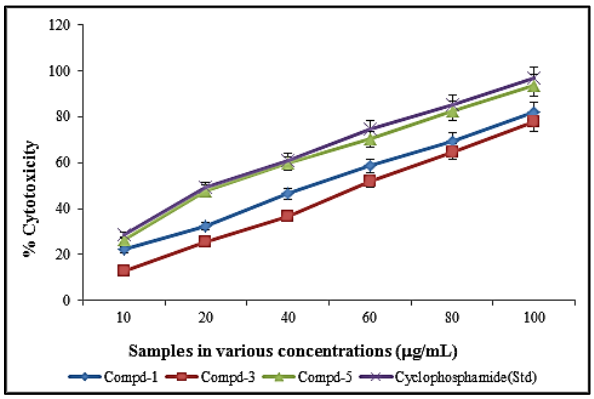
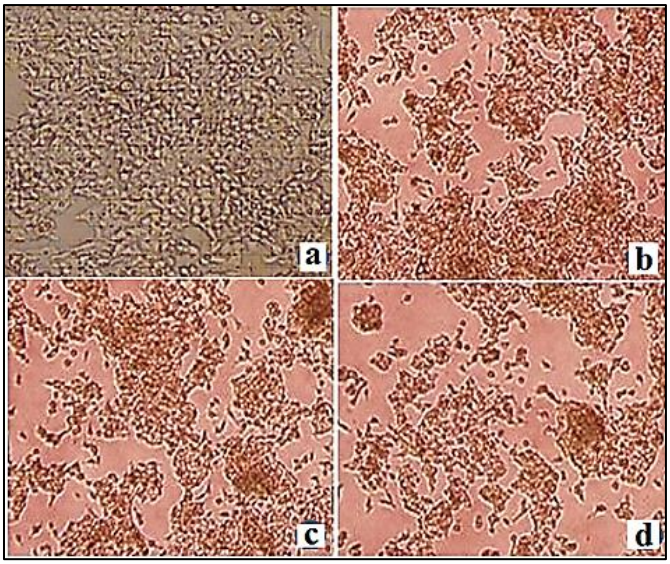


Figure 5: *In vitro* cytotoxicity against cancer cells of compounds-1, 3 and 5



Note: a) Control; b) Treated with Compd-1 (48.61 µg/mL); c) Treated with Compd-3 (58.53 µg/mL); d) Treated with Compd-5 (26.05 µg/mL)

Figure 6: Cell damage/cell death observed in compds 1, 3 and 5 against HepG2 cell lines (Apoptotic morphological changes observed in the cells)

The naphthoquinone derivative lawsone is structurally similar to juglone and also used for the synthesis of pharmacologically active compounds such as Lapachol, α-Lapachone and β-Lapachone [37]. Another naphthoquinone derivative found in walnut is plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), which is a potential drug candidate and has been shown to have anticancer, antileishmanial, antibacterial and antifungal

properties [38-40]. Investigations indicate that the activities such as antioxidant, antiinflammatory, anticancer and antimicrobial arise mainly out of plumbagin's ability to undergo redox cycling, generating reactive oxygen species and chelating trace metals in biological system [41].

Investigations on Ehrlich ascetic tumor activity showed that only juglone inhibited tumor development and lawsone was inactive [42]. Juglone has been reported to inhibit intestinal carcinogenesis [43] and it was also proven to be a potent cytotoxic agent in human tumor cell lines by *in vitro*, including human colon carcinoma (HCT-15) cells, human leukemia (HL-60) cells and doxorubicin-resistant human leukemia (HL-60R) cells [44,45]. Moreover, juglone inhibits the growth and induce apoptosis of sarcoma 180 cells [46] and human gastric cancer SGC-7901 cells [47]. Cytotoxicity of Juglone and its underlying mechanisms against melanoma cells using MTT and clonogenic assays was reported [48]. It was found that Plumbagin inhibits growth and invasion of androgen independent prostate cancer (AI PCa) cells [49]. As supported by the literature Juglone and Plumbagin shows similar activity in the present investigation. Furthermore, we are the first to report that compound **5** have anti-proliferative activity against HepG2 lines.

CONCLUSION

In the present study, the naphthofurans 6, 7 and 8 displayed almost equivalent scavenging activity on DPPH assay and higher activity on ABTS assay relative to ascorbic acid. On the other hand naphthoquinones 1 and 3 showed lesser antioxidant activity but higher cytotoxic activity than naphthofurans except compound 5 which showed promising anticancer activity. *In vitro* cytotoxicity screening of juglone (1), plumbagin (3) and compound (5) in human hepatoma cells (HepG2) showed toxicity at 10 µg/mL concentration, whereas lawsone (2) showed no toxicity to the cells even up to 100 µg/mL concentration. Among the compounds 4-8, the compounds 4, 6, 7 and 8 did not show any toxicity against HepG2 cells up to 100 µg/mL, however 5 showed highest toxicity against cancer cell line at lowest (10 µg/mL) concentrations. Compound 5 without any hydroxyl functionality exhibited significant anticancer and can be considered further in the on-going drug discovery program. The molecular designs and rationalization presented with this can be a tool to process for further modification in the molecular systems to obtain an advanced level of therapeutic potencies.

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