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Synthesis of antioxidative anthraquinones as potential anticancer agents

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ABSTRACT

Antioxidant and antibacterial activities of natural anthraquinones namely chrysophanol (**1**) and emodin (**2**), and synthesized anthraquinones *viz.* 2-methylanthraquinone (**3**), anthraquinone (**4**), 2-bromoanthraquinone (**5**), rubiadin (**6**), chrysophanol diacetate (**7**), rubiadin diacetate (**8**) and 1,8-dimethoxy-3-methylanthraquinone (**9**) were investigated. Anthraquinones **9**, **3**, **6**, **5** and **2** exhibited a high DPPH• radical scavenging capacity (IC_{50} = <500 µg/mL) showing their therapeutic potentiality for the treatment of cancers. These anthraquinones **1-9** have also displayed a weak to moderate antibacterial activity against *Bacillus subtilis*. Chrysophanol diacetate (**7**) including emodin (**2**) have been appeared as the valuable antibacterials.

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1. Introduction

Anthraquinones are important members of the quinone family that have profound therapeutic values as antibacterial, antifungal, anticancer, antioxidant, laxative, diuretic, estrogenic, anti-inflammatory, antiarthritic, etc. [1-3]. Anthraquinone structural motif is present in many natural dyes, pigments, vitamins, enzymes etc [4]. Among 700 natural anthraquinones identified, about 200 compounds were isolated from plants and remaining from lichens, fungi and others [5-6]. Anthraquinones are widely distributed in *Aloe species* of Liliaceae, *Semen cassiae* and *Folium sennae* of Leguminosae, *Radix rubiae* of Rubiaceae, *Cascara sagrada* of Rhamnaceae, and also widely exist in a number of species of Polygonaceae family especially in the genuses *Rheum*, *Rumex* and *Polygonum*. They are more commonly available in the important herbs like *Rheum officinale*, *Polygonum cuspidatum*, *Polygonum multiflorum* and *Polygonum bistorta*.

Antioxidant molecules inhibit oxidation of other compounds. Reactive free radicals, particularly

reactive oxygen species such as superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and nitric oxide ($NO^{\bullet}$), are produced in living organisms during metabolisms. An imbalance between the production of such free radicals and inability of the body to eliminate them through the use of endogenous and exogenous antioxidants result oxidative stress [7]. Oxidative stress causes oxidative damage which eventually leads to a large number of diseases including cancers, mutagenesis, cardiovascular diseases, neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases), immunodeficiency syndrome, diabetes, cellular aging, etc [8-10].

The important reaction used for the laboratory preparation of anthraquinones is Friedel-Crafts acylation between phthalic anhydrides and benzene derivatives. In conventional two steps process, *o*-aroylbenzoic acids are first prepared in the presence of $AlCl_3$ by the Friedel-Crafts acylation [11]. In the second step, intramolecular condensation of aryl and *o*-aroylbenzoic acid is performed to produce anthraquinones using H_2SO_4 or oleum [12], benzoyl chloride and concentrated H_2SO_4 [13-14], benzoyl chloride and $ZnCl_2$ [15], $POCl_3$ and $P_2O_3Cl_4$ [16], or $B(HSO_4)_3$ [17]. A eutectic mixture of $AlCl_3$ and NaCl melt has also been used to prepare various anthraquinones by the Friedel-Crafts acylation between phthalic anhydrides and benzene derivatives [18-19]. Montmorillonite K10 clay [20], alum ($KAl(SO_4)_2.12H_2O$) [4] and $M^{IV}PWs$ (M^{IV} = Zr, Ti and Sn; and PW = phosphotungstate) [21] as heterogeneous solid catalysts have also been explored, but these catalyst are not easily available.

The synthesis of anthraquinones has received a great attention in the recent past due to their therapeutic values as well as synthetic challenges. Total syntheses of some complex anthraquinones such as uncialamycin [22], dynemicin A [23], daunomycin [24], marmycin A [25], (±)-brasiliquinone B [26], etc. have been reported (Figure 1). Synthesis of these bioactive complex molecules requires multiple steps and are therefore available in poor quantities diminishing their therapeutic usefulness. Hence, our researches focus on isolation and/or synthesis of simple organic molecules and/or their derivatives (which can be easily obtained and/or synthesized in a large quantity) possessing remarkable medicinal values [27-31]. Recently, we have reported isolation of two anthraquinones *viz.* chrysophanol (**1**) and emodin (**2**) from the rhizomes of *Rheum australe* (Figure 1) [32]. Herein, we studied antioxidant capacity of these anthraquinones **1**-**2** as well as some newly synthesized anthraquinones **3**-**9**. Antibacterial activity of the anthraquinones **1**-**9** is also screened against 6 bacterial strains.

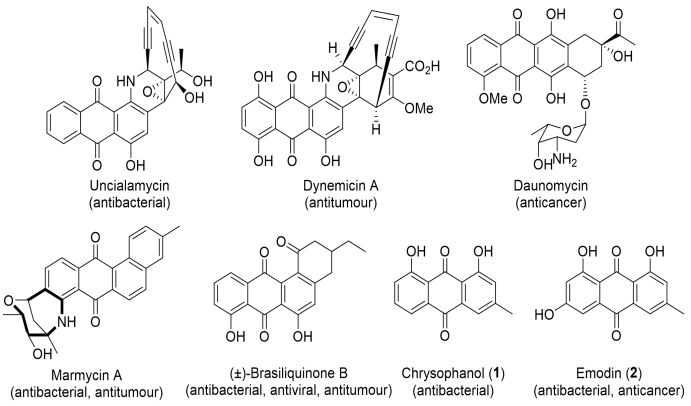


Fig. 1: Structures of some anthraquinones with their medicinal values.

2. Experimental

Chemicals and equipments

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was procured from Sigma-Aldrich. Gallic acid was purchased from Sisco. Analytical thin layer chromatography (TLC) was performed on 0.2 mm pre-coated Kieselgel 60 F_{254} plate (E. Merck). Melting points (Mp) were determined by using a Thile's tube and are uncorrected. UV-Vis spectra were recorded on Cary 60 UV-Visible Spectrophotometer (Agilent). Spectrophotometry was carried out by using an Elisa microplate reader (EPOCH2, BioTek Instruments). IR spectra were recorded on IR Tracer-100 (Shimadzu) in KBr disc.

Previously isolated chrysophanol (**1**) and emodin (**2**) were also used in this work [32].

Procedures for the synthesis of anthraquinones

Method 1. ***Friedel-Crafts and intramolecular condensation reactions to synthesize anthraquinones 3-5***

Two-steps reaction protocol was followed to synthesize anthraquinones **3-5** [11-12]. Briefly, phthalic anhydride (1.48 g, 10 mmol) and a benzene derivative (10 mL of either toluene, benzene or bromobenzene) were taken in a two-necked round bottom flask equipped with a magnetic stirrer, condenser, recovery bend and receiver adapter (that immersed in water containing conical flask). The content was stirred at 0 °C and then introduced $AlCl_3$ (3.07 g, 23 mmol) slowly. The reaction mixture was refluxed at 90 °C until the vapour of HCl was ceased. Completion of the reaction was monitored by TLC (silica gel, hexane:EtOAc, 1:1). After cooling, the reaction mixture was decomposed by addition of ice water and 3M HCl (25 mL). The content was extracted with Et_2O (20 mL × 3), washed with dil. HCl, dried over Na_2SO_4 and filtered. The solvent was evaporated and the obtained residue was vacuum dried. In the second step, dried intermediate product was refluxed with conc. H_2SO_4 (10 mL) at 100 ^oC for 1.5 hours. After cooling, ice water was added slowly to decompose the reaction mixture and then filtered. The residue on the filter paper was washed with 32% NH_3 solution until neutralization followed by water. Pure anthraquinones **3-5** were obtained after recrystallization using EtOH.

Method 2. ***$AlCl_3$:NaCl melt method to synthesize anthraquinone 6***

Anhydrous $AlCl_3$ (13.33 g, 100 mmol) and NaCl (5.32 g, 91 mmol) were taken in a 500 mL round bottom flask and were melted at 125-130 ^oC. To this was added a mixture of phthalic anhydride (2.66 g, 18 mmol) and 2-methyl resorcinol (2.23 g, 18 mmol) in a small portion at a time. Now the reaction temperature was raised to 165-175 ^oC and stirred for 30 minutes. Thereafter, additional $AlCl_3$ (13.33 g, 100 mmol) was added and stirring was continued for another 30 minutes. A red solid product was formed, which was decomposed by addition of ice water (125 mL) and conc. HCl (125 mL) after cooling. The reaction mixture was extracted with Et_2O (100 mL × 3). The etherial layer was washed with dil. HCl, dried over Na_2SO_4, filtered and concentrated. The residue obtained was purified by flash silica gel column chromatography using mixtures of hexane/EtOAc to give rubiadin **6**.

Method 3. ***Acetylation reaction to synthesize anthraquinones 7 and 8***

A mixture of chrysophanol (**1**) (0.254 g, 1 mmol), acetic anhydride (3.66 mL, 38.8 mmol) and pyridine (3.5 mL, 44 mmol) was stirred at room temperature for 4 hours. Thereafter, ice cooled water was added dropwise to the reaction mixture for the precipitation. The precipitate was collected by filtration and the acetylated product **7** was purified by silica gel column chromatography using mixtures of hexane/EtOAc. To prepare anthraquinone **8**, instead of chrysophanol (**1**), rubiadin (**6**) (0.254 g, 1 mmol) was used as the starting material for the acetylation reaction.

Method 4. ***Methylation reaction to synthesize anthraquinone 9***

A mixture of chrysophanol (**1**) (0.127 g, 0.5 mmol), K_2CO_3 (0.760 g, 5.5 mmol), $(CH_3)_2SO_4$ (0.7 mL, 7 mmol) and dry acetone (100 mL) were taken in a round bottom flask equipped with a reflux condenser and $CaCl_2$ guard tube. The reaction mixture was refluxed for 4 hours. Thereafter, the reaction mixture was cooled, filtered and concentrated. Thus obtained residue was purified by silica gel column chromatography using mixtures of hexane/EtOAc to afford anthraquinone **9**.

Analytical data of the synthesized anthraquinones

All the synthesized anthraquinones **3-9** were analyzed by recording of Mp, UV-Vis spectra and

FT-IR spectra, and the data were compared with the available literature.

*2-Methylanthraquinone; 2-methylanthracene-9,10-dione (**3**)*: Method 1. Yellow solid. Yield 1.58 g, 71.1% in two steps. Mp 176 °C (reported 176-178 °C) [4]. UV-Vis (MeOH) λ_{max} nm: 328, 275(s), 256, 206. IR (KBr) ν cm^{-1}: 3000 (C–H aromatic), 2950 (C–H alkyl), 1720 (C=O), 1700 (C=O), 1575 (C=C aromatic), 1285 (C–H bend) [33].

*Anthraquinone; anthracene-9,10-dione (**4**)*: Method 1. Yellow solid. Yield 1.64 g, 78.8% in two steps. Mp 280 °C (reported 283-287 °C) [34]. UV-Vis (MeOH) λ_{max} nm: 325, 271(s), 251, 204 [35]. IR (KBr) ν cm^{-1}: 1700 (C=O), 1590 (C=C aromatic), 1350, 1325 (C–H bend) [36].

*2-Bromoanthraquinone; 2-bromoanthracene-9,10-dione (**5**)*: Method 1. Colourless solid. Yield 2.26 g, 78.7% in two steps. Mp 203 °C (reported 205-206 °C) [4]. UV-Vis (MeOH) λ_{max} nm: 283, 228, 205. IR (KBr) ν cm^{-1}: 1750 (C=O), 1600 (C=O), 1525 (C=C aromatic), 1250 (C–H bend), 810 (C–Br stretch), 500 [33].

*Rubiadin; 1,3-dihydroxy-2-methylanthraquinone; 1,3-dihydroxy-2-methylanthracene-9,10-dione (**6**)*: Method 2. Yellow needles. Yield 2.35 g, 51.4%. Mp 280 °C (reported 281-282 °C) [37]. UV-Vis (MeOH) λ_{max} nm: 389, 256, 221. IR (KBr) ν cm^{-1}: 3450 (–OH), 2950 (C–H alkyl), 1750 (C=O), 1650 (C=O), 1350 ($–CH_3$) [38].

*Chrysophanol diacetate; 3-methyl-9,10-dioxo-9,10-dihydroanthracene-1,8-diyl diacetate (**7**)*: Method 3. Yellow needles. Yield 0.210 g, 62.1%. Mp 204 °C. UV-Vis (MeOH) λ_{max} nm: 428, 285(s), 256, 223, 202. IR (KBr) ν cm^{-1}: 3000 (C–H alkyl), 1750 (two coalescence peaks, C=O), 1670 (C=O), 1650 (C=O), 1600 (C=C aromatic), 1300, 1275 (C–H bend) (Figure 2A).

*Rubiadin diacetate; 2-methyl-9,10-dioxo-9,10-dihydroanthracene-1,3-diyl diacetate (**8**)*: Method 3. Yellow needles. Yield 0.20 g, 59.1%. Mp 164 °C. UV-Vis (MeOH) λ_{max} nm: 415, 277, 244, 204. IR (KBr) ν cm^{-1}: 2975 (C–H alkyl), 1755 (C=O), 1750 (C=O), 1680 (C=O), 1630 (C=O), 1370 ($–CH_3$), 1275 (C–H bend), 1200 (Figure 2B).

*1,8-Dimethoxy-3-methylanthraquinone; 1,8-dimethoxy-3-methylanthracene-9,10-dione (**9**)*: Method 4. Yellow solid. Mp. 193 °C (reported 195 °C) [39-40]. Yield 0.116 g, 82.2%. UV-Vis (MeOH) λ_{max} nm: 338, 256, 206. IR (KBr) ν cm^{-1}: 2950 (C–H alkyl), 1650 (C=O), 1325 (C=C aromatic), 1275, 1230, 1010 (C–O alkoxy), 955 (C–O alkoxy).

Antioxidant capacity: DPPH• radical scavenging assay

The DPPH• radical solution (concentration 0.1 mM) was prepared by overnight stirring of 3.9 mg of DPPH in methanol (100 mL) at 4 °C. Four working solutions of 25, 50, 250 and 500 µg/mL concentrations were prepared for each anthraquinone sample. To 0.5 mL of the working solution, 2.5 mL of the DPPH• radical solution was added. Control was prepared by adding 2.5 mL of the DPPH• radical solution in methanol (0.5 mL). The solutions were mixed well and were kept in dark at room temperature for 30 minutes. The absorbance was measured at 517 nm against blank solution consisting water (0.5 mL) and MeOH (2.5 mL). The inhibition percentage at different concentrations was calculated by using the equation shown below. Statistical analysis was done by using Microsoft excel program.

$$\text{DPPH• scavenging rate (\%)} = 1 - \frac{\text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Antibacterial activity: Agar well diffusion assay

Three gram-positive bacteria *viz. Bacillus subtilis*, *Staphylococcus aureus* (ATCC 25952), *Enterococcus faecalis*, and three gram-negative bacteria *viz. Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATTCC 27853) and *Salmonella typhimurium* (ATCC 14028) were used for the antibacterial assay. These bacterial strains were obtained from National Endemic Health Care Centre, Teku, Kathmandu, Nepal. The pure bacterial cultures were streaked in the nutrient agar slants for sub-culture. Thus developed each pathogenic bacterium were transferred into a separate nutrient broth by a sterile loop and incubated at 37 °C for 24 hours.

Stock sample solutions of each anthraquinone were prepared by dissolving 0.01 g of the sample in 1 mL of DMSO (concentration 10 mg/mL). Now, the sample solutions (50 µL of each) were charged into the 6 mm wells bored on agar plates. DMSO was used as negative control. Gentamycin (10 µg/dics) was used as positive control. The plates were then incubated at 37 ºC for 24 hours, and the zone of inhibition (ZOI) produced was measured.

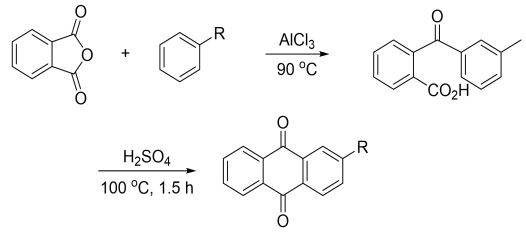
3. Results and Discussion

Anthraquinones synthesis

Following the known protocols, anthraquinones **3**-**6** were synthesized by the Friedel-Crafts acylation reaction between phthalic anhydride and benzene derivatives (toluene, benzene, bromo benzene or 2-methyl resorcinol). For the synthesis of anthraquinones **3**-**5**, a two-steps protocol – the Friedel-Crafts reaction using $AlCl_3$ followed by H_2SO_4-mediated intramolecular condensation – was used (Scheme 1) [11-12]. Anthraquinone **6** was prepared in a single-step by the Friedel-Crafts acylation reaction between phthalic acid and 2-methyl resorcinol using $AlCl_3$:NaCl melt following a known procedure (Scheme 2) [38]. Synthesis of anthraquinones **3**-**6** were confirmed by comparing Mp, UV-Vis spectra and IR spectra reported in the literature.

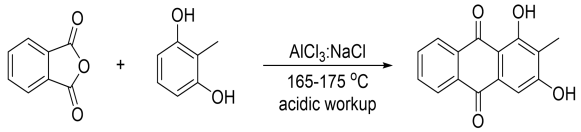
Phenols are commonly acetylated with acetic anhydride in the presence of pyridine [41]. Acetylations of previously isolated chrysophanol (**1**) and newly synthesized rubiadin (**6**) were thus performed under un-optimized conditions to afford the corresponding acetyl derivatives **7** and **8** in 62.1% and 59.1% yields, respectively (Scheme 3). In the IR spectrum of compound **7**, disappearance of a broad peak of the hydroxyl group that appeared in the IR spectrum of chrysophanol (**1**) at 3420 cm^{-1}, and appearance of two coalescence peaks at 1750 cm^{-1} and two sharp peaks at 1670 and 1650 cm^{-1} for four C=O groups confirmed its synthesis (Figure 2A). In a similar way, rubiadin (**6**) had peaks at 3450 (–OH), 1750 (C=O) and 1650 (C=O) in the IR spectrum. On the other hand, its acetyl derivative **8** showed disappearance of the hydroxyl group peak and appearance of the two additional carbonyl peaks (all together four C=O groups at 1755, 1750, 1680 and 1630 cm^{-1}) confirmed its formation (Figure 2B).

Scheme 1



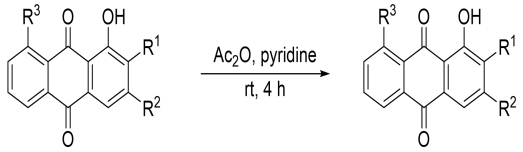
3: R = Me, 71.1% (in two steps)
5: R = H, 78.8% (in two steps)
5: R = Br, 78.7% (in two steps)

Scheme 2



Rubiadin (**6**): 51.4%

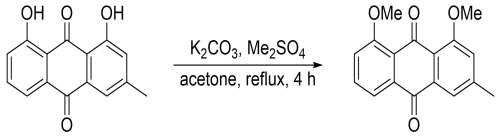
Scheme 3



Chrysophanol (**1**): R^1 = H, R^2 = Me, R^3 = OH
Rubiadin (**6**): R^1 = Me, R^2 = OH, R^3 = H

7: R^1 = H, R^2 = Me, R^3 = OAc; 62.1%
8: R^1 = Me, R^2 = OAc, R^3 = H; 59.1%

Scheme 4



Chrysophanol (**1**)

9: 82.2%

Anthraquinone **9** was synthesized by following a general procedure of methylation using chrysophanol (**1**) as a substrate (Scheme 4) [40, 38]. Strong signals at 1010 and 955 in the IR spectrum

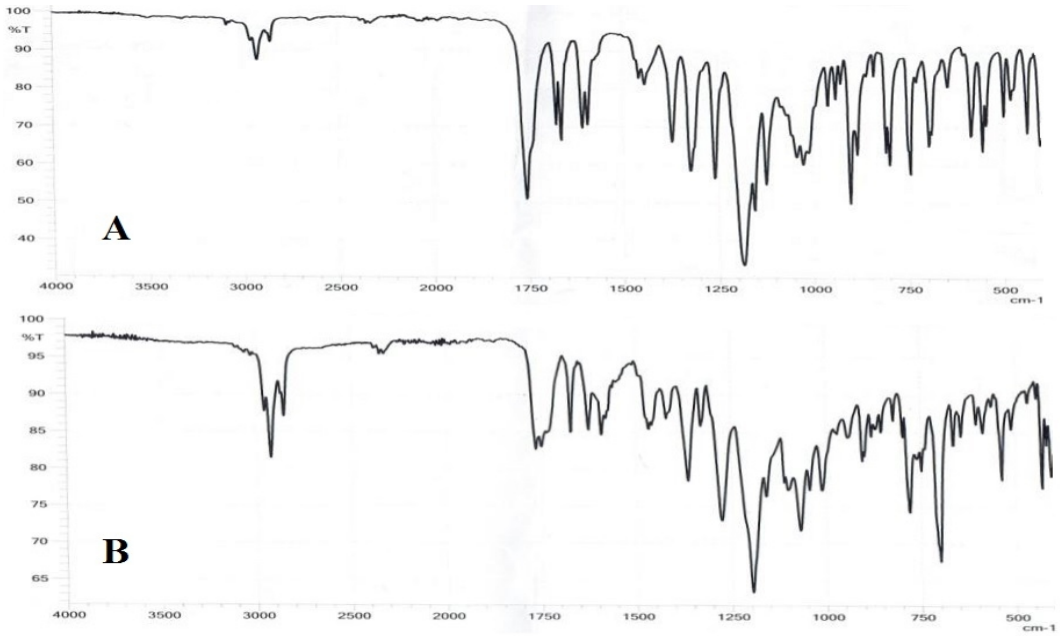


Fig. 2: (A) IR spectrum of chrysophanol diacetate (**7**); **(B)** IR spectrum of rubiadin diacetate (**8**).

of compound **9** were due to the two C–O stretches of newly formed methoxy groups.

Antioxidant capacity

Recently, we have reported isolation and identification of chrysophanol (**1**) and emodin (**2**) from the rhizomes of *R. australe* [32]. Chrysophanol (**1**) is known as an antioxidant [42]; however, it shows no significant effect on the inhibition of cancer cell proliferation [43]. On the other hand, Zhang *et al.* have reported that it is effective against MCF-7 breast cancer, 7901 gastric cancer, A375 melanoma and SKOV-3 oophoroma cells [44]. Emodin (**2**) has more effectively inhibited the proliferation of ovarian, breast, lung, liver and prostate cancer cells [45-47]. More recently, we have studied structure-activity relationship study of quercetin derivatives in the DPPH• radical scavenging capacity, and reported the substituent(s) effect [27]. Since anthraquinones are known for various biological activities including antioxidant and anticancer activities, we were encouraged to synthesize a number of anthraquinones for the screening of their antioxidant potentiality. Thus in the present work, we have synthesized anthraquinones **3**-**9** and together with previously isolated chrysophanol (**1**) and emodin (**2**) were used to determine the antioxidant potentiality using the DPPH assay [48].

By using the experimental absorbance values obtained in the DPPH assay, linear curves of percentage inhibition *versus* concentration for every anthraquinone were plotted and then IC_{50} values were computed. The result of DPPH assay is depicted in Figure 3. As can be seen in the figure, 1,8-dimethoxy-3-methylanthraquinone (**9**) is the most potent antioxidant (IC_{50} = 200 µg/mL) among other anthraquinones tested, followed by 2-methylanthraquinone (**3**) (IC_{50} = 230 µg/mL), rubiadin (**6**) (IC_{50} = 259 µg/mL), and 2-bromoanthraquinone (**5**) (IC_{50} = 383 µg/mL). In this study, anthraquinones **9**, **3**, **6** and **5** were found more effective in scavenging of DPPH• radical than emodin (**2**) (IC_{50} = 400 µg/mL), which has displayed anticancer activity in several studies [45-47]. Rubiadin (**6**) has shown anticancer activity in the 3- (4,5- dimethy lthiazol-2- yl)- 2,5- diphenyl

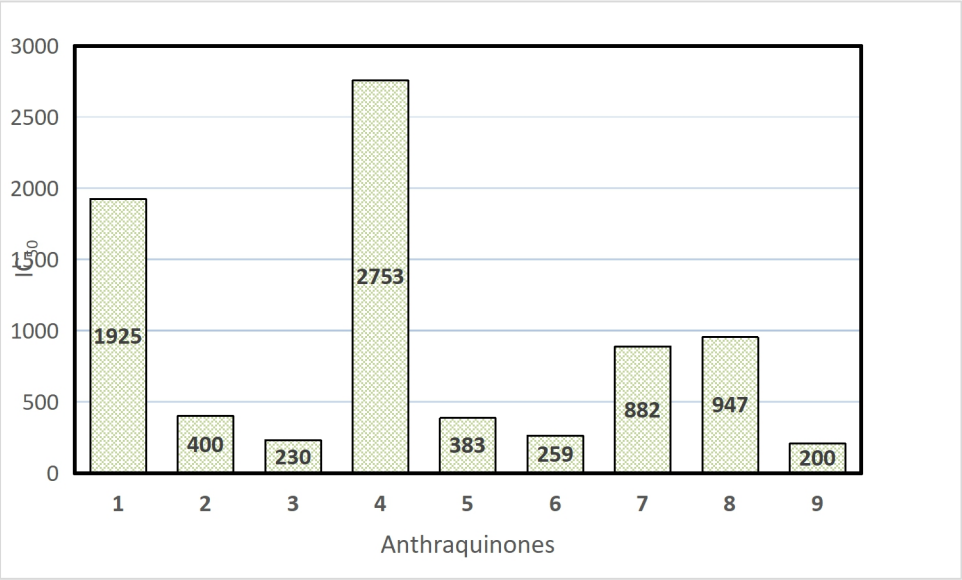


Fig. 3: IC_{50} values of anthraquinones (**1-9**) in the DPPH assay.

Table 1: Antibacterial activity of the anthraquinones **1-9**.

S. N.	Pathogenic bacteria used	ZOI shown by different antharaquinones 1-9 and standard (in mm diameter)[a]									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	Gentamycin
1	*B. subtilis*	9	16	11	11	8	12	10	10	8	16
2	*S. aureus*	–	17	12	–	–	11	13	–	–	24
3	*E. faecalis*	8	–	–	–	–	–	14	–	–	20
4	*K. pneumoniae*	–	–	–	10	–	–	9	–	–	12
5	*P. aeruginosa*	–	–	–	–	–	–	–	–	–	25
6	*S. typhimurium*	–	–	–	–	–	–	–	–	–	18

[a]Values of the ZOI include the diameter of well (6 mm) after 24 hours incubation against different bacterial species in the Agar well diffusion assay. (–)-Sign indicates no significant ZOI was observed.

tetrazolium bromide (MTT) assay against different cancer cells (MCF7 breast cancer, MES-SA human uterine sarcoma, MES-SA/DX5 multidrug-resistant variant human uterine sarcoma, DU145 prostate cancer and H460 lung cancer cells) [37]. Chrysophanol (**1**) was found ineffective in scavenging of DPPH• radical (IC_{50} = 1925 µg/mL). Unsubstituted anthraquinone (**4**) was found least effective among the others.

Antibacterial activity

The isolated and synthesized anthraquinones (**1-9**) were evaluated for the antibacterial activity by using the Agar well diffusion assay [49]. The ZOI produced by these compounds is tabulated in Table 1.

All investigated anthraquinones **1-9** have displayed a weak to moderate antibacterial activity against *B. subtilis* (ZOI = 8-16 mm), while all these compounds were found ineffective against *P. aeruginosa* and *S. typhimurium*. A weak antibacterial activity of chrysophanol (**1**) against *B. subtilis* and *S. aureus* has already been reported [50-51]. Literature search reveals that emodin (**2**) exhibits noticeable antibacterial activity against methicillin-resistant *S. aureus*, *P. aeruginosa*, *Escherichia coli*, *S. typhimurium*, *Bacillus cereus* and *Shigella sonnei* [52-53]. In the present study, emodin (**2**) was found efficient antibacterial against *B. subtilis* (ZOI = 16 mm) and *S. aureus* (ZOI = 17 mm) in accordance with the literature, and in contrast, it was found ineffective against *P. aeruginosa* and *S. typhimurium*.

2-Methylanthraquinone (**3**) (ZOI = 12 mm) and rubiadin (**6**) (ZOI = 11 mm) have displayed noticeable antibacterial activity against *S. aureus*. The growth of *S. aureus* (ZOI = 13 mm), *E. faecalis* (ZOI = 14 mm) and *K. pneumoniae* (ZOI = 9 mm) was remarkably inhibited by chrysophanol diacetate (**7**), which clearly indicated that the biological activity of a compound can be significantly enhanced by the structural modification (compare antibacterial activity of parent chrysophanol **1** *versus* its diacetate derivative **7**). Unsubstituted anthraquinone (**4**) exhibited a weak antibacterial activity against *K. pneumoniae* (ZOI = 10 mm).

4. Conclusion

Anthraquinones are the potential drugs used against different diseases. We have synthesized a number of anthraquinones using known protocols, and evaluated their antioxidant and antibacterial activities. DPPH• radical scavenging capacity of 1,8-dimethoxy-3-methylanthraquinone (**9**), 2-methylanthraquinone (**3**), rubiadin (**6**) and 2-bromoanthraquinone (**5**) were found comparatively more effective than emodin (**2**), a well-known compound for its anticancer activity. Hence these compounds warrant further cancer cell lines studies. In addition, anthraquinones **1-9** have shown a weak to moderate antibacterial activity against *B. subtilis*, while they were found ineffective against *P. aeruginosa* and *S. typhimurium*. Interestingly, a significant enhancement in the antibacterial activity was observed in chrysophanol diacetate (**7**) as compared to its parent compound **1**, which is a known compound with a weak antibacterial activity.

Acknowledgments

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