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Bilkis Jahan Lumbiny
Chemistry Department,
Rajshahi University,
Rajshahi,, Bangladesh.

Mst Rikta Khatun
Chemistry Department,
Rajshahi University,
Rajshahi,, Bangladesh.

M. Azizul Islam
Chemistry Department,
Rajshahi University,
Rajshahi,, Bangladesh.

Correspondence:
Bilkis Jahan Lumbiny
Chemistry Department,
Rajshahi University,
Rajshahi,, Bangladesh.

Effect of Substituents on Coloration and Bioactivity of Some New Synthesized Substituted Chalcones; An Environmentally Safe Natural Dyes

Bilkis Jahan Lumbiny, Mst Rikta Khatun, M. Azizul Islam

Abstract
Claisen-Schmidt condensation reaction were undertaken for synthesizing four substituted chalcones 4′– methyl – 4 – hydroxyl – 3 – methoxy chalcone (3a), 2′ – hydroxyl – 4′ – methoxy – 3,4 – methylenedioxy chalcone (3b), 4′–methyl – 2, 3, 4 – trimethoxy chalcone (3c), 4′ – benzyloxy – 2′ – hydroxyl – 3,4 –methylenedioxy chalcone (3d) and characterized by spectral data. As the number of electrons donating auxochromes increase the color of the compound also deepens. In search of the potency as new antibiotic or chemotherapeutic agents *in vitro* antimicrobial screening of the synthesized chalcones (3a-3d) were done against seven pathogenic bacteria by disc diffusion technique. Compounds are more sensitive against G^- organism than that of G^+ organism at the concentration 250 µg/disc comparing to those of the standard drug (amoxicillin, 40µg/disc). In addition compound 3b showed highest antioxidant activity, IC_{50} is 12.15 (µg/mL) in diphenylpicrylhydrazin (DPPH) model. The compound 3b contains phenolic –OH group in ortho position and suppose to produce phenoxide free radical easily which can be stabilized by electronic group methoxy occupied para position of aromatic ring (A) attached to another aromatic ring containing methylenedioxy moiety.

Keywords: Chalcones, Claisen-Schmidt method, Substituent contribution, Antibacterial, DPPH Model.

1. Introduction

Chalcone [1] (trans-1,3-diaryl-2-propene-1-ones), a biosynthetic color pigment, belonging to flavonoid family are important precursor of open chain flavonoids and isoflavonoids and are abundant in edible plants.[2] Chalcone (polyhydroxylated) bears a very good synthon, especially reactive ketoethylenic group –CO–CH=CH– so that variety of novel heterocyclic with good pharmaceutical profile can be designed. These are yellow-colored compounds because of the presence of that special chromophore which deepens in the presence of other auxochromes. In nature these are widely available in petals of colorful flowers and we also intake from different fruits. [3,4] So these are chosen as an good alternative as non azo dye industrially.[5] Synthetic dye industry has trended to decline with the increasing awareness of toxicity and excessive use of artificial chemical additives. In fact, considerable interest has been emerged relating synthetic additive intolerance with various environmental pollution and adverse toxicological side effects, particularly mental disorders and attention deficit of young generation.[3] Therefore, several policy and restrictions being adopted for a proper replacement of synthetic additive with natural one like food additive, color, fragrance, preservative, dietary antioxidant etc.

Chalcone pigments are safe for food and have curative effects on non-infectious diseases such as lack of oxygen, coronary heart diseases, myocardial infarction, and cerebral and renal thrombosis, diabetes and degenerative diseases. Hence, these dyes were reported to exert antioxidant and radical-scavenging activities and have been recently recommended for use as food colorants.[6] During last sixty years simultaneous effort being carried out to obtain structurally modified chalcones through synthesis as well as isolation works from plant material around the world.[7] Structural modification leads structure activity relationship

(SAR) is also an important factor which has aroused considerable interest to the chemist.

. Claisen-Schmidt condensation reaction were undertaken where aromatic aldehydes can be condensed with aromatic ketones in the presence of aqueous alkali to form α, β unsaturated ketones. Thus, four substituted chalcones; 4′–methyl – 4 – hydroxyl – 3 – methoxy chalcone (3a), 2′ – hydroxyl – 4′ – methoxy – 3,4 – methylenedioxy chalcone (3b), 4′–methyl – 2, 3, 4 – trimethoxy chalcone (3c), 4′ – benzyloxy – 2′ – hydroxyl – 3,4 –methylenedioxy chalcone (3d) were synthesized and characterized by spectral analysis. The present study is aiming to generate new functional natural dyes of basic skeleton of chalcones and their derivatives by introducing electron withdrawing and donating groups like –OBz, –OH, $–CH_3$, $–OCH_3$, $–OCH_2O$ as well as changing their position. Electronic effects of substituent on coloration being observed. Determination of their bioactivity; antimicrobial and antioxidant properties by disk diffusion and diphenylpicrylhydrazin (DPPH) method respectively were done in search of their utility to national and international demand. Thus, newly colored chalcones could be applied as cheap, safe and easy to prepare alternate to replace existing costly natural and toxic azo dyes.

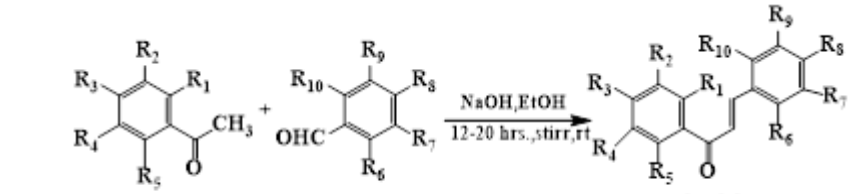
2. Materials and methods

2.1 Chemicals and apparatus

The substituted acetophenones (1a-1c) and substituted benzaldehydes were purchased and substituted acetophenones, 1d was prepared from purchased resorcinol and employed together for the synthesis of substituted chalcones (3a-3d). Other necessary chemicals and reagent grade solvent were purchased from E-Merck or Sigma-Aldrich company and purified if required.

2.2 Synthesis of Substituted Chalcones (3a-3d)[7,8]

One of the most convenient methods is one that involves the Claisen-Schmidt condensation of equimolar quantities of a substituted acetophenone with substituted benzaldehydes in the presence of aqueous alcoholic alkali (Scheme 1). Here 2 – hydroxyl – 4 – benzyloxy acetophenone (1d) was prepared by synthesizing *β*-resacetophenone[7] followed by its benzylation. A mixture of *β*-resacetophenone (10 g), benzylchloride (7.5 mL), anhydrous potassium carbonate (24 g) and potassium iodide in acetone (250 mL) was refluxed for 8 hours. Potassium salts were filtered off and the filtrate evaporated to dryness. The reaction mixture was subjected to column chromatography over silica gel. The elution was done with petroleum spirit and benzene (7:3). From this column chromatography we obtained 2 – hydroxyl – 4 – benzyloxy acetophenone, yield 50%, pale yellow solid; mp 110-111 ^oC, R_f = 0.79 (benzene: acetone) as reported earlier.



1a-1d acetophenone

1a) R_3=-CH_3, R_1=R_2=R_4 = R_5=H
1b) R_1= -OH, R_3 = -OCH_3 R_2= R_4=R_5=H
1c) R_3=-CH_3 R_1=R_2=R_4=R_5 = H
1d) R_1= -OH, R_3 = -OBz R_2=R_4=R_5 =H

2a-2d benzaldehyde

2a) R_8= -OH, R_7 =-OCH_3 R_6 = R_9 = R_{10} = H
2b) R_7, R_8 = -OCH_2O- R_6= R_9=R_{10} = -H
2c) R_8= R_9=R_{10}=-OCH_3, R_6= R_7=H
2d) R_7, R_8 = -OCH_2O- R_6=R_9=R_{10}=-H,

3a-3d chalcone

3a) R_3= -CH_3, R_8= -OH, R_7 =-OCH_3 R_1=R_2=R_4=R_5=R_6=R_9=R_{10}=H
3b) R_1= -OH, R_3 = -OCH_3, R_7, R_8 = -OCH_2O-, R_2=R_4= R_5 =R_6=R_9=R_{10}=H
3c) R_3=-CH_3, R_8= R_9=R_{10}=-OCH_3, R_1= R_2=R_4=R_5= R_6=R_7=H
3d) R_1=-OH, R_3=-OCH_3, R_7, R_8 = -OCH_2O R_2 =R_4=R_5 = R_6=R_9=R_{10}= H

Scheme 1 General synthetic root of chalcone (3a-3d)

2. 3 Characterization of Synthesized Chalcones (3a-3d)

(i) 4′ – methyl – 4 – hydroxyl – 3 – methoxy chalcone, (3a)

$C_{17}H_{16}O_3$; Solid and light brown, yield 58%; m. p. 195-196 ^oC, R_f = 0.69 (pet ether: acetone; 3 : 1), IR (KBr, cm^{-1}); 3339.24 (–OH), 3100 .00 (C=C–H, aromatic, str.) 3000. 00 (C=C–H, olefinic str.), 2944.64 (aliphatic –C–H, asym.-str.), 2800.00 (aliphatic –C–H, sym-str.), 1900.00 (–C=C, olefinic str.), 1647.63 (–C=O, conj. keto group), 1608.84 (–C=C, aromatic), 1579.66, 1515.92, 1453.51, 1428.59 ($–CH_3$, bending), 1336.87, 1304.18, 1275.39, 1221.29 (–C–O–C– str.), 1180.52, 1124.30, 1035.77, 1015.06 (C–O, str.), 984.09, 854.74, 814.79, 726.90 (C–H, bending aromatic), ^1H NMR, δ_H (500 MHz, $CDCl_3$, 16H); 7.930 (d, *J* = 8 Hz, 2H), 7.747 (d, *J* = 15.6 Hz, 1H, C_β–H), 7.383 (d, *J* = 15.6 Hz, 1H, C_α–H), 7.303 (d, 2H, *J* = 7.9 Hz), 7.225 (dd, 1H, *J* = 8.1 Hz, 1.45 Hz) 7.133 (bs, 1H) 6.958 (d, 1H, *J* = 8.2 Hz), 3.967 (s, 3H, $–OCH_3$), 2.439 (s, 3H, $–CH_3$).

(ii) 2′ – hydroxyl – 4′– methoxy – 3, 4 – methylenedioxy chalcone (3b)

$C_{17}H_{14}O_5$; Solid and orange, yield 61%; m. p. 138-140 ^oC, R_f = 0.85 (pet ether : acetone; 3 : 1), IR (KBr, cm^{-1}); 3435.35 (–OH), 3072.88 (–C=C–H, aromatic, str.), 3000.00 (–C=C–H olefinic str.), 2914.85 (aliphatic –C–H, asym.-str.), 2849.99 (aliphatic –C–H, sym-str.), 1866.69 (–C=C olefinic str.), 1633.98 (–C=O, conj. keto group), 1566.13 (–C=C, aromatic str.), 1502.14, 1493.00, 1412.98, 1343.89,

1316.68, (–CH_3, bending), 1032.41(–C–O, str.), 1290.37, 1250.44, 1218.18, 1195.49, 1136.64 (–C–O–C– str.), 1036.16, 1019.25 (–C–O– str.), 977.78, 959.97, 830.28, 795.53 (–C–H, bending aromatic), ^{1}H NMR, δ_H (500 MHz, $CDCl_3$, 14H); 7.808 (d, *J* = 16 Hz, 1H, C_β–H), 7.807 (d, *J* = 9 Hz, 1H), 7.402 (d, *J* =15.4 Hz, 1H, C_α–H), 7.166 (bs, 1H), 7.133 (d, *J* = 7.9 Hz, 1H), 6.850 (d, *J* =7.9 Hz, 1H), 6.488-6.469 (m, 2H), 6.034 (s, 2H, –O_2CH_2), 3.856 (s, 3H, –OCH_3).

(iii) 4′ – methyl – 2, 3, 4 – trimethoxychalcone (3c)

$C_{19}H_{20}O_4$, Solid and brownish yellow, yield 62%; mp 188-190 ºC, R_f = 0.93 (pet ether : acetone; 3 : 1) IR (KBr, cm^{-1}); 3435.80 (–OH), 3000.00 (–C=C–H olefinic str.), 2940.10 (aliphatic –C–H, asym.-str.) 2800.00 (aliphatic –C–H, sym-str.), 1661.48 (–C=O, conj. keto group), 1585.93 (–C=C, aromatic), 1496.59, 1463.04, 1434.28, 1326.50 (–CH_3, bending), 1284.46, 1258.38, 1182.79, 1096.73, 1047.25 (–C–O–C–, str.), 1015.34, 985.13, 811.20 (–C–H, bending aromatic), ^{1}H NMR, δH (500 MHz , $CDCl_3$, 20H); 8.015 (d, *J* = 15.5 Hz, 1H, C_β–H), 7.940-7.880 (m, 2H), 7.595-7.538 (m, 1H), 7.263 (d, *J* = 15.3 Hz, 1H, Cα–H), 6.694 (d, 1H, *J* = 8.2 Hz), 3.848 (s, 9H, –OCH_3), 2.351 (s, 3H, –CH_3)

(iv) 2′ – hydroxyl – 4′ – benzyloxy – 3,4 – methylenedioxy chalcone, (3d)

$C_{23}H_{18}O_5$; solid and yellow, yield 55%; m. p. 140-141 ºC, R_f = 0.92 (pet ether : acetone; 3 : 1): IR (KBr, cm^{-1}): 3400.00 (–OH), 3074.48(–C=C–H, aromatic, str.), 3032.22 (–C=C–H, olefinic, str.), 2911.61 (–C–H, aliphatic, asym.-str.), 2800.00 (–C–H, aliphatic, sym-str.), 1634.08 (–C=O, conj. keto group), 1561.44 (–C=C, aromatic, str.), 1502.93, 1488.53, 1446.70, 1371.72, 1342.90 (–CH_2, bending), 1288.13, 1243.14, 1212.26, 1190.33, 1135.68, 1104.90 (–C–O–C–, str.), 1034.03, 1004.65, 978.07, 933.35(–C–O str.), 841.78, 808.04, 764.00, 739.72 (–C–H, bending aromatic), ^{1}H NMR, δ_H (500 MHz, $CDCl_3$, 18 H); 7.813 (d, *J*=8.5 Hz, 1H), 7.799 (d, *J* = 13.5 Hz, 1H, C_β–H), 7.439-7.334 (m, 5H), 7.157 (d, *J* = 13.0 Hz, 1H, C_α–H), 7.142 (d, *J* = 1.5 Hz, 1H), 6.854 (d, *J* = 8.0 Hz, 1H), 6.570-6.505 (m, 3H), 6.015 (s, 2H, –O_2CH_2), 5.110 (s, 2H, –$CH_2C_6H_5$)

2.4 Antimicrobial activity determination

A biochemical screening to identify the proportions in the clinical use is long overdue. Chalcones and their heterocyclic analogs are potential therapeutic agents in bacterial diseases. In order to detect the antimicrobial activity of a new compound for the development as potential new antibiotic or chemotherapeutic agent *in vitro* antimicrobial screening is a useful technique. In general, antibacterial screening of the test chemicals were studied through primary assay technique, disc diffusion technique [7,9] to classify the organism as susceptible as well as resistance towards particular compounds.

2.4.1 Test Microorganism

The test tube cultures of the seven bacterial pathogens were collected from the Pharmaceutical Microbiology laboratory, Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh and listed in Table-1.

2.4.2 Disk diffusion technique

In the disc diffusion technique [3,4,9.10] dried filter paper discs containing known amount of test material are placed on agar plates seeded with test organisms. These plates were kept at low temperature (4ºC) for 24 hours. Initially the dried discs absorb water from the surrounding test medium and the drug is dissolved. The drug migrates through the adjacent test medium by concentration gradient of the drug according to physical law that governs diffusion of molecules through an agar gel. As a result, there is a gradual change of drug concentration in the agar surrounding each disc. Then the plates are incubated in an incubator at 37.5ºC for 24 hours. After 24 hours incubation the antibacterial activity was carried out by measuring the zone of inhibition in millimeter (mm) by a transparent scale. The zones made by the samples were compared with that of the standard disc. Results obtained from these tests are listed in Tabular form in Table-2.

Table 1: List of the test microorganisms.

Name of microorganism	Gram-positive (G^+) / negative (G^-)
Bacillus cereus, B_1	**G^+**
Staphyllococcus aureus, B_2	**G^+**
Agrobacterium Species, B_3	**G^-**
Escherichia coli, B_4	**G^-**
Shigella dysenteriae, B_5	**G^-**
Shigella sonnei , B_6	**G^-**
Shigella boydii, B_7	**G^+**

2.5 Antioxidant Activity in Natural Product

The main characteristic of an antioxidant is its ability to trap free radicals to inhibit the oxidative mechanisms that lead to degenerative diseases such as cancer, heart disease, osteoporosis, Alzheimer etc. Antioxidant activity is measured by DPPH method. [2, 3, 4, 5]

2.5.1 Antioxidant Activity by DPPH method

Diphenylpicryl hydrazine, DPPH is a stable free radical and the electron in it gives a strong absorption maximum at 517 nm complementary to purple in color. When odd electron of DPPH radical becomes paired with a hydrogen from an antioxidant free radical to form the reduced DPPH-H (Fig. 1) the color fades from purple to yellow and measured by absorbance. A lower absorbance at 517 nm indicates higher percentage scavenging. Schematic description of work procedure is given in Scheme 2.

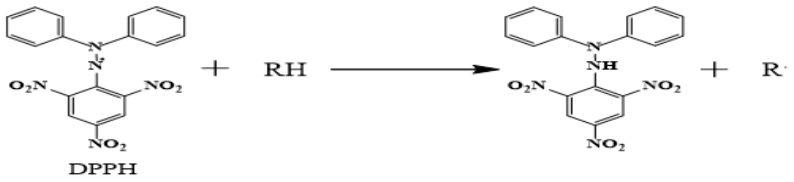
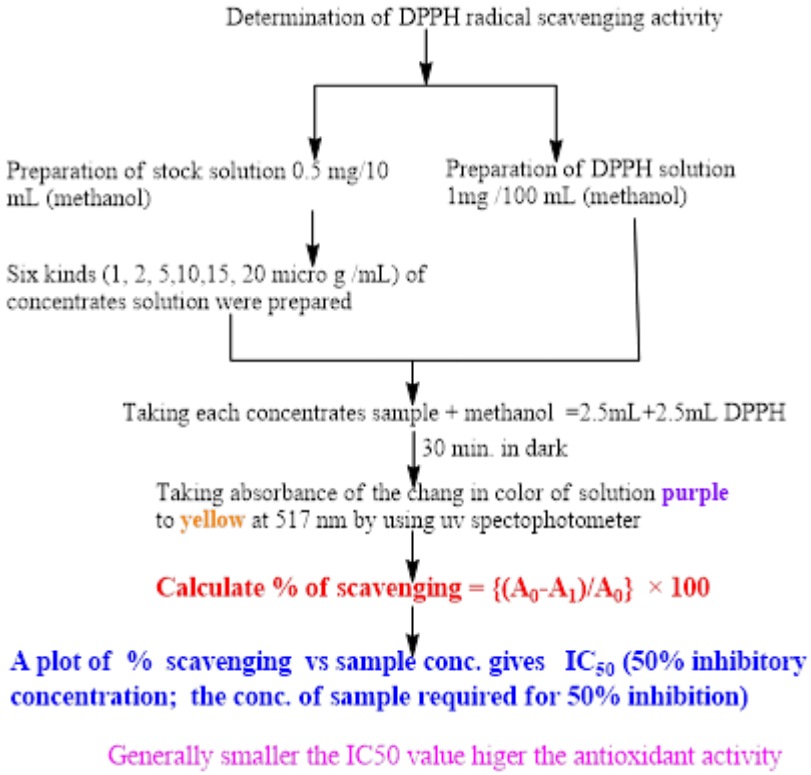


Fig. 1: DPPH scavenges antioxidant free radical by forming DPPH-H.



Scheme 2 Schematic Representation of DPPH Method

3. Results & Discussion

Chalcones, a biosynthetic product, belonging to flavonoid family are precursor of open chain flavonoids and isoflavonoids and are abundant in edible plants. Chalcones are a yellow-orange natural dye obtained as pigments for flower coloration, in fruits and vegetables and can be obtained synthetically as well. These possess interesting structural features, in which two aromatic rings are linked by a three carbon α, β-unsaturated carbonyl system (–CO–CH=CH–), conjugated double bonds and a completely delocalized π-electron system on both benzene rings. Molecules possessing such system have coloration, relatively low redox potentials and have a greater probability of undergoing electron transfer reactions. Different functional groups which are attached to the benzene ring of chalcones can be varied to enhance activity. Four substituted chalcones; 4′– methyl – 4 – hydroxyl – 3 – methoxy chalcone (3a), 2′ – hydroxyl – 4′ – methoxy – 3,4 – methylenedioxy chalcone (3b), 4′–methyl – 2, 3, 4 – trimethoxy chalcone (3c), 4′ – benzyloxy – 2′ – hydroxyl – 3,4 –methylenedioxy chalcone (3d). The drug potency of the synthesized chalcones (3a-3d) were investigated by *in vitro* antibacterial activity against seven pathogenic bacteria viz. (Table-1). The primary assay was performed by disc diffusion technique to classify the microorganism susceptible as well as resistant towards particular compounds. The bioactivity is expressed by the diameter of zone of inhibition in mm as presented in Table 2.

Table-2: Results of the antibacterial activity of the compounds (3a-3d) at the concentration of 250 (μg $disc^{-1}$).

Microorganism	**ShiCo**Compound				
	3a	**3b**	**3c**	**3d**	**A-40***
Bacillus cereus, B_1, G^+	**10**	**10**	**9**	**8**	**22**
Staphyllococcus aureus, B_2, G^+	**No Activity**				
Agrobacterium Species, B_3, G^-	**10**	**8**	**9**	**11**	**31**
Escherichia coli, B_4, G^-	**13**	**14**	**9**	**13**	**19**
Shigella dysenteriae, B_5, G^-	**13**	**11**	**12**	**7**	**24**
Shigella sonnei, B_6, G^-	**10**	**7**	**10**	**12**	**20**
Shigella boydii, B_7, G^+	**10**	**11**	**8**	**9**	**20**

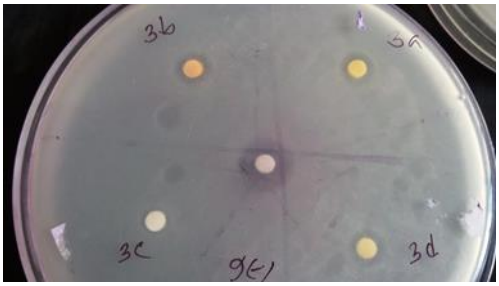


Fig. 2: Photographic representation of the zone of inhibition at the concentration of 250 µg disc^{-1} against *Escherichia coli* (G^-, B_3).

Compounds 3a, 3b, 3d exhibited fairly good potentialities against (G^-) bacteria than that of (G^+) and in some cases about near to that of the standard drugs. Among all these compound 3b showed highest activity (14 mm) against G^- bacteria Escherichia coli in comparison with standard drug (19 mm). This led us to conclude that the presence of electron releasing hydroxyl (–OH) and methoxy group (–OCH_3) are responsible for the antimicrobial effects. The presence of methylenedioxy also enhancing this effect in 3b. Also due to the presence of these auxochromes 3b shows dark orange color whereas other compounds are similar to parent chalcone, yellow. On the other hand steric effect is expected in 3c, where three methoxy groups are occupied ring B and in three adjacent carbons. Besides hydroxyl group is also absent. All these causes relatively poor activity in 3c. Drug resistant bacteria *Staphyllococcus aureus* was tested with 3a-3d but these compounds failed to show any sensitivity towards this organism even with the standard Drug.

In addition, the synthesized chalcones (3a-3d) were evaluated for *in vitro* antioxidant activity using DPPH model. Observation for antioxidant activity is expressed in terms of percent scavenging of DPPH radical and the inhibitory concentration 50% (IC_{50}) as presented in Table-3.

Table- 3: DPPH radical scavenging data of synthesized chalcones **(3a-3d)** and their corresponding IC_{50} values.

Compound No.	**Conc. µg /mL**	**Absorbance at 517 nm**	**% Inhibition**	**IC_{50}* (µg /mL)**
3a	**1**	**0.170**	**14.14**	**17.26**
	2	**0.168**	**15.15**	
	5	**0.149**	**24.75**	
	10	**0.130**	**34.34**	
	15	**0.110**	**44.44**	
	20	**0.099**	**50.00**	
3b	**1**	**0.164**	**17.17**	**12.15**
	2	**0.154**	**22.22**	
	5	**0.124**	**37.37**	
	10	**0.110**	**44.44**	
	15	**0.079**	**60.10**	
	20	**0.059**	**70.20**	
3c	**1**	**0.192**	**3.03**	**162.43**
	2	**0.191**	**3.54**	
	5	**0.187**	**5.56**	
	10	**0.186**	**6.06**	
	15	**0.208**		
	20	**0.184**	**7.07**	
3d	**1**	**0.188**	**5.05**	**50.81**
	2	**0.184**	**7.07**	
	5	**0.171**	**13.64**	
	10	**0.167**	**15.66**	
	15	**0.164**	**17.17**	
	20	**0.153**	**22.73**	
DPPH control	**0.02%**	**0.198**		

* **Ascorbic acid is the standard and IC_{50} 0.08 (µg /mL)**

The trend for antioxidant activity (**IC_{50}**, µg/mL)

Ascorbic acid (0.08) > 3b (12.15) > 3a (17.26) > 3d (50.81) > 3c (162.43)

Highest activity is in case of 3b **as IC_{50}** is lowest for structure 3b, 12.15 (µg/mL) and increasing as 3a, 3d and 3c at DPPH conc. 0.02% compared to the standard, ascorbic acid, IC_{50} 0.08 (µg/mL). Gradual increase percent inhibition with the increment of concentration is shown in Fig. 3 and IC_{50} values are determined from the linear regression of % inhibition vs conc. Plots (Fig. 3). Then the IC_{50} values are represented in Bar diagram for a clear comparison of activity of these compounds and standard ascorbic acid. Numerical digit 5 is added with the IC_{50} values to make the diagram more visible and attractive (Fig. 4).

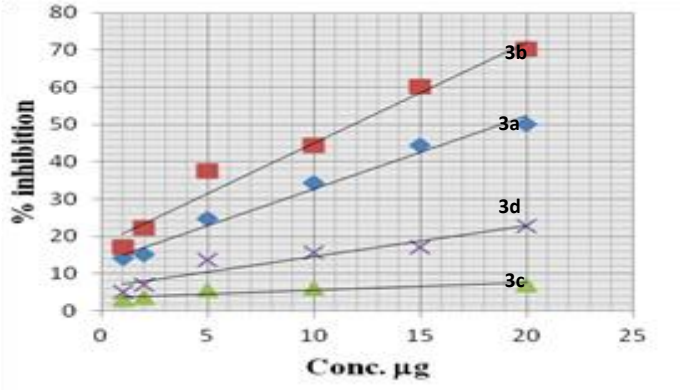


Fig. 3: DPPH radical scavenging activity of (3a-3d).

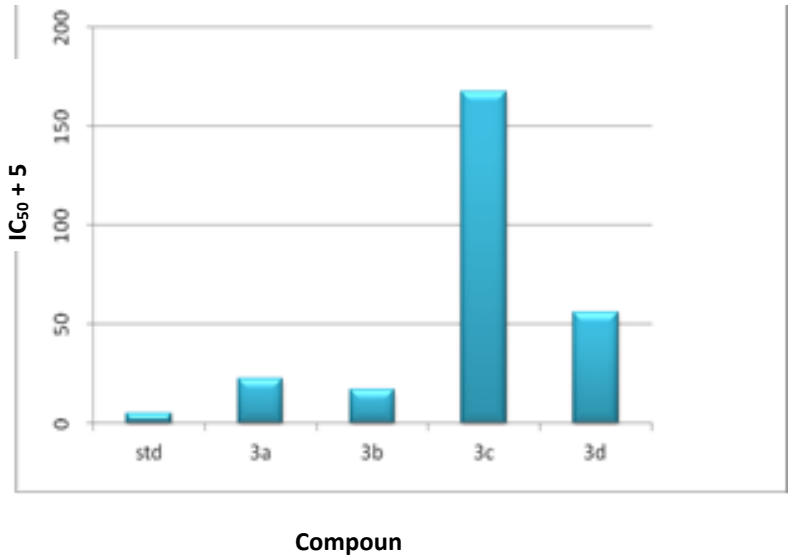


Fig. 4: Bar Diagram of IC_{50} values of (3a-3d).

The compound 3b contains ortho phenolic –OH group and suppose to produce phenoxide free radical easily and stabilized by electronic group methoxy occupied para position of aromatic ring (A) attached to another aromatic ring containing methylenedioxy moiety. On the other hand compound 3c showed least activity due to the absence any phenolic –OH group.

4 Conclusions

The present study includes design a suitable method for the synthesis of chalcones, with varying hydroxy, methoxy, methyl, benzyloxy and methylenedioxy substituents and to provide SAR through coloration, antibacterial, antioxidant activity.

Synthesized chalcones of concentration 250 µg/disc showed high activity against **G⁻** organism and less active towards G+ organism comparing to those of the standard drug (amoxicillin, 40 µg/disc). Among all these compound 3b showed highest activity (14 mm) against G^- bacteria *Escherichia coli* in comparison with standard drug (19 mm). This led us to conclude that the presence of electron releasing hydroxyl (–OH) and methoxy group ($–OCH_3$) are responsible for the antimicrobial effects. The presence of methylenedioxy is enhancing this effect in 3b. Also due to the presence of these auxochromes 3b shows dark orange color whereas other compounds are similar to parent chalcone, yellow.

In addition, the synthesized chalcones (3a-3d) were evaluated for *in vitro* antioxidant activity using DPPH model. Observation for antioxidant activity is expressed in terms of percent scavenging of DPPH radical and the inhibitory concentration 50% (IC_{50}) is lowest for structure 3b, 12.15 (µg/mL) and increasing as 3a (17.26 µg/mL), 3d (50.81 µg/mL) and 3c (162.43 µg/mL) at DPPH conc. 0.02% as compared to the standard, ascorbic acid, IC_{50} 0.08 (µg/mL). The compound 3b contains ortho phenolic -OH group and suppose to produce phenoxide free radical easily, stabilized by electronic group methoxy occupied para position of aromatic ring (A) attached to benzaldehyde part. So compound 3b has a high potency to apply as an alternative toxic synthetic dyes.

5 Acknowledgments

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