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Synthesis and characterization of 2-formylthymol-based azo-aldehyde dyes: Probing their efficacy as a radical scavenger in antioxidant applications

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ABSTRACT

A new series of azo-aldehyde dyes has been derived by performing a diazocoupling reaction between 2-isopropyl-5-methyl phenol and diazonium salts obtained by diazotization of differently substituted aromatic amines. The structures of newly synthesized azo-aldehydes were confirmed by modern analytical spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, FT-IR, and Mass. Thereafter, all synthesized azo-aldehyde dyes were evaluated for their *in vitro* antibacterial activity against *S. aureus*, *S. typhumurium*, *E. coli*, and *P. aeruginosa* strains using the plate method. The formed compounds were also evaluated for antioxidant activity. The present results provide new data to support that the thymol-based azo-aldehyde dyes have a potential to explore a variety of applications in the modern field of molecules and materials of high biological relevance.

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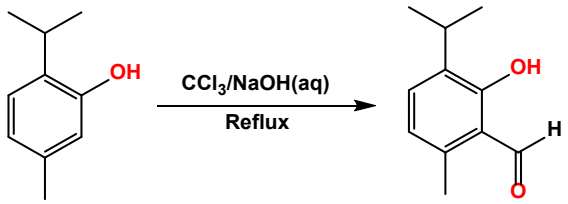
1. Introduction

Natural products play a crucial role in the development of drugs and medicines, and it is also a fact that a remarkable number of drugs available on the market are being derived from naturally occurring substances [1,2]. The present database on the consumption of drugs and medications shows that even today approximately 80% of the world's population uses traditional medicine for primary health care [3], and most of these therapies are based on the use of plant extracts or their active compounds, such as terpenoids as a medicine [4]. Thymol was first discovered by Neumann in 1719 and further purified by M. Lalleman in 1853 [5,6]. Thymol is one of the very crucial natural medicinal assets [7] that has excellent pharmacological activities in the treatment of respiratory and digestive disorders [7-11], has an oral disease [12,13], in food science, thymol is a recognized antioxidant [14,15], antibacterial [16-18], antifungal [19,20] and antimicrobial agent [21,22], *etc*. In addition to that, commercial formulations of thymol are well-known insecticides, [23] acaricides, [24] insect and animal repellents, [25] fungicides, and medical disinfectants, which present itself as an alternative to reduce the employment of synthetic fungicides [26,27].

The azo group (-N=N-) containing compounds are attracting great interest from the scientific community due to their properties, including textile dyeing and coloring [28], leather technology [29], optical switching [30], printing and paper industries [31], cosmetics [32], food industries [33,34]. Azo compounds play a subtle role in many biological operations, such as the inhibition of DNA and RNA, in protein synthesis, carcinogenesis, and in the nitrogen fixation process [35,36]. In addition, azo compounds and their metal chelates have shown exceptional antibacterial activity [37,38]. Furthermore, metal combinations of these organic frameworks have been explored with great care in analytical chemistry as indicators in pH, redox, or complexometric titrations [39,40]. In this regard, taking advantage of the expertise of our group in the functionalization of thymol, as well as the evaluation of the biological activities of substituted thymol [41-43], we execute the possibility to perform diazotization of 2-formylthymol with differently substituted diazonium salts obtained from aromatic amines via diazotization.



Scheme 1. Synthesis of 2-hydroxy-3-isopropyl-6-methylbenzaldehyde (2-formylthymol).

In this way, an exceptional amalgamation of key azo-dye features is introduced by coupling the diazonium salt with the biological activity exhibited by 2-formylthymol. Here in, our results of the formylation of thymol, followed by diazo-coupling of the formyl-thymol with diazonium salt for an innovative thymol-functionalization, are presented, highlighting the biological activities of the functionalized thymol *i.e.* azo-aldehyde.

2. Experimental

2.1. Material

Thymol, chloraniline, bromoaniline, fluoroaniline, 3-tri fluoromethyl aniline, 4-methyl aniline, 2-trifluoromethyl aniline, sodium hydroxide used in this study were purchased from Merck and Sigma-Aldrich and were used without further purification. Ethanol, hexane, chloroform, ether, ethyl acetate, and dimethylsulfoxide were used without distillation prior to use. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was procured from Sigma-Aldrich. Chemical reactions for the synthesis of azo-aldehyde dyes were monitored by TLC (Merck, silica gel $60F_{254}$). TLC visualization was achieved in a UV light chamber.

2.2. Instrumentations and methods

All melting points were measured using an open capillary tube method. UV spectral analysis of the azo-aldehyde dyes was performed on a spectrophotometer (Shimadzu, UV 1900i) at the School of Chemical Sciences, Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon. Infrared (IR) analysis of the synthesized compound was performed on the Shimadzu Spectrophotometer (IRAffinity-1) at the School of Chemical Sciences, KBC-NMU, Jalgaon. Chemical shifts (both ^{1}H- and ^{13}C-NMR spectra) were recorded on a ppm (δ) scale on a Bruker Avance Neo spectrometer operating at 500 and 125 MHz, respectively, using TMS as an internal reference standard in DMSO-d_6 as a solvent at SAIF Chandigarh. Mass spectrometry analysis of the synthesized compounds was performed on a Waters Q-TOF Micro mass at SAIF Chandigarh. After confirming the structures and purity of the synthesized compounds, the pure compounds were evaluated for in vitro antibacterial, antioxidants and amylase activity at the School of Life Sciences Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon.

2.3. Synthesis

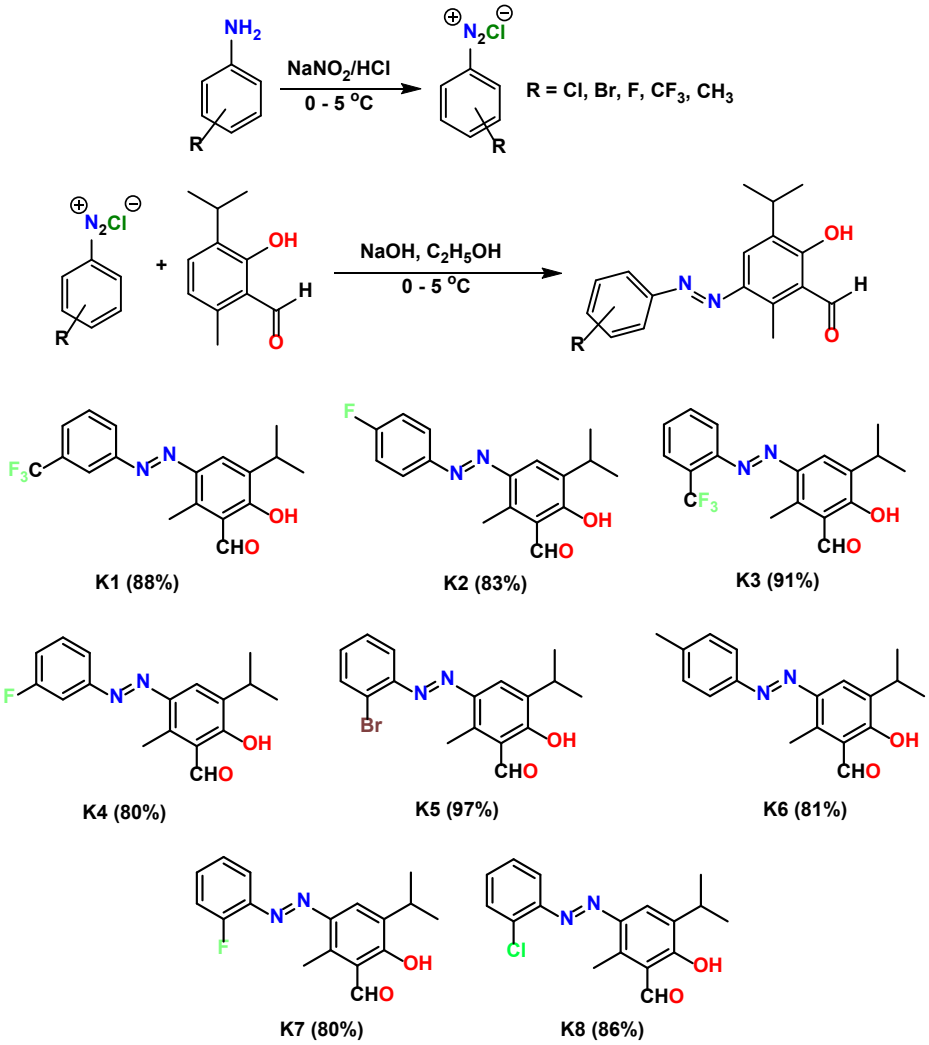
2.3.1. Synthesis of 2-hydroxy-3-isopropyl-6-methylbenzaldehyde (2-formylthymol)

A round bottom flask fitted with a water reflux condenser and a thermometer is charged with a ternary solution of sodium hydroxide (6.66 g, 0.16 M) and phenolic monoterpene (0.02 mol) in 7 mL of distilled water. The temperature of the reaction mixture was maintained approximately 60-65 °C, and then to the hot reaction mass chloroform (0.04 mol) was added through the condenser very slowly in small portions with constant stirring [*Note: Although chloroform is volatile and highly flammable, it still maintained the temperature of the reaction at 60-65 °C during addition*]. Once the addition of chloroform was complete, the reaction mixture was further stirred for 2 hours at 60-65 ° C. The progress of the reaction was monitored using TLC. After confirming the consumption of the reactant by TLC, the excess chloroform was removed from the alkaline solution by steam distillation. After removal of chloroform, the resulting solution was cooled and was acidified with dilute hydrochloric acid (1%). Thereafter, the acidified solution was steam distilled until no more oily drops were collected. The collected distillate was extracted with ether (3 × 30 mL), the distillation of ether gives a residue of unreacted phenolic monoterpene and orthoformylated phenolic monoterpene, the residue was transferred to a small glass stopper flask containing approximately twice the volume of saturated sodium metabisulfite solution. The solution was stirred vigorously for half an hour and allowed to settle for 1 hour. The resulting paste of the bisulfite compound was filtered and washed with ice-cold ethanol and finally with a small amount of ice-cold ether. After washing, the bisulfite compound was treated with dilute sulfuric acid to decompose the bisulfite over warm water bath. Finally, the mixture was cooled and then the residue was extracted with ether (3 × 30 mL), followed by the removal of ether to obtain orthoformylated phenolic monoterpene (Scheme 1) [41-44].

2.3.2. General procedure for synthesis of azo-aldehyde dyes

In a 100 mL round bottom flask taken (0.01 mole) substituted aniline and dissolved it in 2 mL of concentrated hydrochloric acid and 20 mL of distilled water. The solution was cooled (0-5 °C) in an ice-water bath. Sodium nitrite (0.69 g, 0.01 mole) dissolved in 10 mL of distilled water was added dropwise to the solution with stirring until the diazotization was complete. A cooled (0-5 °C) solution of 2-formylthymol (1.78 g, 0.01 mole) [41] was dissolved in (20 mL) ethanol and (5 mL) (10%) sodium hydroxide solution was added dropwise to the above diazo solution dropwise and the reaction mixture was stirred under cold conditions (0-5 °C) for 1.5-2 hours. The precipitate obtained was filtered, washed with cold water, and dried. The crude product was recrystallized with ethanol to yield the pure compound, and the TLC was verified using two solvents (ethyl acetate:hexane, 2:8, v:v) (Scheme 2).

2-Hydroxy-3-isopropyl-6-methyl-5-((3-(trifluoro methyl) phenyl)diazenyl) benzaldehyde (K1): Color: Red. Yield: 88%. M.p.: 126-128 °C. UV/Vis (Methanol, λ_{max}, nm): 362.17. FT-IR (ν_{max}, cm^{-1}): 3175, 3072, 2875, 1635, 1616, 1433, 1060. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.09 (bs, 1H, -OH,), 10.47 (s, 1H, R-CHO), 8.13-8.15 (d, 2H, J = 7.35 Hz, ArH, Azo- Ring), 7.88-7.89 (d, 1H, *J* = 7.9 Hz, ArH), 7.79-7.82 (t, 1H, *J* = 7.7 Hz, ArH), 7.94 (s, 1H, ArH), 3.23-3.28 (m, 1H, Ar-isopropyl), 3.00 (s, 3H, CH_3), 1.21-1.22 (d, 6H, *J* = 6.95 Hz, CH_3-CH-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.73, 21.76, 25.86, 117.89, 120.59, 122.69, 124.86, 125.71, 126.91, 130.02, 130.27, 130.71, 135.09, 142.44, 152.18, 163.65, 197.61. TOF-MS (*m/z*) cal. for $C_{18}H_{17}F_3N_2O_2$, $[M-H]^-$ 349.1164; found: 349.1035.



Scheme 2. Diazotization of substituted anilines and diazo coupling reaction between 2-hydroxy-3-isopropyl-6-methylbenzaldehyde and diazonium salt.

3-((4-Fluoro phenyl) diazenyl)-6-hydroxy-5-isopropyl-2-methylbenzaldehyde (2): Color: Golden yellow. Yield: 83%. M.p.: 196-198 °C. UV/Vis (Methanol, λ_{max}, nm): 360.33. FT-IR (ν_{max}, cm^{-1}): 3242, 3057, 2872, 1633, 1581, 1367, 1041. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.03 (bs, 1H, OH), 10.48 (s, 1H, R-CHO), 7.94-7.97 (t, 2H, *J* = 6.27 Hz, ArH), 7.90 (s, 1H, ArH), 7.52-7.57 (t, 2H, *J* = 8.37 Hz, ArH), 3.25-3.33 (q, 1H, *J* = 16.87 Hz, Ar-isopropyl), 3.00 (s, 3H, CH_3), 1.21-1.22 (d, 6H, *J* = 6.65 Hz, CH_3-C*H*-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.68, 21.80, 25.91, 117.89, 120.53, 124.63, 124.70, 134.91, 141.56, 141.93, 149.04, 159.30, 163.07, 197.69. TOF-MS (*m/z*) cal. for $C_{17}H_{17}FN_2O_2$: $[M-H]^-$ 299.1196; found: 299.0902.

2-Hydroxy-3-isopropyl-6-methyl-5-((2-(trifluoro methyl) phenyl) diazenyl) benzaldehyde (3): Color: Orange. Yield: 91%. M.p.: 146-148 °C. UV/Vis (Methanol λ_{max}, nm): 357.12. FT-IR (ν_{max}, cm^{-1}): 3275, 3078, 2873, 1647, 1585, 1487, 1033. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.14 (bs, 1H, OH), 10.49 (s, 1H, R-CHO), 7.93-7.96 (t, 2H, *J* = 6.5 Hz, ArH), 7.73 (s, 1H, ArH), 7.84-7.85 (q, 2H, *J* = 3.2 Hz, ArH), 3.23-3.28 (m, 1H, Ar-isopropyl), 3.04 (s, 3H, CH_3), 1.19-1.21 (d, 6H, *J* = 6.9, Hz, CH_3-C*H*- CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.69, 21.68, 25.62, 117.88, 120.72, 122.93, 125.11, 126.31, 126.55, 130.75, 133.60, 135.14, 142.44, 142.70, 148.73, 163.88, 197.61. TOF-MS (*m/z*) cal. for $C_{17}H_{17}BrN_2O_2$: $[M-H]^-$ 349.1164; found: 349.0838.

3-((3-Fluoro phenyl) diazenyl)-6-hydroxy-5-isopropyl-2-methylbenzaldehyde (K4): Color: Red. Yield: 80%. M.p.: 112-114 °C. UV/Vis (Methanol, λ_{max}, nm): 365.31. FT-IR (ν_{max}, cm^{-1}): 3247, 3076, 2872, 1629, 1593,1487, 1593. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.07 (bs, 1H, OH), 10.46 (s, 1H, R-CHO), 7.89 (s, 1H, ArH), 7.75-7.77 (m, 1H, ArH), 7.59-7.64 (m, 2H, ArH), 7.35-7.39 (m, 1H, ArH), 3.22-3.27 (m, 1H, Ar-isopropyl), 2.99 (s, 3H, CH_3), 1.20-1.21 (d, 6H, *J* = 6.9 Hz, CH_3-C*H*-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.70, 21.75, 25.89, 117.88, 120.09, 120.10, 120.48, 131.01, 134.98, 141.24, 142.24, 153.66, 161.64, 163.59, 197.62. TOF-MS (*m/z*) cal. for $C_{17}H_{17}FN_2O_2$: $[M-H]^-$ 299.1196; found: 299.1085.

3-((2-Bromo phenyl) diazenyl)-6-hydroxy-5-isopropyl-2-methylbenzaldehyde (K5): Color: Yellow. Yield: 97%. M.p.: 230-232 °C. UV/Vis (Methanol, λ_{max}, nm): 358.12. FT-IR (ν_{max}, cm^{-1}): 3244, 3064, 2870, 1635, 1581, 1458, 711. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.09 (bs, 1H, OH), 10.49 (s, 1H, R-CHO), 7.96 (s, 1H, ArH), 7.85-7.87 (q, 1H, *J* = 1.2 Hz, ArH), 7.65-7.67 (q, 1H, *J* = 1.6 Hz, ArH), 7.51-7.54 (m, 1H, ArH), 7.43-7.46 (m, 1H, ArH), 3.25-3.32 (q, 1H, Ar-isopropyl, *J* = 6.9 Hz), 3.03 (s, 3H, CH_3), 1.21-1.23 (d, 6H, *J* = 6.85 Hz, CH_3-CH-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.72, 22.76, 25.80, 117.89, 117.91, 120.92, 124.22, 128.60, 132.17, 133.15, 133.58, 135.03, 142.37, 148.86, 163.63, 197.62. TOF-MS (*m/z*) cal. for $C_{17}H_{17}BrN_2O_2$: $[M-H]^-$ 359.0395; found: 359.0274.

2-Hydroxy-3-isopropyl-6-methyl-5-(p-tolyl diazenyl) benz aldehyde (K6): Color: Brown. Yield: 81%. M.p.: 122-124 °C. UV/Vis (Methanol, λ_{max}, nm): 355.12. FT-IR (ν_{max}, cm^{-1}): 3257,

3030, 2870, 1639, 1577, 1458. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.00 (bs, 1H, OH), 10.46 (s, 1H, R-CHO), 7.89 (s, 1H, ArH), 7.76-7.78 (d, 2H, *J* = 8.2 Hz, ArH), 7.36-7.37 (d, 2H, *J* = 8.05 Hz, ArH), 3.24-3.27 (t, 1H, *J* = 6.9 Hz, Ar-isopropyl), 2.98 (s, 3H, CH_3), 2.39 (s, 3H, Azo-ring CH_3) 1.21-1.22 (d, 6H, *J* = 6.9 Hz, CH_3-CH-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.57, 20.85, 21.75, 25.87, 117.78, 120.48, 122.36, 129.73, 134.71, 140.86, 141.06, 141.97, 150.18, 162.77, 197.54. TOF-MS (*m/z*) cal. for $C_{18}H_{20}N_2O_2$: [M-H]$^-$ 295.1447; found: 295.0602.

3-((2-Fluoro phenyl) diazenyl)-6-hydroxy-5-isopropyl-2-methylbenzaldehyde (K7): Color: Dark brown. Yield: 80%. M.p.: 156-158 °C. UV/Vis (Methanol, λ_{max}, nm): 356.18. FT-IR (ν_{max}, cm^{-1}): 3273, 3041, 2872, 1647, 1589, 1485, 1028. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.08 (bs, 1H, OH), 10.47 (s, 1H, R-CHO), 7.87 (s, 1H, ArH), 7.70-7.73 (m, 1H, ArH), 7.55-7.59 (m, 1H, ArH), 7.44-7.48 (m, 1H, ArH), 7.32-7.34 (q, 1H, *J* = 3.97 Hz, ArH), 3.22-3.28 (m, 1H, Ar-isopropyl), 3.00 (s, 3H, CH_3), 1.21-1.22 (d, 6H, *J* = 6.9 Hz, CH_3-C*H*-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.66, 21.68, 25.91, 120.39, 124.84, 124.87, 132.53, 132.59, 134.94, 139.95, 140.01, 142.17, 142.37, 157.74, 163.46, 197.58. TOF-MS (*m/z*) cal. for $C_{17}H_{17}FN_2O_2$: [M-H]$^-$ 299.1196; found: 299.0294.

3-((2-Chloro phenyl) diazenyl)-6-hydroxy-5-isopropyl-2-methylbenzaldehyde (K8): Color: Orange. Yield: 86%. M.p.: 166-168 °C. UV/Vis (Methanol, λ_{max}, nm): 358.79. FT-IR (ν_{max}, cm^{-1}): 3258, 3082, 2870, 1635, 1581, 1463, 758. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 13.12 (bs, 1H, OH), 10.49 (s, 1H, R-CHO), 7.93 (s, 1H, ArH), 7.68-7.71 (m, 2H, ArH), 7.52-7.55 (m, 1H, ArH), 7.47-7.50 (m, 1H, ArH) 3.23-3.29 (m, 1H, Ar-isopropyl), 3.03 (s, 3H, CH_3), 1.21-1.23 (d, 6H, *J* = 6.9 Hz, CH_3-C*H*-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 10.74, 21.72, 25.86, 117.80, 117.91, 120.74, 128.00, 130.56, 131.90, 133.33, 135.05, 142.36, 142.42, 147.99, 163.73, 197.63. TOF-MS (*m/z*) cal. for $C_{17}H_{17}ClN_2O_2$: [M+H]$^+$ 317.1057; found: 317.1035

2.4. Biological activity

2.4.1. Antibacterial assay for synthesized compounds

The antibacterial activity of the synthesized compounds (K1-K8) was evaluated against common pathogens such as *Escherichia coli* (NCIM-2065), *Staphylococcus aureus* (ATCC-6538), *Salmonella typhimurium* (NCIM-2501) and *Pseudomonas aeruginosa* (NCIM-2242) using the well diffusion method. Thymol and synthesized compounds were diluted in dimethyl sulfoxide (5 mg/mL). The prepared dilutions were inoculated in a well on media plates. The zone of inhibition (in mm) was measured against each pathogen. The dimethylsulfoxide was run as blank and antibiotic streptomycin (5 mg/mL) was kept as positive control [45,46].

2.4.2. α-Amylase/Starch hydrolysis activity of synthesized compounds

The α-amylase is an enzyme which is commonly used for the degradation of starch. Therefore, attempts have been made to assess the synthesized compounds (K1-K8) for starch degradation using 1% starch agar medium [47,48]. The prepared dilutions (5 mg/mL) in dimethyl sulfoxide were inoculated in prepared well on starch agar plate. At the α-amylase (Hi-Media) was used as positive control while dimethylsulfoxide was used as blank. The plates were allowed to incubate at room temperature (30-35 °C) for approximately 6 hours. After incubation, plates were flooded with 10% iodine solution to check the clearance zone around the well. The formed zones were measured in mm.

2.4.3. Radical scavenging activity of synthesized compounds

The radical scavenging activity of the synthesized compounds was ensured using 1,1-diphenyl-2-picrylhydrazyl (DPPH). For this purpose, a methanolic solution of DPPH (12 mg of DPPH was dissolved in 50 mL of methanol) was used as a stock solution. The optical density (OD) of stock solution was adjusted to 0.973 at 517 nm and the adjusted solution was used as a working solution. Diluted 1 mL samples (5 mg/mL of DMSO) were mixed with 0.5 mL of working DPPH solution and kept in dark at room temperature for 30 minutes. After incubation the OD of each tube was measured at 517 nm [49,50]. The samples were diluted in dimethylsulfoxide, hence the dimethyl sulfoxide was run as a control, while methanol was treated as a blank. Ascorbic acid (5 mg/mL) was run as positive standard. The % of radical scavenging activity was calculated by using the Equation 1.

$$\text{The radical scavenging activity} = \left[\frac{AC-AS}{AC}\right] * 100 \quad (1)$$

where, AC: optical density of control and AS: optical density of sample.

3. Results and discussion

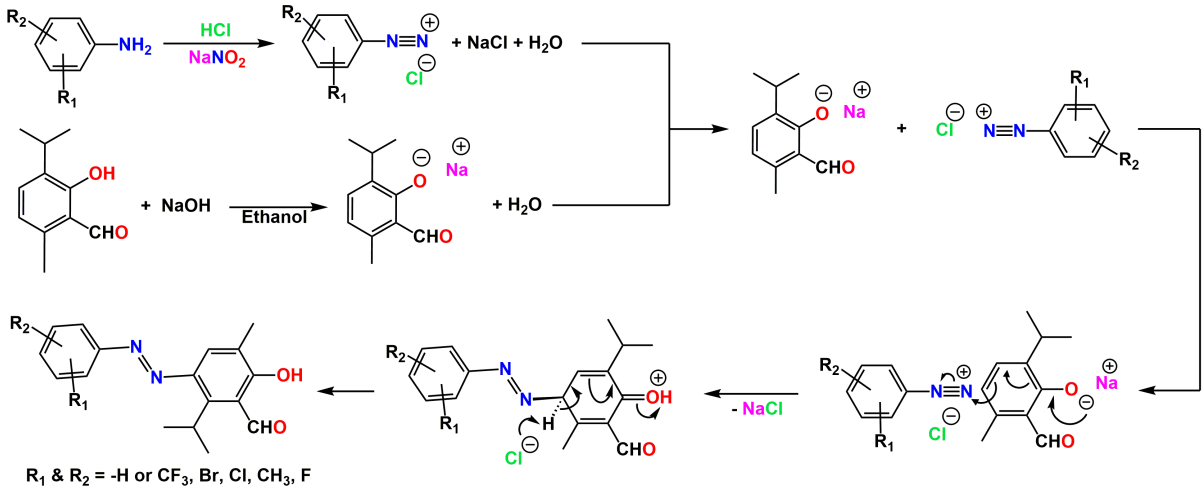
3.1. Synthesis

Aldehyde functions are predetermined for many industrial uses, such as value-added building blocks for the synthesis of quite a large number of molecules which have substantial industrial and academic significance, for example, vanillin is one of the most important flavor molecules used widely in the food and fragrance industry [51-54]. Therefore, the installation of aldehydic function or formylation of important biological aromatic carbocycles or heterocycles is drawing the attention of the scientific community, and as a result a large number of scientific reviews and research articles are available in the literature [55-60] for the formylation of phenols. Numerous applications of formyl thymol have been reported in the past few decades. Taking this in consideration the attempts have been made to perform formylation of the thymol to advance 2-formylthymol (Scheme 1). After the successful characterization of 2-formylthymol (**2**), we explored its potential as a coupling partner for the diazocoupling reaction, between diazonium salts obtained from a number of substituted amines through a process of diazotization and coupling with 2-formylthymol (Scheme 2).

The method goes smoothly even with the presence of electron-attracting groups such as -trifluoromethyl (-CF_3), -fluoro (-F), -chloro (-Cl), and -bromo (-Br), and faster reactions were encountered when the substituents are at *ortho*- and *para*-positions rather than metaposition (Scheme 2). The closure scrutiny of Scheme 2 reveals that the bromo group as a substituent with -I and +m effects at the *ortho*-position has an exceptional impact on the overall yield (97%) of the resulting azo-aldehyde (**K5**). In case of fluorine as a substituent which exerts (-I and +m effects) has no significant impact on the yield of the corresponding azo-aldehydes (80%) when fluorine it is present at o*rtho*- (**K-7**) or *meta*- (**K-4**) position, while fluorine at *para*-position (**K2**) (-I effect) is predominant and have a considerable impact on the overall yield of the resulting azo-aldehyde *i.e.* 83%. In the next derivatives, when chlorine (-I and +m effects) occupies the *ortho*-position it gives yield (86%) of the corresponding azo-aldehyde (**K8**), which is significantly higher than *ortho*-substituted fluorine (**K7**) and comparatively lower than *ortho*-substituted bromine (**K5**), this observation clearly indicates that the size of the halogen is an effective parameter to address reactivity in organic transformations. As we know, fluorine is always inductively electron-withdrawing but electron-donating by resonance, while the perfluoroalkyl group (-CF_3) is always electron-withdrawing [58,61]. Therefore, to examine the impact of field effects on the electronic behaviour of the -CF_3 group, we decorated the *ortho*-

Table 1. α-Amylase activity of synthesized azo-aldehydes.

Entry	Azo-aldehyde	Zone of diameter in mm
1	K1	9
2	K2	No zone
3	K3	No zone
4	K4	No zone
5	K5	No zone
6	K6	11
7	K7	12
8	K8	No zone
9	Thymol	No zone
10	Amylase (5 mg/mL)	15
11	DMSO	No zone



Scheme 3. Plausible mechanism for the formation of azo-aldehyde.

and *meta*-positions with the -CF_3 group, and the experimental data in hand emphasize that at *ortho*-position (**K3**). The -CF_3 group is more electron-withdrawing than *meta*-position (**K1**), leading to the formation of the corresponding azo-aldehydes **K3** and **K1** in 91 and 88%, respectively. Finally, the -CH_3 group at the *para* position furnishes an 81% yield of the corresponding azo-aldehyde (**K6**) and this is due to the electron-donating behavior of the -CH_3 group.

The plausible mechanism for diazotization followed by the diazo-coupling reaction is depicted in Scheme 3. The conclusive scrutiny of Scheme 3 suggests that the diazonium salt obtained from various substituted aromatic primary amines by the action of nitrous acid or sodium nitrite in the presence of a strong acid such as hydrochloric acid, is involved, as the coupling partner with 2-formylthymol gives appropriate azo-aldehyde as a product. After successful synthesis of azo-aldehydes, we confirmed the structure of these heterocycles using modern analytical tools, namely UV-vis, FT-IR, NMR (^1H and ^{13}C), and mass spectral analysis. The experimental data obtained are in harmony with the data reported in the literature. Subsequently, we examined the potential biological activities of these synthesized azo-aldehydes, which are discussed in Section 3.2.

3.2. Biological activity

3.2.1. Antibacterial assay for synthesized azo-aldehydes

The antibacterial activity of the synthesized azo aldehydes (K1-K8) was evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. All synthesized compounds (K1-K8) exhibited no inhibition zones against the tested bacterial strains. In contrast, the reference compound thymol showed inhibition zones of 11 mm against *E. coli*, 12 mm against *S. aureus*, 12 mm against *S. typhimurium*, and 10 mm against *P. aeruginosa*. The positive control streptomycin (5 mg/mL) exhibited stronger activity, with inhibition zones of 17 mm (*E. coli*), 16 mm (*S. aureus*), 15 mm (*S. typhimurium*), and 14 mm (*P. aeruginosa*). The negative control DMSO showed no inhibition zones against any of the tested bacteria.

3.2.2. In vitro α-amylase/starch hydrolysis activity of synthesized azo-aldehydes

Generally, the α-amylase is a useful enzyme for hydrolysis of starch in various food and processing industries. The hydrolytic activity of starch is indicated on the plate using simple technique. The hydrolytic zone does not react with iodine, and hence no coloration was observed. Synthesized azo-aldehydes were subjected to evaluation of their starch hydrolytic activity, like α-amylase, the experimental results are shown in Table 1. The closure scrutiny of Table 1 revealed that azo-aldehyde K1, K6, and K7 show promising starch destruction, while the positive result was indicated by standard amylase enzyme.

3.2.3. In vitro radical scavenging activity of synthesized azo-aldehydes

Free radicals are formed as by-products of normal biological processes and have a substantial effect on the life-governing processes positively or negatively carried out by the body [62,63]. The current study was carried out to evaluate the DPPH free radical scavenging effect of the synthesized azo-aldehydes and the results are tabulated in Table 2. In the present study, the radical scavenging effect was evaluated using a modified method [49] using the free radical 2,2-diphenyl-1-picrylhydrazyl. The DPPH assay is routinely used in laboratories to determine the free radical scavenging potential of purified phenolic compounds and natural plant extracts, as the assay is rapid, easy, and inexpensive [63-71]. As we know,

Table 2. Radical scavenging activity of synthesized azo-aldehydes.

Entry	Compound	Radical scavenging activity
1	K1	9.80±0.21
2	K2	49.67±0.07
3	K3	10.54±0.15
4	K4	53.44±0.15
5	K5	71.09±0.03
6	K6	18.17±0.14
7	K7	41.59±0.30
8	K8	80.18±0.45
9	Blank	-
10	Thymol	43.12±0.96
11	Ascorbic acid	90.57±0.30

the DPPH assay measures the ability of a compound to act as a free radical scavenger or hydrogen donor [49,63]. Our results demonstrated the scavenging effect of the synthesized azo-aldehydes and compared with ascorbic acid, a potential radical scavenger. The azo-aldehyde K-8 exhibits excellent radical scavenging in comparison with other azo-aldehydes.

4. Conclusions

In this work, the new azo-aldehyde dyes were prepared and characterized by different spectroscopic techniques. After successful characterization, studies related to examining the radical scavenging effect of synthesized azo-aldehyde dyes were performed and the azo-aldehyde dye K8 was found to exhibit an excellent radical scavenging effect. While the azo-aldehyde dyes K1, K6, and K7 exhibited appreciable α-amylase activity.

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Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest. Ethical approval: All ethical guidelines have been adhered.

CRediT authorship contribution statement

Conceptualization: Kishor Jaysing Girase; Methodology: Kishor Jaysing Girase, Santosh Venkatrao Dandge; Software: Kishor Jaysing Girase, Santosh Venkatrao Dandge, Mangal Arun Chaudhari and Ratnamala Subhash Bendre Validation: Kishor Jaysing Girase, Santosh Venkatrao Dandge; Formal Analysis: Mangal Arun Chaudhari and Ratnamala Subhash Bendre; Investigation: Ratnamala Subhash Bendre; Resources: Ratnamala Subhash Bendre; Data Curation: Santosh Venkatrao Dandge and Ratnamala Subhash Bendre; Writing - Original Draft: Kishor Jaysing Girase; Writing - Review and Editing: Santosh Venkatrao Dandge and Ratnamala Subhash Bendre; Visualization: Santosh Venkatrao Dandge and Ratnamala Subhash Bendre; Supervision: Ratnamala Subhash Bendre; Project Administration: Ratnamala Subhash Bendre.

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