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MICROWAVE-ASSISTED AND CONVENTIONAL SYNTHESIS OF HALOGENATED COUMARIN-AZO DERIVATIVES AND STRUCTURAL-ACTIVITY RELATIONSHIP STUDY FOR ANTIMICROBIAL POTENTIAL

(Sintesis Berbantukan Gelombang Mikro dan Konvensional Derivatif Coumarin-Azo Berhalogen dan Kajian Hubungan Struktur-Aktiviti untuk Potensi Antimikrob)

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Abstract

Untreatable bacterial infectious diseases have become a leading cause of mortality due to the emergence of drug-resistant bacteria. The search for a new effective pharmaceutical drug can be time-consuming and expensive. Therefore, structural chemical modification of natural product-based compounds with known biological properties for potential drug candidates has gained a great interest among researchers. Microwave-assisted synthesis is quickly becoming the method of choice in modern organic synthesis for drug discovery due to benefits such as higher yield and shorter reaction time. In this study, a series of coumarin derivatives have been synthesized by incorporating halogenated azo moieties in the molecular network *via* diazo-coupling, Knoevenagel condensation, and hydrolysis reactions. Microwave-assisted organic synthesis reaction has produced overall higher yields of products (74-94 %) in 6-17 mins compared to the conventional reflux method (56-85 %) in 6-18 h. The structural activity relationship of all compounds was initially evaluated *via in silico* (molecular docking) for potential antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus*. The synthesized compound gave a higher binding affinity (-6.3 to -8.9 kcal/mol) compared to ampicillin (-6.7 to -7.3 kcal/mol) and coumarin (-6.0 to -6.2 kcal/mol). *In vitro* evaluation (agar well diffusion), nevertheless, gave weak to no bacterial inhibition activity. This study is significant in searching for potential drug precursors to benefit mankind.

Keywords: diazo, docking, *In silico*, Knoevenagel, microwave

Abstrak

Penyakit berjangkit bakteria yang tidak dirawat disebabkan oleh kemunculan bakteria kerintangan ubat telah menjadi punca utama kematian. Proses pencarian ubat farmaseutikal baharu yang efektif boleh mengambil masa yang lama dan memerlukan kos yang tinggi. Oleh itu, pengubahsuaian struktur kimia sebatian yang berasaskan produk semula jadi dengan sifat biologi yang diketahui sebagai calon ubat yang berpotensi telah menarik minat dikalangan para penyelidik. Kaedah sintesis berbantukan gelombang mikro dengan cepatnya telah menjadi kaedah pilihan dalam sintesis organik moden untuk pencarian ubat berpotensi kerana kelebihannya seperti mampu menghasilkan produk yang lebih tinggi dan mengurangkan masa tindak balas. Dalam kajian ini, siri derivatif kumarin telah disintesis dengan menggabungkan moieti azo halogen dalam rangkaian molekul melalui tindak balas gandingan

diazo, pemeluwapan Knoevenagel, dan hidrolisis. Tindak balas sintesis organik berbantukan gelombang mikro telah berjaya menghasilkan produk keseluruhan yang lebih tinggi (74-94 %) dalam masa 6-17 minit berbanding kaedah refluks konvensional (56-85 %) yang memerlukan 6-18 jam. Penilaian hubungan aktiviti struktur semua sebatian untuk potensi aktiviti antimikrob terhadap Escherichia coli dan Staphylococcus aureus dinilai melalui kaedah *in silico* (dok molekul). Sebatian yang disintesis memberikan afiniti pengikatan yang tinggi (-6.3 hingga -8.9 kcal/mol) berbanding ampicillin (-6.7 hingga -7.3 kcal/mol) dan kumarin (-6.0 hingga -6.2 kcal/mol). Walaubagaimanapun, penilaian *in vitro* (penyebaran perigi agar) menunjukkan tiada aktiviti perencatan tumbuhan bakteria. Kajian ini penting dalam mencari prekursor ubat yang berpotensi untuk memberi manfaat kepada manusia.

Kata kunci: diazo, dok, *In silico*, Knoevenagel, gelombang mikro

Introduction

Untreatable infectious bacterial disease is a leading cause of mortality due to the rapid emergence of drug-resistant bacteria [1]. Antibiotics are crucial to combat many advent diseases and treat immune-compromised patients [2]. However, current antibiotics are becoming less effective against drug-resistant bacteria due to daily antibiotic consumption [3] and bacterial mutation [4]. There is an urgent need to develop new antimicrobial agents with high efficacy and effectiveness. The search for an effective pharmaceutical drug can be time-consuming and costly. Natural product-based compounds with known biological potentials have become a great source of inspiration in drug design [5]. Chemical modification of natural product-based compounds for potential drug candidates has gained significant interest among researchers [6-7]. Microwave-assisted synthesis has gained much interest in modern drug development programs due to its efficiency, shorter reaction times, and higher yields [8]. Furthermore, it is more cost-effective and environmentally friendly than the conventional approach via heating [9].

Coumarin is an example of a natural product-based compound that is naturally found in many plants [10]. Naturally occurring coumarin derivatives has many biological activities in plants especially in controlling plant growth, respiration, photosynthesis, and defense against various infections [11-12]. Coumarins' excellent biological properties have led to the development of synthetic coumarin derivatives with medical properties namely anti-bacterial, anti-cancer, anti-inflammation, anti-viral, and many others [11-14]. The biological properties of coumarin are due to its basic molecular structure, which noncovalently interact with many receptors in living organisms and resulting in a diverse range of physical activities [12&15]. Numerous ways are reported to synthesize coumarins namely Perkin reaction [16], Knoevenagel condensation [17], Pechmann condensation [18], and more [19].

In recent years, drugs which comprise two or more pharmacophore groups binding together covalently in one molecular framework have received great interest. The multi-targeted strategy has led to the development of a number of bioactive hybrid molecules with desired pharmacokinetic profiles and less tendency to drug-resistant bacteria [20]. Molecular hybridization of several pharmacophore groups acts by inhibiting two or more conventional targets at once. Structural hybridization of coumarin with other biologically active moieties is envisaged to increase their biological properties as new potential drugs [20-21]. Similar to coumarin, azo dyes are another biologically active moiety found in many natural products [22] with a wide spectrum of biological properties [23]. Azo dyes participate in various biological processes, including DNA inhibition, RNA and protein synthesis, carcinogenesis, and nitrogen fixation [24]. The incorporation of electronegative halogen substituents, on the other hand, improves the penetration of lipid membranes and increases the biological properties of molecules by making the structure more lipophilic [25-26].

This paper reports on the synthesis of coumarin incorporated with halogenated azo moiety, which is further evaluated for antibacterial properties against *E. coli* ATCC 25922 and *S. aureus* S48/81. The reaction involved a diazo-coupling reaction of halogenated azo-benzaldehyde intermediate **1a-d** followed by

Knoevenagel condensation reaction and hydrolysis (MW–assisted and conventional method) to achieve halogenated azo-coumarin derivatives **2a-d** and **3a-d**, respectively. The structure-activity relationship of the compounds for potential bacterial growth inhibition was preliminarily studied *via in silico* (molecular docking) followed by agar well diffusion (*in vitro*).

Materials and Methods

Materials and instrumentation

All purchased reactants and solvents of analytical grade were used without further purification. Microwave-assisted synthesis (MW) (was conducted using Anton Paar Monowave 300 (Austria). ^{1}H NMR and ^{13}C NMR spectra were recorded with DMSO-d6 as a solvent on a JEOL JNM-ECZ500R (Japan). Chemical shifts are given in parts per million (δ). FTIR spectra were obtained using Thermo SCIENTIFIC NICOLET iS10 (United States).

Synthesis of intermediate azo-benzaldehyde derivatives (1a-d)

A mixture of aniline derivatives (10 mmol) was dissolved in 10 mL of 25 % hydrochloric acid, HCl solution and cooled in an ice bath. Sodium nitrite, $NaNO_2$ solution (1.5 eq. mol) was added and reacted under constant stirring for 15 min. 2-Hydroxybenzaldehyde (10 mmol) dissolved in 20 mL of 1M sodium hydroxide, NaOH solution was then added to the mixture and reacted for a few minutes. The pH of the reaction was adjusted to 8-9. The reaction continued to react for 1-2 h. The reaction mixture was acidified using an HCl solution to precipitate out the crude product. The crude product was vacuum filtered, rinsed with distilled water, dried overnight, and purified *via* fast recrystallization from hot ethanol. The data analysis of **1a-d** can be obtained from supplementary file S1.

Synthesis of 3-ethylcarboxylatecoumarin-azo derivatives (2a-d)

The azo intermediate **1a-d** and diethyl malonate with a 1:1 ratio was dissolved into 20 ml of ethanol in a round bottom flask. Piperidine (0.25 eq mol) and a few drops of acetic acid were added to the reaction mixture, refluxed for 8-18 h, and confirmed *via* TLC analysis. Warm 50% ethanol (20 ml) was added to the reaction mixture and allowed to rest in ice water for 1 h. The solid product was filtered and rinsed with ethanol solution. The solid was recrystallized from ethanol to obtain a pure product. The conventional reflux heating method (I) was replaced with microwave irradiation (II) to reduce reaction time and increase product yield. The data analysis of **2a-d** can be obtained from supplementary file S2.

Synthesis of 3-ethylcarboxylatecoumarin-azo derivatives (3a-d)

Coumarin-azo **2a-d** dissolved in ethanol (15 ml) in a reaction flask followed by the addition of 1 M NaOH (15 ml). The reaction mixture was reacted for 3 h and confirmed *via* TLC analysis. The reaction mixture was then acidified with concentrated HCl and allowed to react for 15-30 min at room temperature. The solid precipitate was filtered and rinsed with hot distilled water and purified *via* hot filtration from ethanol. The conventional reflux heating method (I) was replaced with microwave irradiation (II) to reduce reaction time and increase product yield. The data analysis of **3a-d** can be obtained from supplementary file S3.

In silico study via molecular docking

Docking studies were performed using Autodock Tools and Vina. The X-ray structure of *E. coli* GyrB24 with inhibitor (PDB entry: 6yd9) and *S. aureus* GyrB ATPase domain in complex with a small molecule inhibitor (PDB entry: 3u2k) was retrieved from Protein Data Bank. The cubic grid box of 40 Å size (x, y, and z) with a spacing of 0.5 Å was centered on the active sites of the protein.

In-vitro study via agar well diffusion

In vitro screening of **1a–d**, **2a–d**, and **3a–d** was performed against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) *via* the agar well diffusion method. *E. coli* and *S. aureus* were cultured in Mueller-Hinton Broth (MHB) as inoculum and incubated at 37.5 ℃ with continuous shaking overnight at 180 rpm. The bacteria were spread on Mueller-Hinton Agar (MHA) plates using a bacteria suspension made with a sterilized cotton-tipped swab. A hole with a diameter of 6 to 8 mm is punched aseptically with a sterile tip and a volume of 10 µL of the synthesized compound in DMSO. The

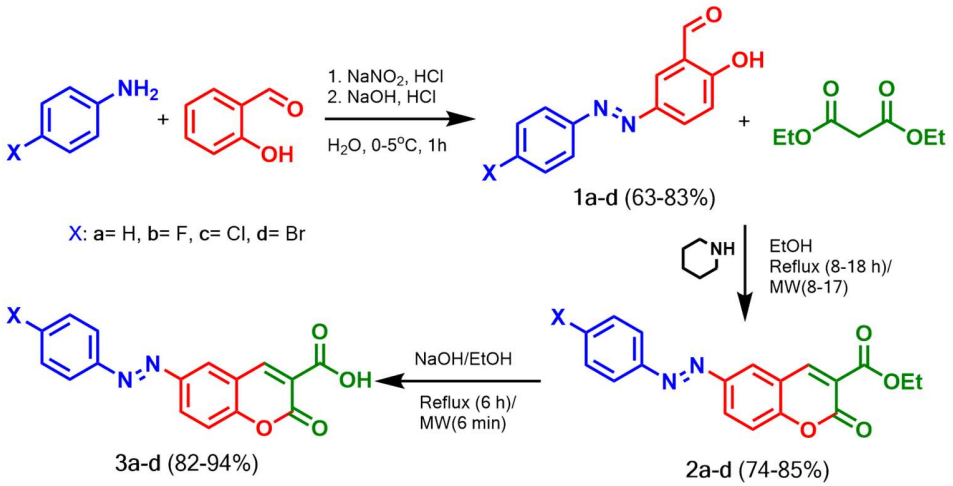
plates were incubated at 37.5 °C for 24 h. The zone of inhibition was measured in millimeters to evaluate the effectiveness of the synthesized compounds.

Results and Discussion

Chemistry

Prior to the synthesis of halogenated azo-coumarin **2a-d** and **3a-d,** an intermediate halogenated azo-benzaldehyde **1a-d** with a high yield (63-83%) was synthesized *via* diazotization of halogenated aniline in the presence of hydrochloric acid and sodium nitrite followed by coupling with 2-hydroxybenzaldehyde in basic condition. The formation of halogenated azo ethyl coumarin-3-carboxylate **2a-d** was achieved *via* Knoevenagel condensation of **1a-d** with diethyl malonate in piperidine and acetic acid as catalyst. Due to the long refluxing hour in conventional protocol employing hazardous solvent, microwave irradiation has become a great interest to introduce green and facile synthesis of one pot Knoevenagel condensation with higher yield [8]. The synthesis was comparatively performed using conventional heating and microwave irradiated (MW) synthesis. The microwave-assisted Knoevenagel condensation in MW was completed in shorter times of 8-17 min (74-85%) compared to the conventional reflux in 8-18 h (56-79%). The subsequence microwave irradiated hydrolysis of **2a-d** afforded halogenated azo coumarin-3-carboxylic **3a-d** with a higher yield and a shorter time (82-94% in 6 min) compared to the conventional procedure (76-85% and 6 h). The synthetic reactions of **1a-b**, **2a-d**, and **3a-d** are shown in Scheme 1. The yields and reaction times of conventional and microwave-assisted synthesis of **2a-d**, and **3a-d** is depicted in Table 1.

The molecular structure of all the synthesized compounds was characterized and confirmed using FTIR and NMR spectroscopy. Based on Figure 1, the formation of the N=N group of **1a-d** *via* diazo-coupling was confirmed by the strong absorption peak corresponding to the *v*(N=N) bend at 1483–1475 cm^{-1} [6&26]. The formation of **2a-d** *via* Knoevenagel reaction was indicated by the presence of *v*(C-H) stretch at 3051-3031 cm^{-1}. In addition, a strong signal at 1741–1732 cm^{-1} contributed by the *v*(C=O) lactone confirmed the formation of the coumarin lactone ring [27]. IR spectra of **3a-d** showed the presence of a strong signal in the range of 1745-1727 cm^{-1} contributed by *v*(C=O) lactone stretch and the disappearance of *v*(C-H) stretch at 3051-3031 cm^{-1} which confirmed successful hydrolysis of the ethyl group [27].



Scheme 1. Synthesis of **1a-d**, **2a-d**, and **3a-d**

Table 1. Comparison of conventional heating and MW irradiated synthesis of **2a-d** and **3a-d**

Product	Temperature (°C)		Time (min)		Yield (%)	
	Reflux	[MW]	Reflux	[MW]	Reflux	[MW]
2a	80	[140]	480	[8]	79	[85]
2b	80	[140]	600	[9]	74	[80]
2c	80	[140]	720	[11]	68	[83]
2d	80	[140]	1080	[17]	56	[74]
3a	80	[140]	360	[6]	80	[94]
3b	80	[140]	360	[6]	85	[85]
3c	80	[140]	360	[6]	77	[83]
3d	80	[140]	360	[6]	76	[82]

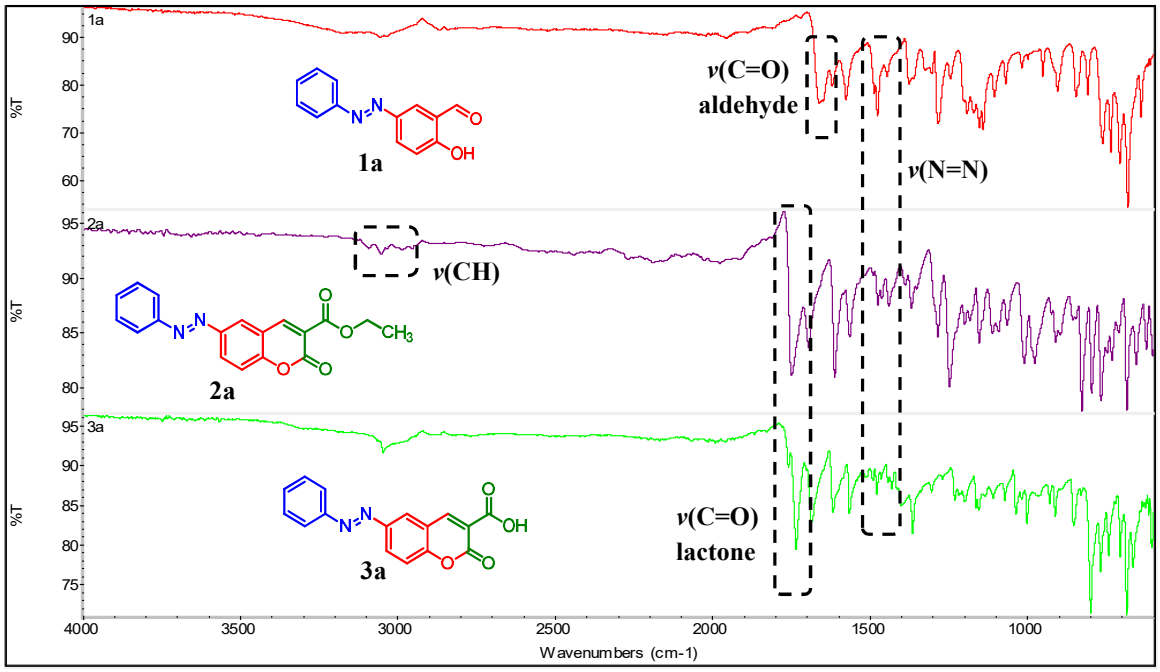


Figure 1. IR spectra of **1a**, **2a**, and **3a**

The ^{1}H NMR spectra of **1a-d**, **2a-d**, and **3a-d** showed the resonances attributed to aromatic protons at 8.93–7.20 ppm integrated with the corresponding number of aromatic protons. Figure 2 illustrated the ^{1}H spectra of **1a**, **2a**, and **3a**. The successful synthesis of -N=N- azo of **1a-d** *via* diazo coupling was confirmed by the duplet (H_a) and duplet-duplet (H_b) peaks at 8.17-8.19 ppm and 8.08-8.10 ppm, respectively. In addition, ^{1}H NMR spectra of **1a-d** showed the presence of proton for aldehyde as a singlet (H_c) at 10.37–10.36 ppm. The ^{1}H NMR spectra of **2a-d** indicated the formation of the coumarin *via* Knoevenagel condensation by the disappearance of the **1a-d** singlet aldehyde proton (H_c) peaks and the appearance of a new singlet (H_d) at 8.93 ppm. The appearance of the ethyl protons as a quartet (H_e) and triplet (H_f) at 4.34-4.55 and 1.34-1.33 ppm, respectively was also depicted by **2a-d**. The disappearance of peaks corresponded to -CH2CH3- at 4.34-1.33 ppm in the ^{1}H NMR spectra has clearly confirmed the formation of **3a-d** *via* hydrolysis.

The ^{13}C NMR spectra of **1a-d**, **2a-d,** and **3a-d** showed the resonances corresponding to aromatic carbons at 163.8–116.2 ppm. Compounds **1a-d** showed the

resonances attributed to *ν*(C=O) aldehyde at 190.6-190.4 ppm. The ^{13}C NMR spectra of **2a-d** showed the peak attributed to $–C_2H_5$ at 61.7–14.0 ppm. The resonance corresponding to *ν*(C=O) of **2a-d** and **3a-d** was observed at 163.8–156.0 ppm.

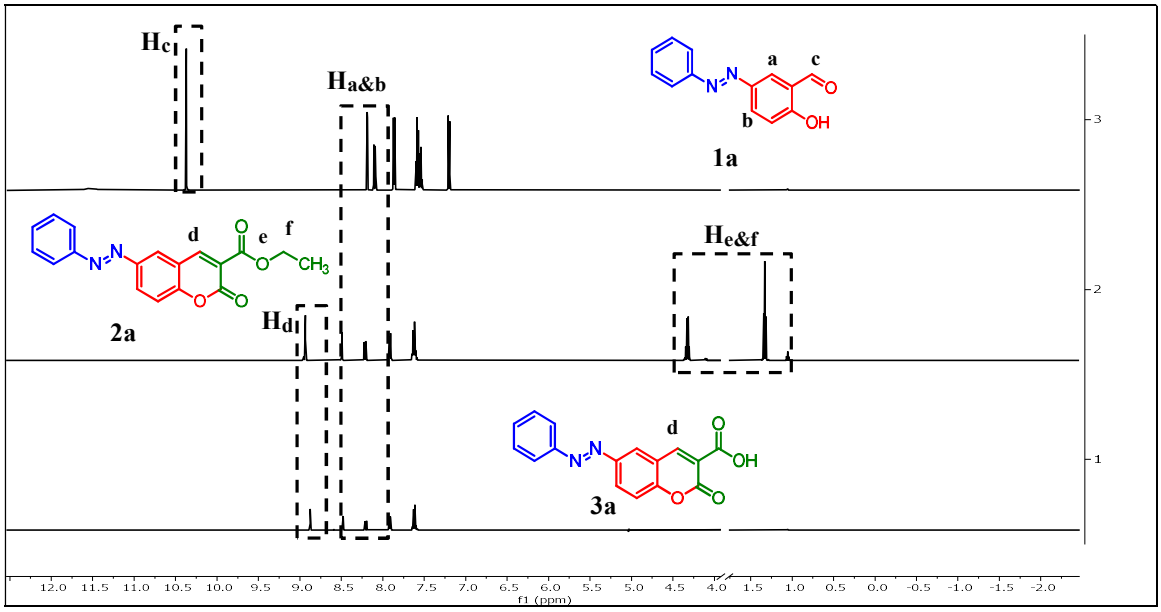


Figure 2. ^{1}H NMR spectra of **1a**, **2a**, and **3a**

Molecular docking study (*In silico*)

A preliminary study on the structural-activity relationship analysis of **1a-d**, **2a-d**, and **3a-d** as ligands was conducted to evaluate the possible binding interactions of the potential antibacterial agent. The binding affinity and interaction visualization between the ligands with DNA gyrase protein of *E. coli* GyrB24 (PDB entry: 6yd9) and *S. aureus* GyrB ATPase (PDB: 3u3k) were performed *via* Autodock Vina [28-29]. The binding affinity of **1a-d**, **2a-d**, and **3a-d** against DNA gyrase protein of *E. coli* and *S. aureus* is depicted in Table 2, where ampicillin was set as the standard drug. The drug candidates are usually chosen from the ligands that able to bind strongly to the target protein [30]. Higher binding affinity of the ligand indicates the ligand's ability to form strong binding interactions and influence the physiological function of target proteins [30].

The binding energies of **2a-d** and **3a-d** were -7.6 to -8.7 kcal/mol and -7.5 to -8.9 kcal/com, respectively for *E. coli* and *S. aureus*. The depicted binding energies of all the ligands were higher compared to the binding energy of the ampicillin (-6.7 to -7.3 kcal/mol) and coumarin (-6.0 to -6.2 kcal/mol). This indicates that coumarin azo derivatives have potential and ability to form stronger interaction, thus inhibit the bacterial enzyme activity. The intermediate **1a-d** scored the lowest (-6.3 to -6.6 kcal/mol). Among all, **3b** gave the highest binding energy -7.9 kcal/mol (*E. coli*) and -8.9 kcal/mol (*S. aureus*). Figure 3(a) depicted the interaction of **3b** to the active site of the enzyme for *E. coli*. The highly lipophilic characteristic of the compound formed hydrophobic interaction at the active site *via* π-alkyl and π-sigma bonding with the π-orbital of the alkyl group of various amino acid residues such as ILE78, PRO79, ARG76, and VAL120. An electrostatic interaction *via* the π-anion bond was also shown between the coumarin nucleus of **3b** with GLU50 residues.

The interaction of **3b** towards the active site of the *S. aureus* DNA gyrase enzyme is shown in Figure 3(b). Hydrophobic interaction *via* π-alkyl and π-sigma was observed between the benzene ring of **3b** with ILE86 and VAL131 residues. Hydrogen bond interaction was observed between the COOH of **3b** and GLU58 residue.

The presence of fluorine enables the ligand to form halogen bond interaction with GLU50 residues. In addition, van der Waals interaction between **3b** and various amino acid residues in the active site of the enzyme has also contributed to the strong ligand enzyme interaction.

Table 2. Binding affinity of **1a-d**, **2a-d**, and **3a-d** against DNA gyrase protein of *E. coli* and *S. aureus*

Compound	Binding Affinity (kcal/mol)	
	E. coli	***S. aureus***
1a	-6.5	-6.6
1b	-6.5	-6.6
1c	-6.4	-6.3
1d	-6.3	-6.3
2a	-7.8	-8.4
2b	-7.6	-8.7
2c	-7.6	-8.6
2d	-7.6	-8.6
3a	-7.9	-8.5
3b	-7.9	-8.9
3c	-7.9	-7.7
3d	-7.5	-8.7
Coumarin	-6.2	-6.0
Ampicillin	-7.3	-6.7

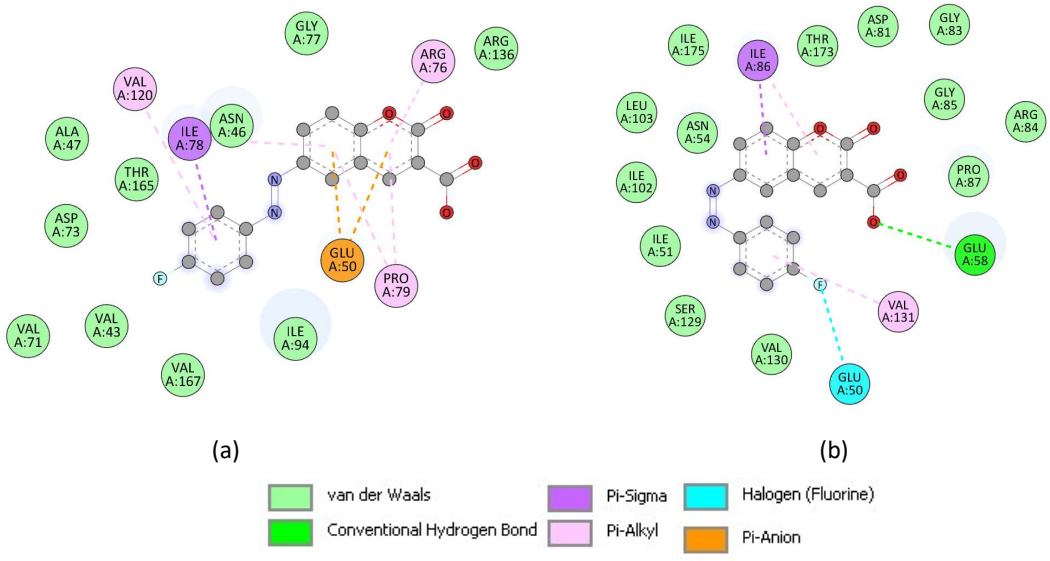


Figure 3: Interaction between **3b** and (a) *E. coli* and (b) *S. aureus*

The majority of DNA enzyme active binding sites has at least one hydrophobic pocket, and the lipophilic property makes it possible for lipophilic ligands to bind to hydrophobic protein binding sites [31].

Agar well diffusion study *(In vitro)*

Based on *in silico* strong interaction of the ligands against the DNA gyrase protein of *E. coli* and S. aureus, *in vitro* evaluations were conducted to evaluate the bacterial inhibition activity of the synthesized compounds against *E. coli* and *S. aureus via* agar well diffusion [32]. Ampicillin was used as the positive control and DMSO as the negative control. The results are depicted in Figure 4.

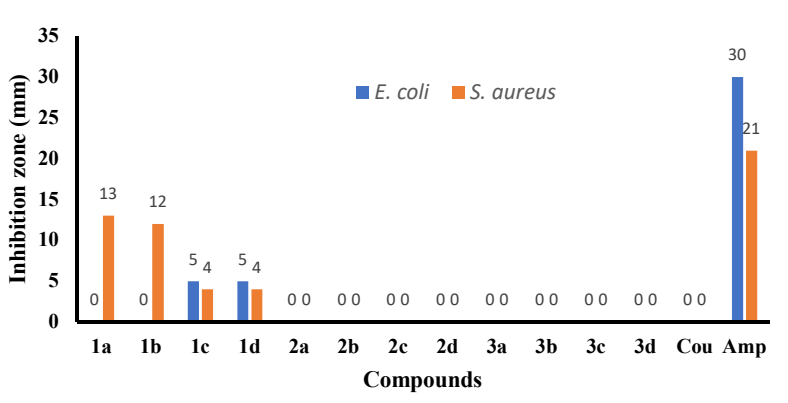


Figure 4. Inhibition activities of **1a-d**, **2a-d**, and **3a-d** against *E. coli* and *S. aureus*

Based on the results, all the synthesized azo **1a-d** showed inhibition activities against *S. aureus*. Among them **1a-b** demonstrated excellent activities on Gram positive bacteria (12-13 mm), while **1c-d** showed bacterial inhibition against both *E. coli* (5 mm) and *S. aureus* (4 mm), respectively. The bacterial inhibition activities of **1a-d** contributed to the presence of the N=N and halogens in the molecular network. The azo has the ability to protonate in acidic conditions and interact with the phosphate group on the peptidoglycan layer of bacteria, thus hindering the formation of the cell wall [6&33]. The biological activities of azo are well known for their ability to inhibit the DNA gyrase enzyme [34]. Additionally, azo can undergo tautomerization enabling it to act as both hydrogen bond acceptor and donator [24&35-36], hence increasing the biological activity. The presence of the OH group has also contributed to the inhibition activity of **1a-d** due to its ability to form hydrogen bond interaction with the biological target receptor [37-38].

The subsequence one pot cyclization of coumarin **2a-d** and **3a-d** via Knoevenagel condensation, however, reduced the inhibition activity against both bacteria strains. The compounds were inactive due to the competitive resonance and reduced electrophilicity of the lactone with the presence of azo group [9]. The formation of lactone further increases the lipophilicity of the compound, decreasing the compound permeability [39]. Poor dissolution properties of a compound could also contribute to the reduces antibacterial activity [40]. The combined interaction of hydrophilic and lipophilic characteristics in molecule structure plays a significant role in antibacterial activities [41-42].

Conclusion

The synthesis of coumarin-azo derivatives bearing halogens **2a-d** and **3a-d** has been successfully synthesized with great yield from **1a-d** *via* MW assisted Knoevenagel condensation followed by hydrolysis. Microwave-assisted synthesis promotes green chemistry by using less solvent and reaction times compared to the conventional heating method. Molecular docking evaluation of **2a-d** and **3a-d** showed higher binding affinity (-7.5 to -7.9 kcal/mol) than ampicillin (-7.2 to -7.3 kcal/mol) which indicates the potential ligands to affect enzyme activity and inhibit bacterial growth. The azo intermediate **1a–d** showed moderate inhibition activity with **1a** depicted the highest inhibition zone of 13 mm against *S. aureus*, whereas **1c-d** gave 5 mm against *E. coli* due to involvement of electron

delocalization and resonance stability. Nevertheless, the **2a-d** and **3a-d** showed no inhibition to the bacterial growth against *E. coli* and *S. aureus*. The halogenated azo intermediates exhibited a better binding tendency with target proteins as compared to the coumarin-azo derivatives. This research is essential in pharmaceutical industry for the drug development of lead chemicals.

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