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Synthesis and molecular docking simulation on the antimicrobial effects of halogenated vanillin-azo dyes and schiff base derivatives

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Abstract

Lead compounds containing nitrogen pharmacophores from natural resources have garnered interest among researchers due to their potential for drug development. However, the extractions of the active metabolites are usually labor-intensive and time-consuming. In this study, halogenated vanillin derivatives featuring azo dyes (N=N) (**1a-1 h**) and Schiff base (C=N) (**2a-2 h**) have been synthesized via diazonium coupling and nucleophilic substitution reaction, respectively. The comparative effect of N=N and C=N moieties was evaluated for antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* via disc diffusion method. Incorporating C=N (8–13 mm) into the vanillin network showed excellent inhibition against *S. aureus* compared to N=N (7–8 mm) and the standard ampicillin (12 mm). While the halogenated vanillin featuring N=N (7–9 mm) and C=N (7–8 mm) moieties showed excellent zone of inhibitions against *E. coli* compared to the parent vanillin. The in-silico screening using AutoDock Vina, showed **2c-h** (inhibition zone > 10 mm) with a high binding affinity against DNA gyrase enzyme with binding energy ranging from − 7.3 to − 7.9 kcal/mol, similar to re-docking of ampicillin − 7.6 kcal/mol and co-crystalize compounds BPH651 with − 7.5 kcal/mol. This research contributes a significant milestone in drug design, especially for the development of new antibacterial drugs with outstanding properties.

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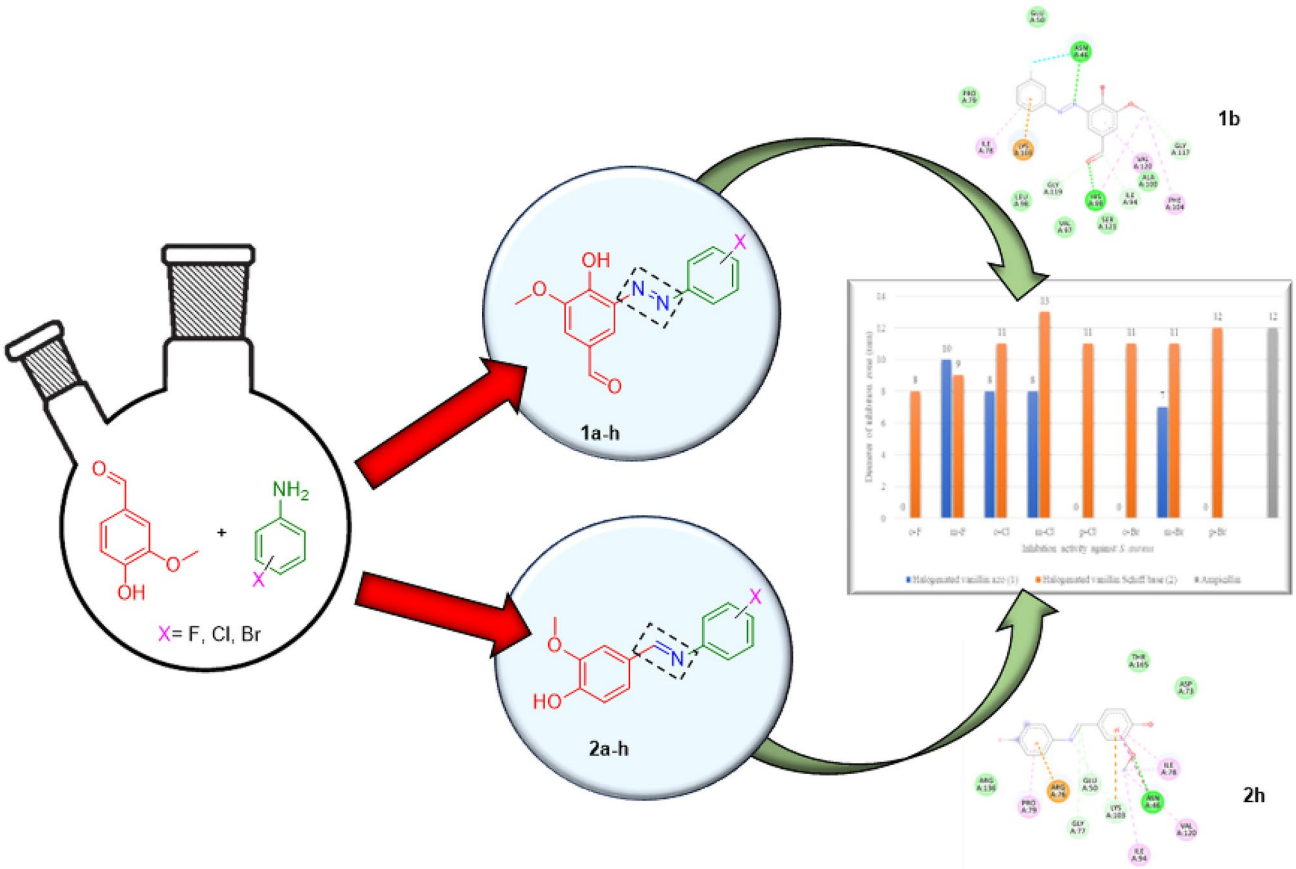
Graphical abstract



Article Highlights

- A synergistic effect of functional groups in vanillin-Schiff base (C=N) and vanillin-azo dyes (N=N) contributed to the hydrogen bond interaction with the biological receptors.
- Structure–Activity relationship of halogenated vanillin derivatives supported the inhibition activity against *Staphylococcus aureus* and *Escherichia coli*.
- The incorporation of nitrogen chromophores and halogen into vanillin moieties improved binding interactions and binding affinity.

Keywords Vanillin · Nitrogen · Imine · Molecular docking · In silico

1 Introduction

The rise of pathogenic microbe resistance has prompted researchers to search for effective pharmaceutical drugs. The prescription against nonbacterial diseases, such as viral infections and uncontrolled consumption of drugs has led to microbial resistance [1]. It has become challenging for researchers due to the rising expenses in drug development with a limited time [2–4]. Traditional medicine has utilized natural resources such as penicillin [5], coumarin [3], and aspirin [4] to relieve pain and fever. Modifying active scaffolds from natural product-based compounds can potentially develop novel drugs with enhanced efficacy against drug-resistant microbes with less cost and time.

Chemical modification of compounds derived from natural sources offers a promising approach as it enhances the likelihood of diverse interactions with biological targets and improves their biological activity [2, 3]. Vanillin is an example of an active scaffold derived from the natural product *Vanilla planifolia*, a common food-grade additive that is widely used in food [6] and pharmaceutical products [7]. However, its extraction process is labor-intensive and lower in yield [8]. Many studies reported on the chemical alteration of vanillin's structure to enhance its biological properties for anticancer [9, 10], antiviral [11], antifungal [12] and antibacterial [13]. Trimethoprim is an example of a known pharmaceutical drug containing vanillin to treat upper respiratory tract infections [14].

Many active chromophores with nitrogen moieties, including azo dyes (N=N) and Schiff base (C=N) have been exclusively integrated into the vanillin network to increase their biological activities. The presence of an active moiety of azomethine (C=N) in Schiff base contributed to a broad range of biological effects in the pharmaceutical industry such as anticancer [15], antimicrobial [16] and antibacterial properties [17]. Schiff bases have also been utilised as ligands to form metal complexes due to their stronger coordinative ability, which makes them useful for catalytic reactions [16], fluorescent chemosensors [15] and dyes [17]. While, Azo dyes have been widely used in textile, fiber, cosmetics, leather and paints [3, 17].

There are many drugs containing azo dyes (N=N) that have been commercially used to treat bacterial infections such as sulfasalazine and phenazopyridine [18]. The presence of an azo group (N=N) that is easily protonated under acidic conditions contributes to improving the biological activity of a compound [3]. Nevertheless, a synergistic incorporation of nitrogen moieties and halogen substituents in the vanillin is scarcely reported. The presence of halogen moieties can enhance the lipophilic characteristics of molecules, facilitating their ability to penetrate the outer membrane of bacteria [19, 20]. The inclusion of halogens into the molecular framework of vanillin, which bears nitrogen chromophores, is anticipated to synergistically enhance the antimicrobial effectiveness of the parent vanillin.

This work reports the formation of halogenated vanillin-azo dyes (**1a-1 h**) from a series of vanillin derivatives comprising nitrogen chromophores and halogens at *ortho, meta, and para* positions by the diazo coupling reaction. Another series of halogenated vanillin-Schiff base derivatives (**2a-2 h**) was prepared using a nucleophilic substitution reaction. The in vitro antibacterial activities against *E. coli* ATCC 25922 and *S. aureus* S48/81 were comparatively evaluated for potential antimicrobial drugs, using ampicillin as the reference drug. The structure–activity relationship of the molecules and evaluation of the binding affinity via in-silico molecular docking on DNA gyrase enzyme (PDB ID: 4DUH) as a potential therapeutic target were performed to postulate the molecular basis of the antibacterial action of the synthesized compounds.

2 Results and discussion

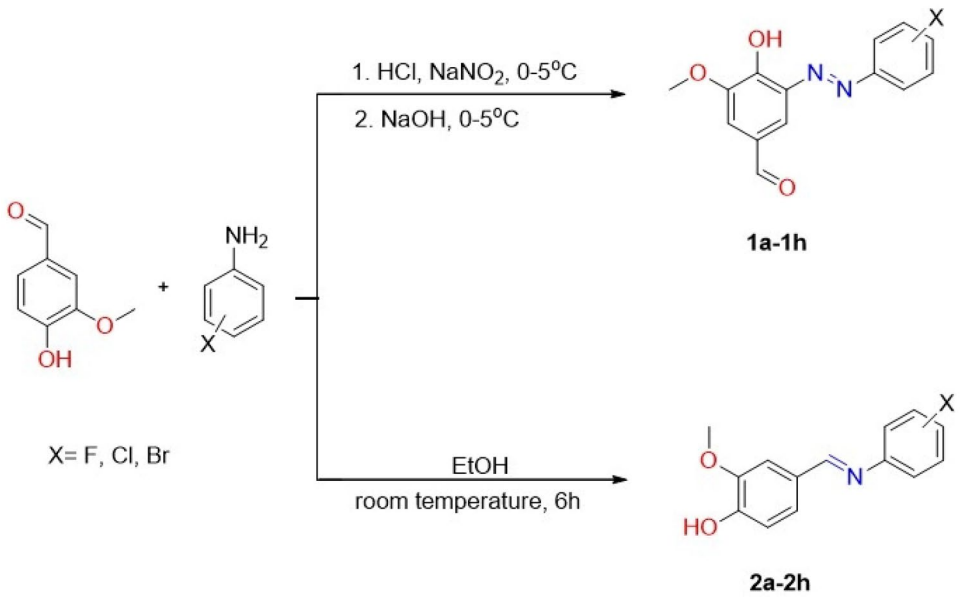
2.1 Chemistry

A series of vanillin-azo dyes **1a-1 h** containing halogen and nitrogen moieties (N=N) as an active pharmacophore was prepared from the reaction of vanillin with anilines featuring halogens. The diazo coupling reaction was conducted in the presence of NaOH. A comparative nitrogen active pharmacophores of vanillin-Schiff base derivatives (C=N) **2a-2 h** featuring halogens were synthesized via nucleophilic substitution reaction of halogenated aniline with vanillin in ethanol at the ambient temperature. The schematic diagram for the synthesis of **1a-1 h** and **2a-2 h** is shown in Scheme 1.

The chemical structures of **1a-1 h** & **2a-2 h** were elucidated via FTIR, ^{1}H and ^{13}C NMR spectroscopy (Figure S2, S3 & S4). The characterization **1a-1 h** using FTIR depicted a strong absorption peak at 1490–1464 cm^{-1} indicating the formation of *v*(N=N) [3]. The *v*(C=O) peaks were observed at 1649–1685 cm^{-1} [21] while v(O–H) presence as a broad peak at 3426–3453 cm^{-1} [22]. The ^{1}H and ^{13}C NMR spectroscopy of **1a-1 h** showed the presence of aromatic protons at 7.33–8.13 ppm for the aromatic protons in the compound. The peaks at 3.90–3.91 ppm represent the -OCH_3 [22] while the resonance at 9.88–9.91 ppm is attributed to the aldehyde peak [23]. The ^{13}C NMR peak at 190.8–192.2 ppm and 56.1–56.8 ppm attributed to the C=O and –OCH_3 [23], respectively, which confirmed the formation of **1a-1 h.**

The FTIR spectra of **2a-2 h** showed the disappearance of *v*(C=O) at 1650–1680 cm^{-1} and the appearance of strong absorption at 1617–1622 cm^{-1} for *v*(C=N) [24], indicating the formation of the imine group. The broad peak at 3055–3175 cm^{-1} corresponded to *v*(O–H). ^{1}H and ^{13}C NMR spectroscopy of **2a-2 h**, depicted peaks at 6.50–7.62 ppm for the Ar–H in **2a-2 h,** while the peak at 3.89–3.93 ppm attributed to the –OCH_3. The disappearance of the aldehyde peak at 9–10 ppm and the appearance of a singlet peak at 8.42–8.45 ppm represented the imine peak [24], which confirmed

Scheme 1 Synthesis of compounds **1a-1h** and **2a-2h**



the formation of **2a-2 h**. The ^{13}C NMR spectra depicted peaks at 160.6–162.3 ppm and 55.4–55.5 ppm for C=N and $-OCH_3$, respectively.

2.2 Antibacterial activities of 1a-1 h and 2a-2 h

The antibacterial activity of the halogenated vanillin-azo series (**1a-1 h**) and halogenated vanillin-Schiff base series (**2a-2 h**) were carried out against *S. aureus* and *E. coli* using the turbidimetric kinetic method. However, several compounds encountered solubility restrictions and dissolution in the culture media caused the precipitation of the drug[2, 21]. This occurrence has interfered with the measurement of biological activities. It was envisaged that the relatively lipophilic molecular characteristics of the compounds may restrict their solubility in the culture media [2].

Kirby-Bauer disc diffusion assay was alternatively used in this study to evaluate the inhibitory efficacy of the compounds against *E. coli* and *S. aureus* (Figure S1 and Fig. 1)**.** Both series of halogenated vanillin derivatives featuring C=N moiety (**2a-2 h)** and N=N **(1a-1 h)** showed moderate to excellent zone inhibition of *S. aureus* and *E. coli*. The incorporation of C=N into the vanillin network demonstrated superior inhibition against *S. aureus* (zone of inhibition 8–13 mm) compared to N=N (7–8 mm) and the standard ampicillin (12 mm). In comparison to the parent vanillin, the halogenated vanillin bearing C=N and N=N moieties showed excellent zones of inhibitions against *E. coli* with 7–8 mm and 7–9 mm, respectively. It is also worth mentioning that low lipid solubility can reduce antimicrobial efficacy due to their impermeability towards the bacterial surface such as *S. aureus* [25]. The introduction of halogen has remarkably increased the lipophilic characteristic of the synthesized compounds, enabling them to penetrate the outer membrane of the bacteria [26].

The remarkable inhibition activity of **2a-2 h** was aligned with the recent similar findings [27] on the superior antimicrobial properties of Schiff bases compared to azo dyes due to their stronger binding affinity and better hydrogen bonding interactions with specific protein sites, effectively disrupting bacterial growth [17]. However, the antimicrobial activity of vanillin-bearing Schiff base moieties was relatively lower against *E. coli* due to the rigid lipopolysaccharide bilayer at the outer membrane of bacteria which therefore reduced the ability of the compound for penetration [19]. Interestingly, the inhibition activity against *E. coli* could only be observed on compounds featuring bromine (**2f**, **2 g**, & **2 h**). This could be attributed to the polarizability of the bromine atom which possesses a larger atomic size. The high polarization effect of bromine compared to fluorine and chlorine influenced the binding interactions of the modified scaffolds with the target protein, which in turn impacted the overall antibacterial efficacy [20].

The *meta*-halogenated vanillin derivatives (**1b**, **1d**, **1 g**) exhibited significant activity against *S. aureus*, consistent with previous findings demonstrating the antimicrobial efficacy of organic compounds containing halogen substitutions at the *meta* position [4, 28]. Nevertheless, *meta*-substituted chlorine (**1d**) showed no inhibition against *E. coli* due to the less lipophilic [20] compared to *meta*-substituted bromine (**1 g**). Excellent inhibition of *meta*-fluoro (**1b**) was due to the formation of hydrogen bonds with the target receptor [29]. The presence of the active moiety N=N that is easily protonated

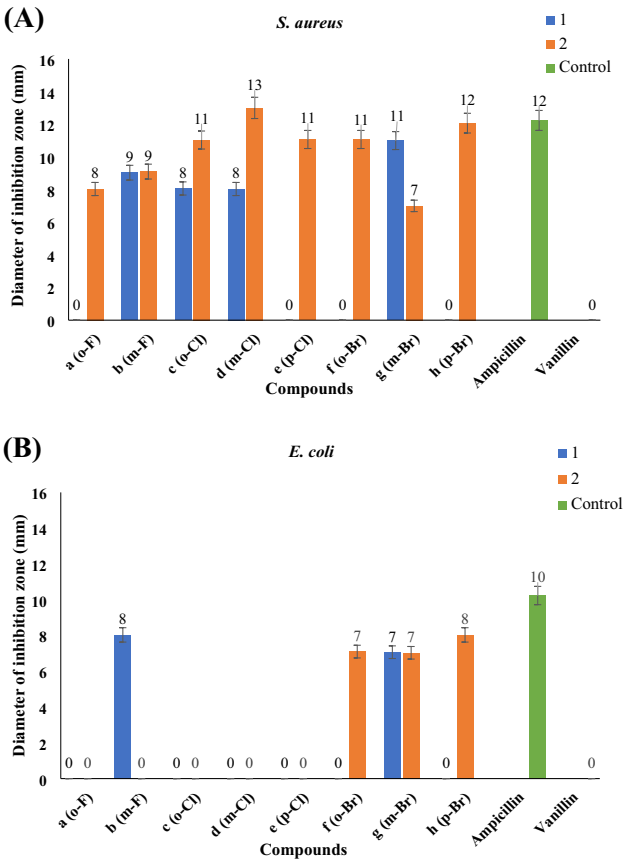


Fig. 1 In vitro antibacterial screening of compounds **1a-1 h** and **2a-2 h** against **A** *S. aureus* as Gram-positive bacteria and **B** *E. coli* as Gram-negative bacteria

under acidic conditions contributes to the antibacterial properties of the compound [30–32]. Additional interaction of the carbonyl and phenyl ring with the phosphates moieties on the bacteria's surface has also increased the antibacterial properties of a compound [33, 34]. Besides, the presence of halogen atoms as substituents has also significantly increased the biological activity of the compounds by maintaining the non-hydrophobic interaction between halogen and the amino acid of the bacteria, [21]. It is worth mentioning that the integration of halogen substituents may result in steric hindrance of the molecule and increase its capacity to interact efficiently with the bacteria [34]. Less inhibition on both series against *E. coli* was reported due to the thicker outer membrane of the bacteria containing lipopolysaccharide which could reduce the compound's ability to penetrate [19].

A synergistic effect of functional groups presence on vanillin-Schiff base (C=N, –OH) and vanillin-azo dyes (N=N, C=O, –OH) has contributed to the antibacterial of the compounds via hydrogen bond interaction with the biological receptors compared to the parent vanillin alone. Among all the synthesized compounds, the *meta*-halogenated vanillin showed good inhibition against both Gram-positive and Gram-negative bacteria. This is due to the synergistic effects of hydrophilic-lipophilic characteristics in the molecule thus contributing to the antibacterial properties [35]. The aromatic rings in the molecular network have also played a significant role in enhancing the lipophilic properties which also can potentially interact with the hydrophobic areas of bacterial enzymes [21].

2.3 Molecular docking studies

Molecular docking screening was used to postulate the molecular basis of the antibacterial action of the synthesized compounds. DNA gyrase enzyme (PDB ID: 4DUH) was chosen as a potential therapeutic target to examine the antibacterial action. One of the topoisomerase classes (topoisomerase II) is represented by the DNA Gyrase enzyme. The DNA is wound and unwound by these enzymes during transcription and replication. Due to its ability to change the topological state of DNA and act as a model for other DNA topoisomerases, the gyrase enzyme is anticipated to be a crucial intracellular target for antibacterial drugs [36]. Re-docking was performed to the crystal structures of the DNA gyrase enzyme using the inhibitors BPH651 to validate the docking parameter used in this work for the docking outputs to be replicated. The

orientation of the inhibitor's optimal docking pose was superimposed with the binding site of the inhibitor's original crystal structure. The conformation (1.0292) was set for BPH651 and BPH651 (Fig. 2). In a re-docking study, both docked inhibitor conformations showed the same orientation with the inhibitor, indicating that AutoDock Vina has excellent accuracy in predicting the ligand the binding interactions with the DNA gyrase enzyme [37].

To investigate the mechanism of action and the inhibition of the DNA gyrase enzyme at the molecular level, a molecular docking study was performed on all compounds **(1a-1 h** and **2a-2 h)** (Table S1). Halogenated vanillin-Schiff Base **2c-2 h** with inhibition zone > 10 mm showed excellent binding affinity for the enzyme with a binding energy of – 7.3 to

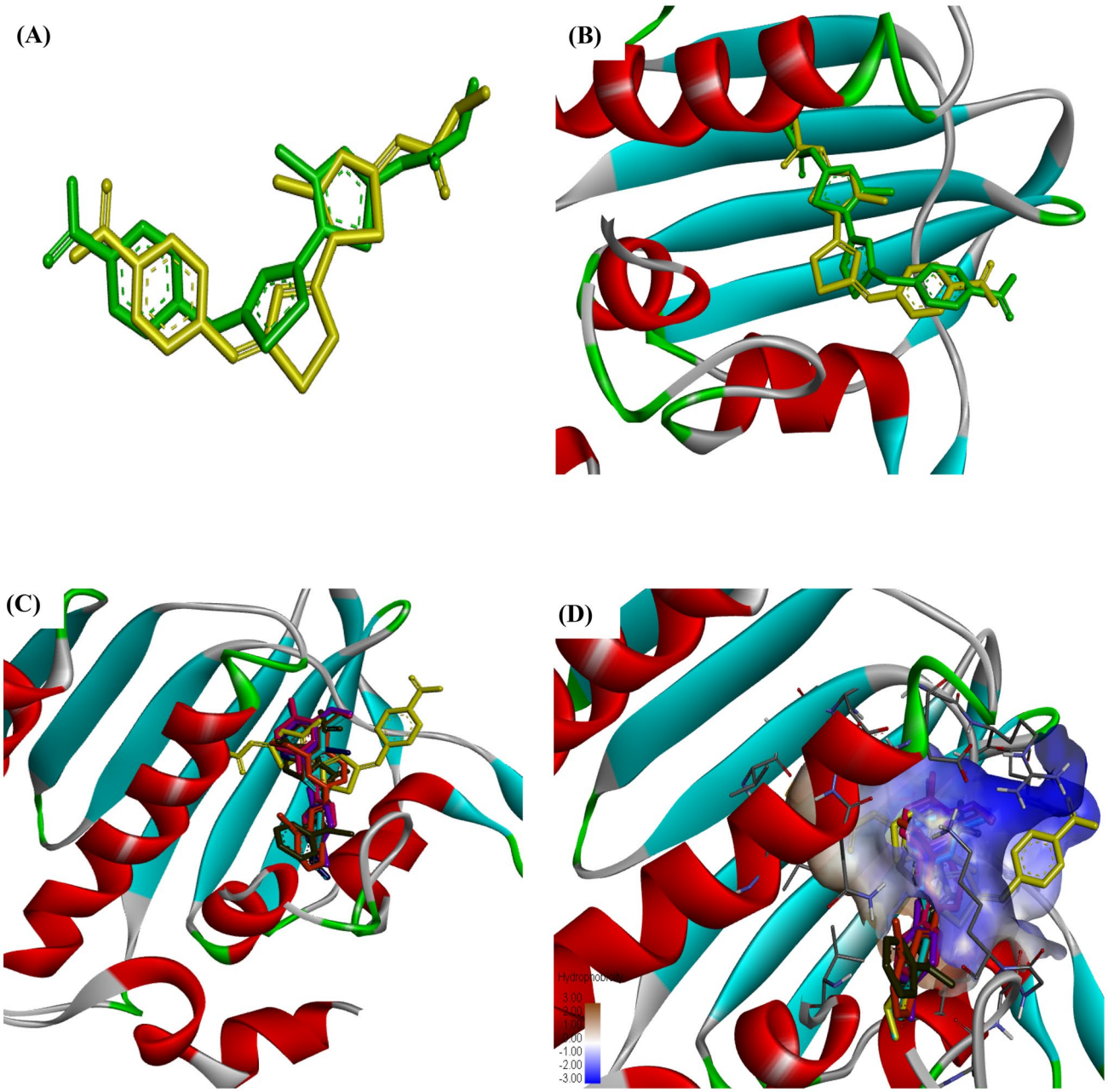


Fig. 2 Molecular docking simulation of binding interaction with the DNA gyrase enzyme using stick model. **A** The alignment of the redocked BPH651 (green stick model) and co-crystallized BPH651 (yellow stick model) within the binding site of DNA gyrase enzyme. **B** Superimposition of redocking BPH651 (green stick model) and co-crystallized BPH651 (yellow stick model). **C** The alignment of **2c** (blue stick model), **2d** (brown stick model), **2e** (purple tick model), **2f** (orange stick model), **2g** (turquoise stick model), **2h** (pink stick model), ampicillin (silver stick model) and BPH651 (yellow stick model) within the binding site of DNA gyrase enzyme. **D 2c** (blue stick model), **2d** (brown stick model), **2e** (purple tick model), **2f** (Orange stick model), **2g** (turquoise stick model), **2h** (pink stick model), ampicillin (grey stick model) and BPH651 (yellow stick model) in the hydrophobic pocket of DNA gyrase enzyme

– 7.9 kcal/mol, similar to re-dock of ampicillin – 7.6 kcal/mol and co-crystalize compounds BPH651 with – 7.5 kcal/mol. The hydrogen bond interaction of **2c-2 h** with amino acid in binding pocket enzyme makes a key contribution to the inhibitory activity. Compounds **2c-2 h** show hydrogen bonds with ASN46 similar to the co-crystalize ligand and ampicillin. Notable, the Schiff base derivatives contribute to the binding interaction between nitrogen and amino acids in the enzyme, thereby leading to bacterial inhibition activity. The hydrophobicity of **2c-2 h** and the hydrophobic pocket in the enzyme's binding side are other factors that contribute to the excellent interaction of the ligand and receptor. The active compounds in this study showed similar amino acid interaction with BPH65 and ampicillin. Examples of the amino acid are ASN46, PRO79, ILE94, LYS103 and VAL120.

3 Conclusion

In conclusion, the incorporation of azo **1a-h** and Schiff base **2a-h** on the molecular network of vanillin has surpassed the antibacterial efficacy compared to the parent vanillin. Compounds **2a-2 h** exhibited pronounced effectiveness against *S. aureus* as compared to **1a-1 h**. Among newly synthesized compounds, compound **2d** exhibited better inhibition against *S. aureus* compared to the reference drug, ampicillin. The incorporation of halogenated azo and Schiff base groups into the vanillin structure allowed for the synergistic effects of several functional groups, which enhanced binding interactions with the bacterial surface. This finding concludes that the addition of nitrogen and halogen elements enhances the antibacterial properties of vanillin, rendering its derivatives promising candidates for further exploration and potential drug development. Further research and optimization of these derivatives hold the potential to yield antibacterial medications with wide-ranging applications in the medical field.

4 Experimental section

The chemicals were analytical grade with no purification. The melting point (Stuart MP3) was used with an open tube capillary. ^{1}H NMR and ^{13}C NMR spectra were recorded using JEOL ECA 500 at 500 MHz (^{1}H) and 126 MHz (^{13}C) with the chemical shift relative to DMSO-d_6/acetone-d_6 as standard reference (in δ ppm).

4.1 Synthesis series of halogenated vanillin-azo (1a-1 h): general procedure

Halogenated vanillin-azo was synthesized following the procedure reported by Mortadza & Ngaini [23]. A solution of $NaNO_2$ (2 M, 5 mL) was added to an aniline derivative (5 mmol) in HCl solution (2 M, 10 mL) at 0–5 °C. The mixture was slowly added into vanillin (5 mmol) in NaOH(0.4 g, 10 mmol) in distilled water (10 mL) and continued stirring for 1 h. The mixture was acidified with HCl (2 M). The precipitate was filtered, washed and recrystallized in ethanol to give **1a-1 h**. The supporting data (**1a-1 h)** is accessible in the supplementary files Data S1**.**

4.2 Synthesis of halogenated vanillin-Schiff base (2a-2 h): General procedure

The synthesis of halogenated vanillin-Schiff base was adapted from previous literature by Chigurupati [24]. Halogenated aniline (0.5 mmol) and vanillin (0.5 mmol) were stirred in ethanol at room temperature for 10 h. Distilled water was added and the mixture was cooled to obtain the crude solid, filtered, washed, and recrystallized using ethanol: hexane (1:2) to afford **2a-2 h**. Data for **2a-2 h** is accessible in the supplementary files Data S2.

4.3 Antibacterial studies

Kirby-Bauer disc diffusion method was employed for the antibacterial evaluation of **1a-1 h** and **2a-2 h** against Gram-positive and Gram-negative bacteria (*S. aureus* and *E. coli*) [21]. DMSO served as a negative control and reference drug, ampicillin was chosen. Compounds **1a-1 h** and **2a-2 h** in DMSO (100 ppm) were introduced to the sterile paper discs and put on the surface of microorganism-inoculated media. The inhibition zone diameter was measured after 24 h and recorded.

4.4 Molecular docking studies

In-silico screening using molecular docking was applied to all the synthetic derivatives to postulate the molecular mechanism behind the antibacterial characteristics of the compounds. The 2D structures of each chemical were created using Accelrys, San Diego, USA (Discovery Studio® 4.0). The Avogadro program was used to optimize the ligand structures (shape and energy) using the MMFF-94 force field's steepest descent and conjugate gradient approach (5,000 steps) [38]. The structure of the selected macromolecules was obtained from the Protein Data Bank (PDB). DNA gyrase enzyme (PDB ID: 4DUH) was chosen as a potential therapeutic target. The target protein contains an inhibitor with a resolution of less than 3.0 [39]. The hydrogen atoms were introduced into protein structures employing AutoDockTools [40]. AutoDock Vina was used to perform the docking [41]. Discovery Studio® 4.0 (Accelrys, San Diego, USA) was used to visualize the binding interactions where the highest binding affinity was selected.

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Author contributions Conceptualization, research design and funding acquisition by Z.N. and A.N.. M. A., F. N. A. and F. S. conducted the experiment. N. H. performed the computational molecular docking analysis. M. A. and N. H. wrote the original draft of the manuscript and prepared the supplementary materials. Z.N. reviewed and edited the manuscript. All authors reviewed the manuscript.

Data availability All data generated or analyzed during this study are included in this published article as supplementary data.

Declarations

Competing interests No potential conflict of interest was reported by the authors.

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