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Synthesis of *E*-stilbene azomethines as potent antimicrobial and antioxidant agents

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Abstract: A series of new extensively conjugated *E*-stilbene azomethines (**5a**–**5h**) were synthesized and screened for their antioxidant and antimicrobial activity. The compounds were tested against bacterial (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Bacillus subtilis*) and fungal strains (*Aspergillus niger*, *A. flavus*, and *Trichoderma harzianum*) using the agar well diffusion method. Among the tested compounds, *N*'-(4-nitrobenzylidene)-2-((*E*)-styryl)benzohydrazide (**5g**) was found to possess potent antimicrobial activity higher than some drugs with significant activity reported in the literature, e.g., cefradine and terbinafine hydrochloride. Additionally, compounds **5a**–**5h** were also evaluated for antioxidant potential using DPPH free radical scavenging and ferric thiocyanate (FTC) assays. Among these, *N*'-(4-hydroxybenzylidene)-2-((*E*)-styryl)benzohydrazide (**5e**) exhibited significant antioxidant potential by both assays. Compound **5e** demonstrated higher DPPH free radical scavenging activity (IC_{50} = 22 ± 0.19 µ g/mL) than the standard, butylated hydroxytoluene (BHT; IC_{50} = 28 ± 0.10 µ g/mL). A similar trend was observed for compound **5e** in FTC assay, which exhibited 86 ± 0.19% inhibition, whereas the BHT control showed 81 ± 0.21% inhibition of linoleic acid peroxidase. The structure elucidation of the synthesized compounds was carried out by UV-Vis, FT-IR, ^{1}H NMR, ^{13}C NMR, and elemental analysis and mass spectrometry. These results suggest possible use of these compounds for the rational design of new antimicrobial and antioxidant agents.

Key words: *E*-Stilbenes, azomethine analogues, Mizoroki–Heck reaction, antimicrobial activity, antioxidant activity

1. Introduction

Phytoalexins are naturally occurring stilbenes reported to act as secondary metabolites in numerous plants.[1] One example of such compounds is resveratrol (3,4,5-trihydroxystilbene), which was first isolated from the Japanese plant *Veratrum grandiflorum.*[2] This compound has demonstrated significant antineoplastic activity,[3] angiogenesis,[4] action against certain microbes[5] and inflammation,[6] activity against acquired immunodeficiency syndrome,[7] radical scavenging,[8] and platelet aggregation.[9] Other reported biological activities of resveratrol include antagonist action against aryl hydrocarbons receptors (AhR)[10], enhancement of estrogenic potency,[10] neuroprotective effects,[8] cAMP phosphodiesterase inhibition,[11] and slowed progression of atherosclerosis.[12]

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Another example of naturally occurring phytoalexins is pterostilbene, which has been shown to be effective in treating resistant hematological malignancies[13] and diabetes.[13] Thus, the multifarious biological effects of stilbene analogues of phytoalexins may have potential for expansion into other application domains.

Reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxide ($OH^\bullet$), and neutral radicals such as nitric monoxide (NO) are believed to be the key players in damaging lipids, proteins, and DNA in living bodies.[14] These free radicals may divert cells to abnormal physiological functions, leading to disease states such as cardiovascular complications, diabetes, or cancer.[15] Antioxidants work to scavenge the ROS in biological systems through reductive mechanisms.[16] Overproduction of ROS in humans resulting from excessive electromagnetic radiation exposure, extensive muscular work, and overconsumption of certain foods suppresses the endogenous antioxidant-mediated redress. The detrimental effects of oxidants can be cured by using exogenous natural and synthetic antioxidants.[14] Antioxidant activity has become a topic of growing interest.

According to a report, the third most significant causes of mortality and morbidity worldwide are infectious diseases.[14] Microbial strains such as *Pseudomonas aeruginosa*, *Escherichia coli*, Enterobacteriaceae, *Klebsiella pneumoniae*, and *Staphylococcus aureus* have been recognized as pathogens.[17] Multiresistant microbial species are known to cause serious global health problems.[18] Hydrazide derivatives are present in numerous biologically active structures and display a wide diversity of biological activities. Among them, antimicrobial activity is most frequently met in the literature.[19–21] Drug design by structural modifications of the known antimicrobial agents is a feasible way to address these issues. Keeping these literature findings in view, a small library of Schiff bases of *E*-stilbene hydrazide analogues was synthesized and screened for in vitro antimicrobial and antioxidant potential.

2. Results and discussion

A small library of eight *E*-stilbene azomethines (**5a**–**5h**) was synthesized to evaluate its potential for antimicrobial and antioxidant activity. The chemical structures of the target compounds were analyzed by UV-Vis, FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopic techniques. The UV-Vis spectra of the *E*-compounds (**5a**–**5h**) are shown in Figure 1. The λ_{max} (maximum absorption bands) values of compounds **5a**–5**h** were in the range of 361–387 nm (Figure 1). A literature search of the λ_{max} values of related stilbene analogues revealed a range of 290–360 nm.[18] The bathochromic shifts observed in compounds **5a**–**5h** can be explained on the basis of extended π-conjugation and the presence of auxochromes (chloro, hydroxyl, nitro, and methoxy groups). The FT-IR absorption peaks ranging from 960 to 982 cm^{-1} are assigned to the out-of-plane bending vibration of the C-H bond of the *E*-ethylene bridge of the stilbene molecule.[18] The FT-IR spectrum of compound **5a** is shown in Figure 2.

^{1}H NMR spectra also confirmed the identity of specific protons in the synthesized compounds. The chemicals shift (δ) values (6.85–8.20 ppm) are assigned to the aromatic and olefinic protons of the synthesized compounds.[22,23] The *trans*-geometry of olefinic protons in the stilbene structure is confirmed by the presence of two doublets in spectra with large coupling constants (J = 16–17 Hz). A singlet observed in the range of δ 9.14–9.35 ppm is assigned to the -CH proton of the azomethine moiety of compounds **5a**–**5h**. The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **5a** is shown in Figure 3. ^{13}C NMR and mass spectral studies also confirmed the synthesis of targeted compounds. The ^{13}C NMR (75 MHz, $CDCl_3$) and mass spectra (electrospray ionization mode) of compound **5a** are shown in Figure 4 and Figure 5, respectively.

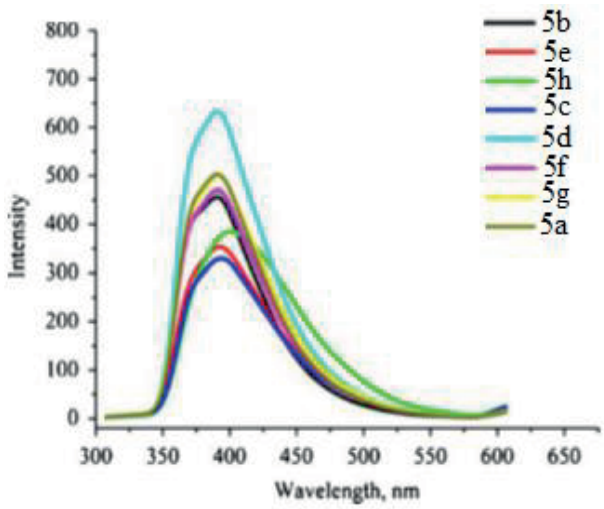


Figure 1. The UV-Vis spectra of the compounds **5a**–**5h**.

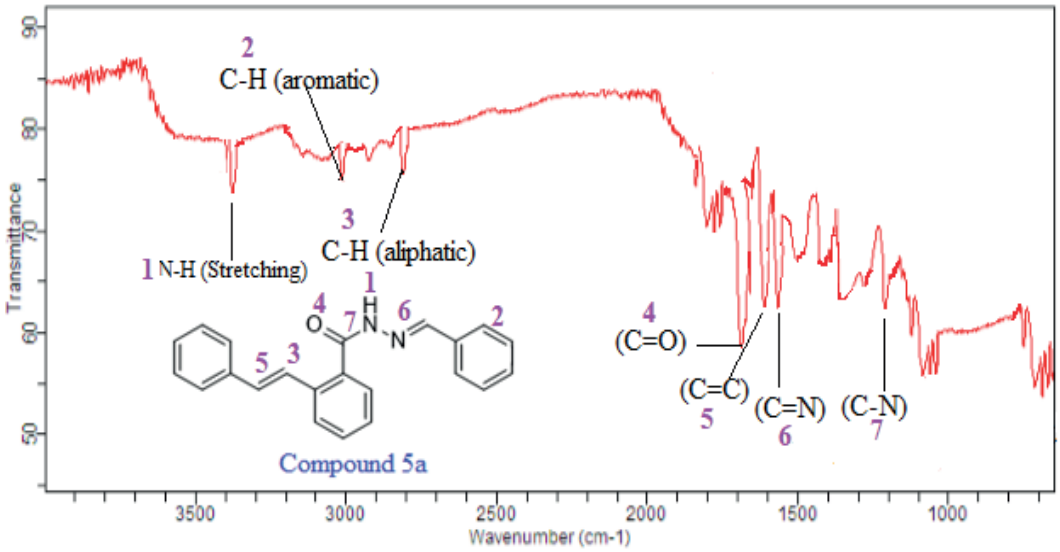


Figure 2. FT-IR spectrum of compound **5a**.

2.1. Biological activities

2.1.1. DPPH radical scavenging assay

A variety of mechanisms have been proposed for the antioxidant action in the body. Antioxidants have attracted special attention since the discovery of age-related oxidative stress.[24] The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of resveratrol was found to be IC_{50} = 33.79 µ M.[25] The antioxidant activity of resveratrol is thought to be linked to the presence of hydroxyl groups located at specified positions in its structure.[26]

The results for DPPH free radical scavenging activity (expressed in terms of IC_{50} values) of compounds **5a**–**5h** are collected in Figure 6. These values range from 22 ± 0.19 to 75 ± 0.21 µ g/mL compared to the standard butylated hydroxytoluene (BHT) with IC_{50} = 28 ± 0.10 µ g/mL. The tested compounds (**5a**–**5h**)

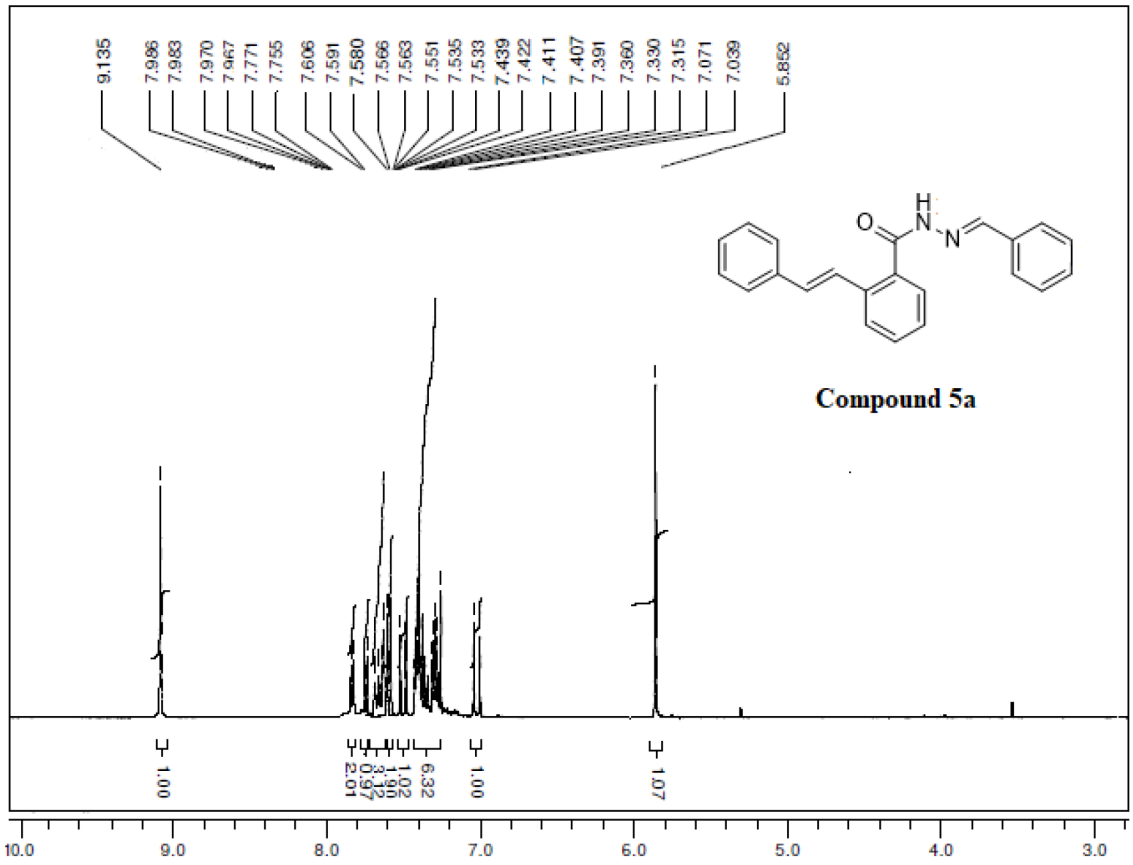


Figure 3. ^{1}H NMR spectrum of compound **5a**.

showed decreasing order of DPPH activity as follows: **5e** > **BHT** > **5d** > **5h** > **5a** > **5c** > **5b** > **5f** > **5g**. The highest DPPH activity was shown by compound **5e** with IC_{50} = 22 ± 0.19 µ g/mL compared to the standard BHT (IC_{50} = 28 ± 0.10 µ g/mL). The enhancement of DPPH activity may be due to the increased conjugation mediated by the electron-donating 4-hydroxyphenyl moiety as well as the amide-iminol tautomerism in compound **5e** where a push-pull mechanism operates within the molecule. The notion of the antioxidant activity being associated with the push-pull electronic effect in compound **5e** is supported by the observed decrease in antioxidant activity for compound **5g**, where the electron-withdrawing 4-nitrophenyl moiety exerts the opposite electronic effect (IC_{50} = 75 ± 0.21 µ g/mL). Similarly, compounds **5b** and **5c** with 2-chlorophenyl and 4-chlorophenyl moieties, respectively, showed reduced antioxidant activity compared to compound **5e** (Figure 6). As expected, compound **5a**, having unsubstituted phenyl moiety, exhibited slightly higher antioxidant activity than compounds **5b** and **5c** that have 2-chlorophenyl and 4-chlorophenyl moieties, respectively (Figure 6).

The proposed mechanism for the DPPH free radical scavenging activity of the most potent compound (**5e**) is illustrated in Figure 7, where an amide-iminol tautomerization promoted by the solvent is shown.[27] The amide-iminol tautomerism in compound **5e** helps to promote extensive delocalization of the single electron, resulting in greater stability of the DPPH-generated radical.[28] The enhanced stability caused by the electron-enriched extended conjugation of the radical generated in the structure of **5e** is likely the cause of its antioxidant activity.

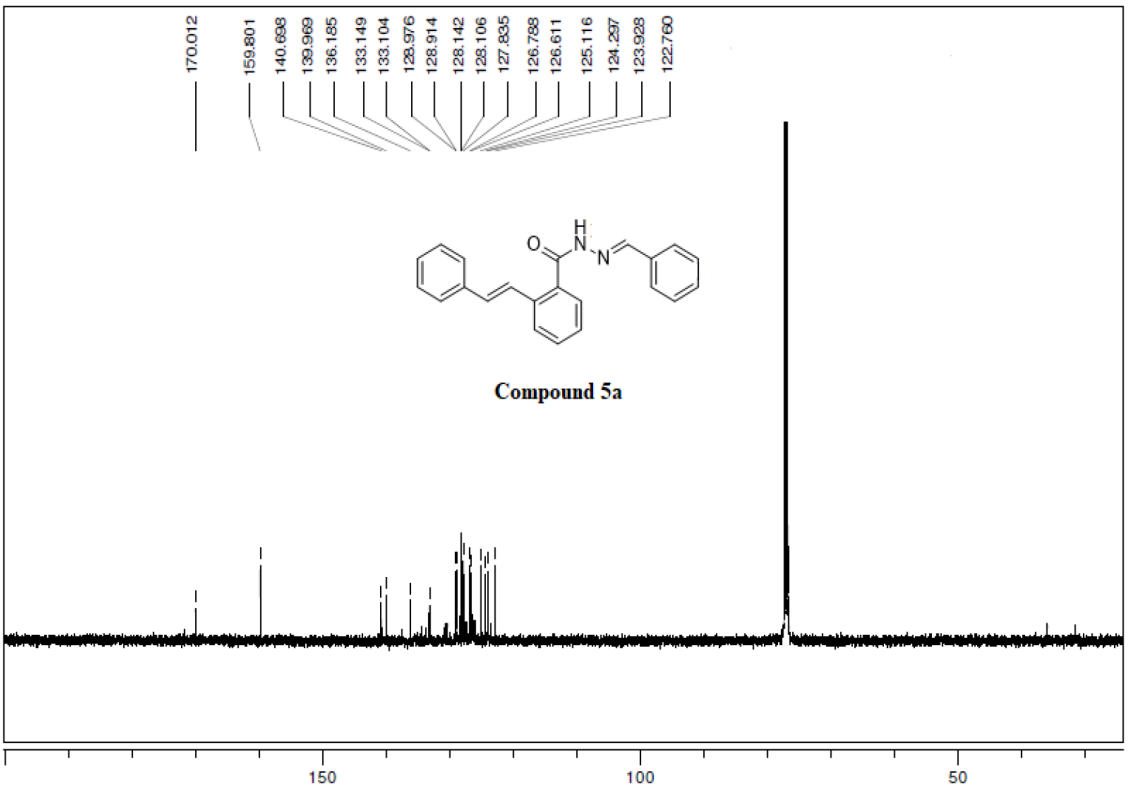


Figure 4. ^{13}C NMR spectrum of compound **5a**.

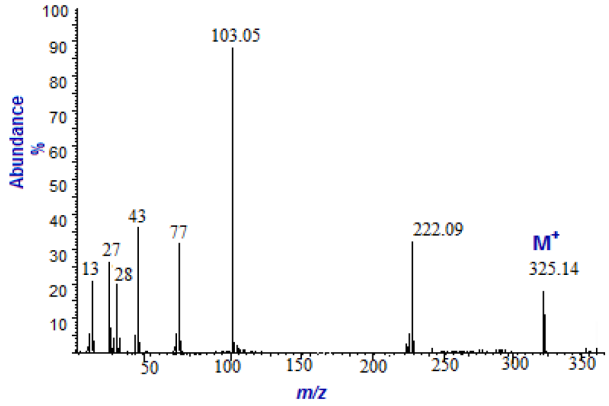


Figure 5. Mass spectrum (MS-ESI) of compound **5a**.

2.1.2. Antioxidant activity via inhibition of linoleic acid oxidation

ROS may attack the polyunsaturated fatty acid chains of cell membranes and start a self-propagation chain reaction, exerting a damaging influence on the cellular and tissue environments. Antioxidants are also effective in scavenging ROS related to the lipid peroxides providing protective health benefits.

The results of the inhibitory effects of the synthesized compounds (**5a**–**5h**) on linoleic acid peroxidase are summarized in Figure 8. Compounds **5a**–**5h** exhibited linoleic acid peroxidase inhibition in the range of 46 ± 0.20% to 86 ± 0.19%. Compounds **5e** and **5d** inhibited linoleic acid peroxidase most effectively (86 ± 0.19%

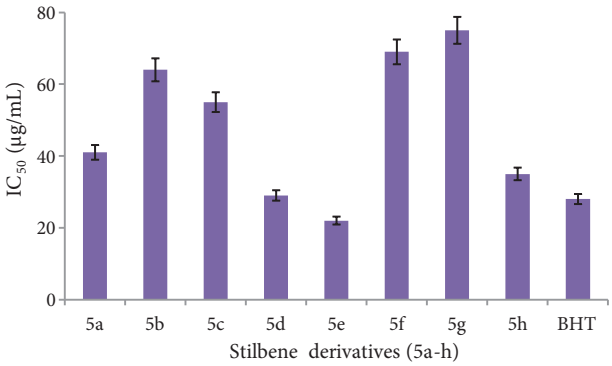


Figure 6. The DPPH free radical scavenging activity of compounds **5a**–**5h**.

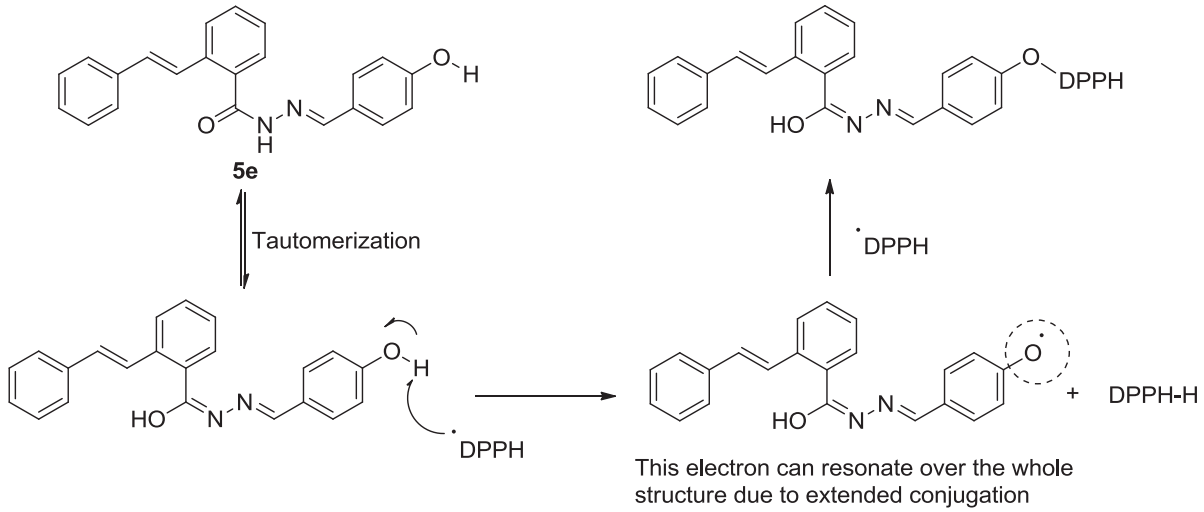


Figure 7. Proposed mechanism for DPPH free radical scavenging activity of compound **5e**.

and 81.5 ± 0.11% inhibition) (Figure 8). Compounds **5e** and **5d** with 4-hydroxyphenyl and 2-hydroxyphenyl moieties, respectively, fared slightly better than the standard BHT (81.0 ± 0.21%), whereas compound **5g**, having 4-nitrophenyl, showed the least inhibition (46 ± 0.20%). The inhibition of linoleic acid oxidation for the remaining compounds (**5h**, **5a**, **5c**, **5b**, and **5f**) was found to be 73.11 ± 0.19%, 71 ± 0.20%, 65 ± 0.11%, 59 ± 0.18%, and 51 ± 0.12%, respectively. The decreasing order of linoleic acid hydroperoxidase inhibition was as follows: **5e** > **5d** > **BHT** > **5h** > **5a** > **5c** > **5b** > **5f** > **5g**.

The antioxidant activity seems to be related to the molecular structure, and more precisely to the presence of both azomethine moiety and the auxochromes, particularly the hydroxyl moiety located at the *ortho*- and *para*-positions of the aromatic ring as well as an extension of the conjugation into the stilbene molecule. These structural features of compounds may contribute to the expected high radical scavenging effect resulting from the higher stability of the generated free radicals.

2.1.3. Antimicrobial activity

The antimicrobial activity is linked to the presence of strongly electron-withdrawing substituents on the stilbene skeleton.[29] This may result in the formation of charge transfer complexes, which play an important role in

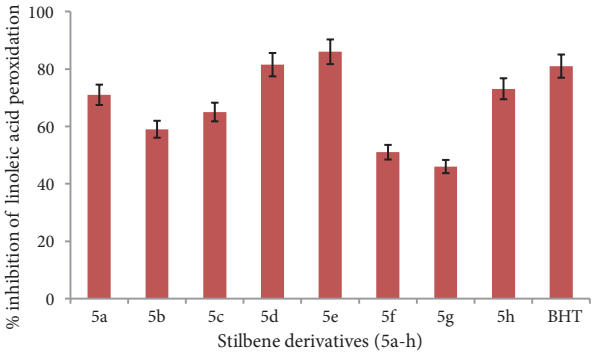


Figure 8. The percentage inhibition of linoleic acid peroxidation of compounds **5a**–**5h**.

enhancing affinity with the membrane proteins.[30] A decrease in activity has been observed for the electron-rich hydroxylated stilbenes, especially against gram-negative bacteria.

Resveratrol detoxifications by extracellular laccases have also been reported.[31] Fungal pathogens may avoid the inhibitory effect of natural stilbenes by oxidative degradation.[31] The natural stilbenes are not suitable for the chemical control of pathogenic fungi. In essence, the compounds that are not prone to oxidative degradation may represent excellent lead structures for developing efficient antimicrobial agents.[32]

Minimum inhibitory concentrations (MICs) of resveratrol were found to be 119.23 µ g/mL, 171 µ g/mL, and 342 µ g/mL against bacterial strains such as *E. faecalis*, *S. aureus*, and *P. aeruginosa,* respectively.[33,12] The simple stilbene structure displayed weak inhibition against *B. subtilis* and *P. syringae*, and against the fungal strains *B. cinerea*, *A. niger*, *C. herbarum*, and *M. aucupariae.*[34] With this in mind, synthesis of *trans*-stilbenes with azomethine moiety (with electron-donating and -withdrawing substituents on the aryl moiety) was carried out to probe their antimicrobial effects.

The results of antimicrobial activities of compounds **5a**–**5h** are given in terms of the zone of inhibition (ZOI) diameter in millimeters (Figures 9 and 10). The antimicrobial activity results in terms of MICs are summarized in Tables 1 and 2. The highest antibacterial activity was shown by compound **5g** with 4-nitrophenyl substituent moiety. The MIC values of compound **5g** against four bacterial strains, *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, and *B. subtilis*, were recorded to be 0.22 ± 0.03 mg/mL, 0.07 ± 0.05 mg/mL, 0.10 ± 0.03 mg/mL, and 0.06 ± 0.04 mg/mL, respectively (Table 1). Compound **5g** also showed strong inhibition with ZOI diameters of 32 ± 0.07 mm, 33 ± 0.04 mm, 32 ± 0.03 mm, and 33 ± 0.08 mm against *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, and *B. subtilis*, respectively (Figure 9). Similarly, compound **5f**, with a nitro group at the *meta*-position of the benzene ring, showed significant antibacterial activity against *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, and *B. subtilis* with ZOI diameters of 27 ± 0.01 mm, 29 ± 0.03 mm, 31 ± 0.03 mm, and 30 ± 0.03 mm (Figure 9) and MICs of 0.29 ± 0.03 mg/mL, 0.09 ± 0.03 mg/mL, 0.12 ± 0.04 mg/mL, and 0.07 ± 0.03 mg/mL, respectively (Table 1). Compound **5a**, having simple phenyl moiety, exhibited only moderate antibacterial activity. The literature reveals that *Z*-stilbenes show higher inhibition potential against gram-negative bacteria as compared to *E*-stilbenes.[35] However, these newly synthesized *E*-stilbene analogues (**5a**–**5h**) were found to be more effective against bacterial strains *E. coli*, *B. subtilis, Klebsiella pneumoniae*, and *S. aureus* in comparison to resveratrol. The standard drug (cefradine) showed good inhibition (ZOI diameters of 28 ± 0.04 mm, 29 ± 0.08 mm, 30 ± 0.07, and 31 ± 0.06 mm) against *E. coli*, *S. aureus*, *Klebsiella pneumoniae*,

and *B. subtilis*, respectively (Figure 9). In comparison with the standard drug, compound **5g**, having a 4-nitrophenyl group, exhibited strong bactericidal activity.

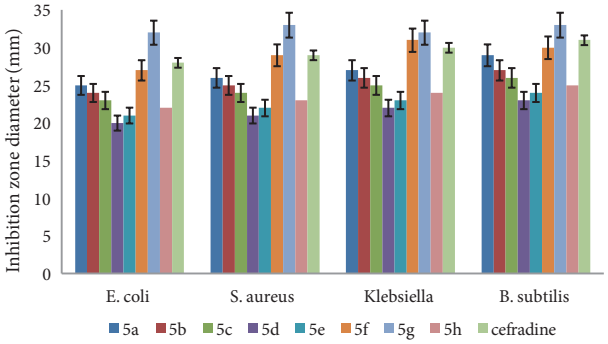


Figure 9. The antibacterial activity of compounds **5a**–**5h** (ZOIs in mm).

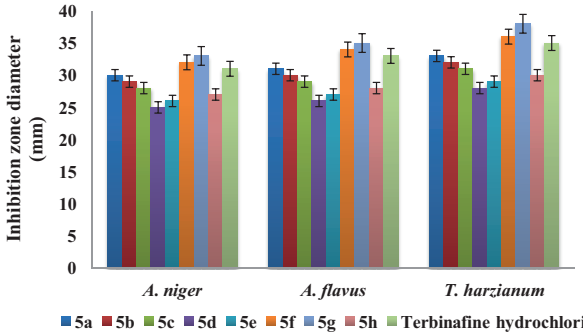


Figure 10. The antifungal activity of compounds **5a**–**5h** against tested fungal strains (ZOIs in mm).

Stilbene azomethines **5a**–**5h** displayed moderate to high antifungal activity against tested fungal strains (*A. niger*, *A. flavus*, and *T. harzianum*) as shown in Figure 10. Compound **5a**, having unsubstituted phenyl moiety, showed similar antifungal activity against *A. niger* compared to the standard drug terbinafine hydrochloride with ZOI diameters of 31 ± 0.06 mm and 31 ± 0.04 mm, respectively. 4-Nitrophenyl-containing compound **5g** demonstrated the highest antifungal activity against the tested fungal strains (Table 2). Compound **5f** with 3-nitrophenyl moiety had reduced antifungal activity in comparison to the 4-substituted compound (**5g**) (Table 2). This could be explained by the powerful electron-withdrawing nature (negative mesomeric and inductive effect) of the polar nitro group.

2.2. Conclusion

In this study, thermodynamically more stable *E*-stilbene azomethines (**5a**–**5h**) were synthesized by using the Mizoroki–Heck reaction as an essential step. It is worth mentioning that most of the compounds showed significant antioxidant and antimicrobial activity. Compounds **5g** and **5f** demonstrated potent antimicrobial activity, whereas compound **5e** with 4-hydroxyphenyl showed strong antioxidant activity. The potent antioxidant activity is linked to the presence of an extended π-conjugated system, which is further enhanced by the presence of auxochromes that promote amide-iminol tautomerism. On the other hand, the antimicrobial effect of the synthesized compounds, e.g., compounds **5g** and **5f**, seems to be promoted by the presence of the electron-withdrawing groups, such as the nitro group. These extended π-conjugated systems are proposed to generate stable free radicals that may act on the ROS. In sum, the newly synthesized *E*-stilbene analogues may hold significant potential for use in the food industry as putative detoxifying additives. The current study provides some pointers for further investigation into many other (*E*)-stilbene azomethines containing various electron-donating and -withdrawing groups that may serve as potent, safe, and cost-effective antimicrobial agents for the pharmaceutical industry.

3. Experimental

3.1. Chemical reagents

All chemicals were procured from Sigma Aldrich or Alfa Aesar and were used without further purification. The solvents were dried using standard procedures. Melting points were determined with a Büchi 434 melting

Table 1. MICs of compounds **5a**–**5h** against tested bacterial strains.

Bacterial strains	Minimum inhibitory concentrations (MICs) (mg/mL)								
	Standard	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**
E. coli	0.30 ± 0.03	0.35 ± 0.06	0.40 ± 0.04	0.42 ± 0.03	0.45 ± 0.04	0.47 ± 0.05	0.29 ± 0.03	0.22 ± 0.03	0.46 ± 0.05
S. aureus	0.10 ± 0.02	0.15 ± 0.06	0.20 ± 0.03	0.22 ± 0.03	0.25 ± 0.05	0.27 ± 0.04	0.09 ± 0.03	0.07 ± 0.05	0.29 ± 0.07
Klebsiella pneumoniae	0.14 ± 0.02	0.19 ± 0.05	0.24 ± 0.06	0.27 ± 0.03	0.29 ± 0.04	0.32 ± 0.07	0.12 ± 0.04	0.10 ± 0.03	0.31 ± 0.03
B. subtilis	0.08 ± 0.04	0.14 ± 0.04	0.18 ± 0.03	0.22 ± 0.05	0.26 ± 0.04	0.28 ± 0.05	0.07 ± 0.03	0.06 ± 0.04	0.29 ± 0.05

Values are mean ± SD of triplicate assays. Standard = cefradine.

Table 2. MICs of compounds **5a**–**5h** against tested fungal strains.

Fungal strains	Minimum inhibitory concentration (MICs) (mg/mL)								
	Standard	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**
A. niger	0.30 ± 0.03	0.31 ± 0.06	0.40 ± 0.08	0.42 ± 0.07	0.45 ± 0.05	0.47 ± 0.04	0.29 ± 0.03	0.22 ± 0.03	0.46 ± 0.02
A. flavus	0.10 ± 0.06	0.15 ± 0.04	0.20 ± 0.06	0.22 ± 0.02	0.25 ± 0.06	0.27 ± 0.04	0.09 ± 0.03	0.07 ± 0.03	0.29 ± 0.03
T. harzianum	0.14 ± 0.04	0.19 ± 0.04	0.24 ± 0.08	0.27 ± 0.05	0.29 ± 0.06	0.32 ± 0.06	0.12 ± 0.03	0.10 ± 0.03	0.31 ± 0.02

Values are mean ± SD of triplicate assays. Standard = terbinafine hydrochloride.

point apparatus. FT-IR spectra (KBr dis ks) were recorded on a Bruker FT-IR IFS48 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ using a Bruker AC400 (400 MHz and 300 MHz) spectrophotometer. Mass spectra were recorded on mass spectrometer ESI Micromass ZMD-2000 in electrospray ionization mode. The ions were observed as quasimolecular ions $[M–H]^-$. The chemical shift δ values are given in ppm. The multiplicity is given as s = singlet, d = doublet, dd = doublet of doublet, t = triplet, etc. The progression of reactions was monitored by thin-layer chromatography (TLC) performed on 2 × 5 cm aluminum sheets preloaded with silica gel $60F_{254}$ to a thickness of 0.25 mm (Merck). The CHN analysis was performed on a Carlo Erba Strumentazione-Mod-1106. The chromatograms were visualized under ultraviolet light.

3.2. Chemistry

The synthesis of *E*-stilbenes **5a**–**5h** is described in the Scheme. 2-Iodobenzoic acid (**1**) was refluxed with absolute ethanol in the presence of sulfuric acid as a catalyst to furnish ethyl-2-iodobenzoate (**2**) in 75% yield.[36] The resulting ester (**2**) was subjected to Mizoroki–Heck reaction to furnish *E*-stilbene (**3**) in good yield.[37,38] The ester functionality of compound **3** was converted to *E*-stilbene hydrazide (**4**) by treatment with hydrazine hydrate (Scheme) in the presence of ethanol as a solvent.[39] The stilbene azomethines (**5a**–**5h**) were synthesized by condensation of *E*-stilbene hydrazide (**4**) with a variety of aromatic aldehydes (Scheme).[40]

3.2.1. Synthesis of ethyl-2-iodobenzoate (2)

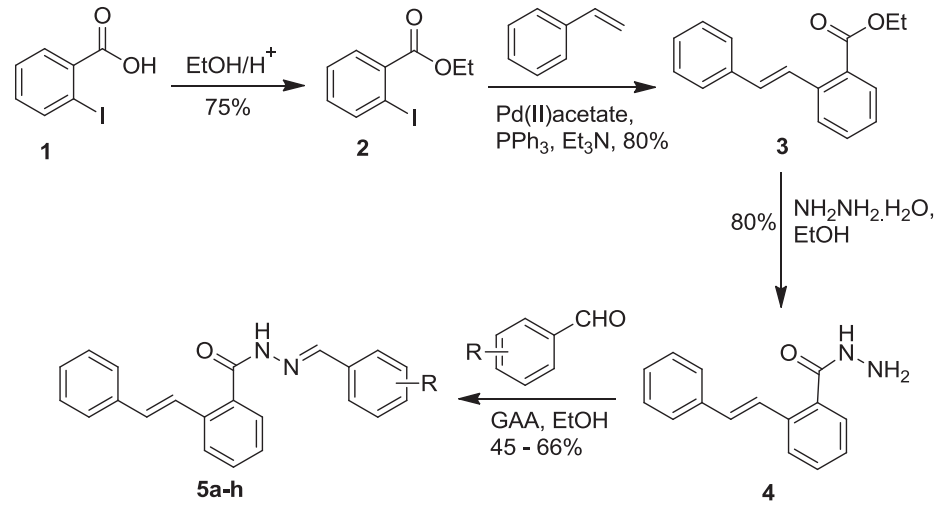
Ethyl-2-iodobenzoate (**2**) was prepared using the reported procedure.[36] Yield: 75%; IR (υ_{max}, KBr, cm^{-1}): 3010 (C-H aromatic), 2890 (C-H aliphatic), 1660 (C=O), 1110 (C-O).

3.2.2. Synthesis of stilbene (3) via Mizoroki–Heck reaction

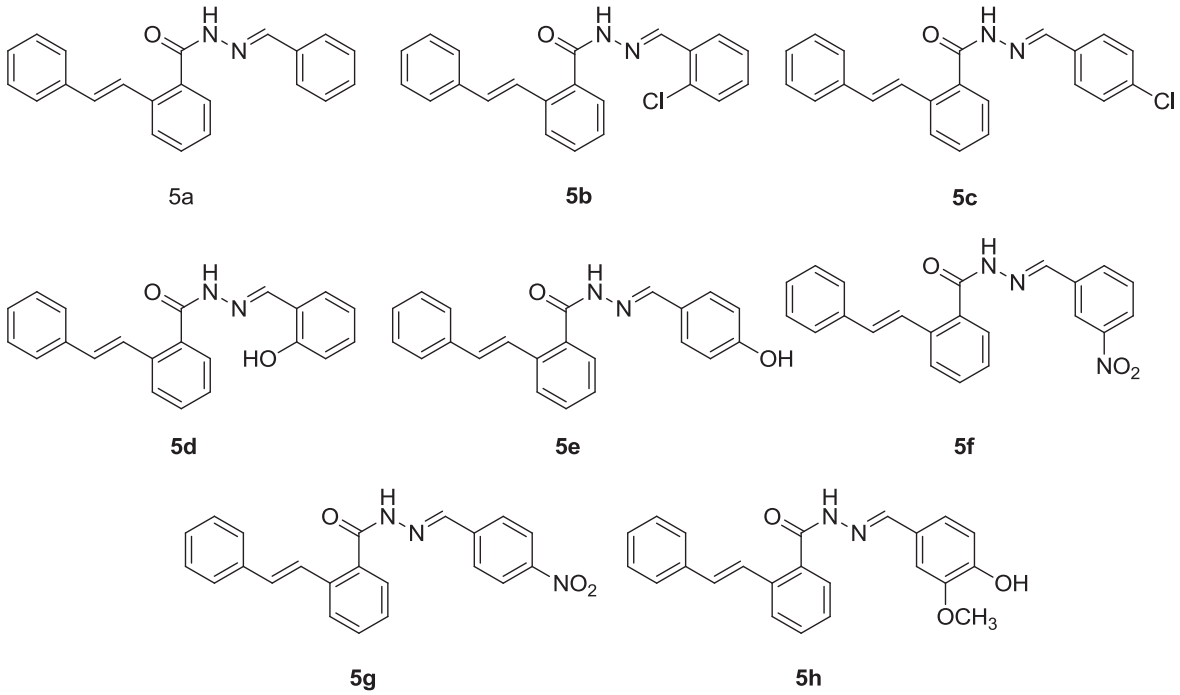
To a 100-mL round-bottom flask, ethyl-2-iodobenzoate (**2**) (5.1 g, 0.018 mol), styrene (2.59 mL, 0.0225 mol), triethylamine (5 mL), $Pd(OAc)_2$ (0.4 g, 0.0018 mol), and triphenylphosphine (0.94 g, 0.0036 mol) were added. The reaction mixture was stirred at room temperature for 30 min. The reaction mixture was subjected to reflux at 120 °C for 12–15 h under argon atmosphere. After completion of the reaction, the reaction mixture was dissolved in ethyl acetate (10 mL). The reaction mixture was adsorbed on silica gel under reduced pressure and purified by column chromatography. The hexane/ethyl acetate mixture (90:10) was used as an eluent. The product was obtained as a colorless solid. Yield: 80%; mp: 92 °C; IR (υ_{max}, KBr, cm^{-1}): 3010 (C-H aromatic), 2860 (C-H aliphatic), 1660 (C=O), 1595 (C=C), 966 (CH=CH).

3.2.3. Synthesis of *E*-2-styrylbenzohydrazide (4)

To a 100-mL round-bottom flask *E*-ethyl 2-styrylbenzoate (**3**) (2.52 g, 0.01 mol) in absolute ethanol (10 mL) and 80% hydrazine hydrate (2.42 mL, 0.04 mol) were added. The reaction mixture was refluxed until the completion of the reaction. Reaction completion was confirmed with TLC. After the completion of reaction, the remaining hydrazine hydrate and ethanol were distilled off under reduced pressure. The crude product was washed with water and crystallized from 30% aqueous ethanol. Yield: 80%; mp: 150 °C; colorless crystals; IR (υ_{max}, KBr, cm^{-1}): 3340 (N-H), 3010 (C-H aromatic), 2860 (C-H aliphatic), 1660 (C=O), 1595 (C=C), 970 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.12, (s, 1H, N-H), 7.21–7.71 (m, 9H, Ar-H), 7.38 (d, 1H,



5a: R = H,
5b: R = 2-Cl,
5c: R = 4-Cl,
5d: R = 2-OH,
5e: R = 4-OH,
5f: R = 3-NO_2,
5g: R = 4-NO_2,
5h: R = 3-OCH_3-4-OH



Scheme. Synthesis of compounds **5a**–**5h**.

HC=CH, J = 16 Hz), 6.98 (d, 1H, HC=CH, J = 16 Hz), 4.23 (broad singlet, 2H, NH_2); $C_{15}H_{14}N_2O$ (MW: 238.28 g/mol): C, 75.61%; H, 5.92%; N, 11.76%; Found: C, 75.62%; H, 5.92%; N, 11.75%.

3.2.4. General procedure (GP-1) for synthesis of compounds 5a–5h

To a 100-mL round-bottom flask *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and aromatic aldehydes (1.2 eq., 0.0024 mol) were added in the presence of absolute ethanol followed by the addition of a few drops of glacial acetic acid. The reaction mixture was refluxed until the completion of the reaction. Reaction completion was monitored by TLC. After the completion of reaction, the reaction mixture was cooled in a refrigerator overnight. The appeared solid was filtered. Finally, the crude product was crystallized from chloroform to furnish stilbene azomethines **5a**–**5h** in 45%–66% yields.

3.3. *N*'-Benzylidene-2-((*E*)-styryl)benzohydrazide (5a)

Compound **5a** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and benzaldehyde (0.24 mL, 0.0024 mol) by following GP-1. Yield: 66%; light brown solid; mp: 240 °C; λ_{max} in nm: 370; FT-IR (υ_{max}, KBr, cm^{-1}): 3395 (N-H), 3035 (C-H aromatic), 2855 (C-H aliphatic), 1680 (C=O), 1590 (C=N), 1620 (C=C), 1195 (C-N), 960 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.14, (s, 1H, C-H), 7.07–7.98 (m, 14H, Ar-H), 7.42 (d, 1H, HC=CH, J = 16.1 Hz), 7.04 (d, 1H, HC=CH, J = 16.1 Hz), 5.85 (s, 1H, -NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 170.1, 159.8, 140.7, 139.9, 136.2, 133.2, 133.1, 129.0, 128.9, 128.2, 128.1, 127.8, 126.8, 126.6, 125.1, 124.3, 123.9, 122.8; ESI-MS: 325.14 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{18}N_2O$ (MW: 326.39 g/mol): C, 80.96%; H, 5.56%; N, 8.58%; Found: C, 80.95%; H, 5.57%; N, 8.56%.

3.3.1. *N*'-(2-Chlorobenzylidene)-2-((*E*)-styryl)benzohydrazide (5b)

Compound **5b** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 2-chlorobenzaldehyde (0.34 g, 0.0024 mol) by following GP-1. Yield: 48%; yellow solid; mp: 212 °C; λ_{max} in nm: 373; FT-IR (υ_{max}, KBr, cm^{-1}): 3350 (N-H), 3030 (C-H aromatic), 2855 (C-H aliphatic), 1680 (C=O), 1620 (C=N), 1610 (C=C), 1170 (C-N), 980 (CH=CH), 743 (C-Cl); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.30, (s, 1H, C-H), 7.21–7.85 (m, 13H, Ar-H), 7.39 (d, 1H, HC=CH, J = 16.5 Hz), 7.01 (d, 1H, HC=CH, J = 16.5 Hz), 5.95 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.1, 159.9, 145.8, 140.1, 139.0, 137.3, 135.3, 133.5, 131.8, 131.3, 129.6, 129.4, 128.9, 128.5, 128.2, 128.0, 127.6, 127.1, 126.4, 120.1; ESI-MS: 359.11 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{17}ClN_2O$ (MW: 360.84 g/mol): C, 73.23%; H, 4.75%; N, 7.76%; Found: C, 73.25%; H, 4.74%; N, 7.76%.

3.3.2. *N*'-(4-Chlorobenzylidene)-2-((*E*)-styryl)benzohydrazide (5c)

Compound **5c** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 4-chlorobenzaldehyde (0.34 g, 0.0024 mol) by following GP-1. Yield: 45%; yellow solid; mp: 230 °C; λ_{max} in nm: 375; FT-IR (υ_{max}, KBr, cm^{-1}): 3340 (N-H), 3045 (C-H aromatic), 2890 (C-H aliphatic), 1690 (C=O), 1630 (C=N), 1605 (C=C), 1160 (C-N), 982 (CH=CH), 760 (C-Cl); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.22, (s, 1H, -CH), 7.25–7.91 (m, 13H, Ar-H), 7.30 (d, 1H, HC=CH, J = 17 Hz), 6.96 (d, 1H, HC=CH, J = 17 Hz), 5.90 (s, 1H, -NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.9, 158.9, 145.9, 140.5, 139.2, 135.6, 133.1, 132.8, 129.8, 129.5, 129.1, 128.7, 128.4, 127.5, 127.3, 127.1, 126.4, 123.6; ESI-MS: 359.12 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{17}ClN_2O$ (MW: 360.84 g/mol): C, 73.23%; H, 4.76%; N, 7.77%; Found: C, 73.21%; H, 4.77%; N, 7.78%.

3.3.3. *N*'-(2-Hydroxybenzylidene)-2-((*E*)-styryl)benzohydrazide (5d)

Compound **5d** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 2-hydroxybenzaldehyde (0.25 mL, 0.0024 mol) by following GP-1. Yield: 55%; yellow solid; mp: 270 °C; λ_{max} (nm): 380; FT-IR (υ_{max}, KBr, cm^{-1}): 3370 (N-H), 3310 (O-H, broad), 3050 (C-H aromatic), 2905 (C-H aliphatic), 1690 (C=O), 1610 (C=N), 1590 (C=C), 1150 (C-N), 965 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.14, (s, 1H, -CH), 7.35–8.20 (m, 13H, Ar-H), 7.29 (d, 1H, HC=CH, J = 16.7 Hz), 6.87 (d, 1H, HC=CH, J = 16.7 Hz), 5.85 (s, 1H, -NH), 4.20 (s, 1H, Ar-OH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 172.5, 161.7, 146.3, 141.5, 139.6, 137.1, 134.8, 134.1, 131.6, 131.3, 130.7, 129.2, 128.7, 128.4, 128.2, 127.9, 127.5, 127.1, 126.8, 122.4; ESI-MS: 341.14 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{18}N_2O_2$ (MW: 342.39 g/mol): C, 77.17%; H, 5.30%; N, 8.18%; Found: C, 77.15%; H, 5.32%; N, 8.17%.

3.3.4. *N*'-(4-Hydroxybenzylidene)-2-((*E*)-styryl)benzohydrazide (5e)

Compound **5e** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 4-hydroxybenzaldehyde (0.3 g, 0.0024 mol) by following GP-1. Yield: 50%; yellow solid; mp: 285 °C; λ_{max} (nm): 383; FT-IR (υ_{max}, KBr, cm^{-1}): 3360 (N-H), 3300 (O-H, broad), 3040 (C-H aromatic), 2890 (C-H aliphatic), 1660 (C=O), 1615 (C=N), 1625 (C=C), 1140 (C-N), 970 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.25 (s, 1H, -CH), 7.40–8.12 (m, 13H, Ar-H), 7.34 (d, 1H, HC=CH, J = 16.5 Hz), 7.10 (d, 1H, HC=CH, J = 16.5 Hz), 5.95 (s, 1H, -NH), 4.25 (s, 1H, Ar-OH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 175.2, 164.5, 147.4, 142.4, 140.3, 136.8, 134.5, 133.8, 131.5, 130.5, 129.8, 129.5, 129.1, 128.7, 128.2, 127.8, 127.2, 119.7; ESI-MS: 341.14 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{18}N_2O_2$ (MW: 342.39 g/mol): C, 77.17%; H, 5.30%; N, 8.18%; Found: C, 77.15%; H, 5.32%; N, 8.18%.

3.3.5. *N*'-(3-Nitrobenzylidene)-2-((*E*)-styryl)benzohydrazide (5f)

Compound (**5f**) was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 3-nitrobenzaldehyde (0.36 g, 0.0024 mol) by following GP-1. Yield: 60%; yellow solid; mp: 205 °C; λ_{max} (nm): 365; FT-IR (υ_{max}, KBr, cm^{-1}): 3320 (N-H), 3070 (C-H aromatic), 2850 (C-H aliphatic), 1650 (C=O), 1620 (C=C), 1610 (C=N), 1350 (Ar-NO_2), 1130 (C-N), 960 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.35, (s, 1H, -CH), 7.30–8.22 (m, 13H, Ar-H), 7.41 (d, 1H, HC=CH, J = 16 Hz), 7.03 (d, 1H, HC=CH, J = 16 Hz), 5.92 (s, 1H, -NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 173.6, 160.4, 145.1, 141.5, 140.7, 138.2, 134.2, 133.5, 132.7, 131.5, 130.7, 129.8, 128.5, 128.3, 128.2, 127.6, 127.4, 127.1, 126.4, 118.9; ESI-MS: 370.13 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{17}N_3O_3$ (MW: 371.39 g/mol): C, 71.15%; H, 4.61%; N, 11.31%; Found: C, 71.14%; H, 4.62%; N, 11.31%.

3.3.6. *N*'-(4-Nitrobenzylidene)-2-((*E*)-styryl)benzohydrazide (5g)

Compound **5g** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 4-nitrobenzaldehyde (0.36 g, 0.0024 mol) by following GP-1. Yield: 58%; yellow solid; mp: 225 °C; λ_{max} (nm): 361; FT-IR (υ_{max}, KBr, cm^{-1}): 3330 (N-H), 3040 (C-H aromatic), 2870 (C-H aliphatic), 1680 (C=O), 1620 (C=N), 1615 (C=C); 1360 (Ar-NO_2), 1120 (C-N), 980 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.30, (s, 1H, -CH), 7.35–8.14 (m, 13H, Ar-H), 7.28 (d, 1H, HC=CH, J = 16 Hz), 6.85 (d, 1H, HC=CH, J = 16 Hz), 5.95 (s, 1H, -NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.9, 158.5, 145.2, 139.3, 138.6, 133.5, 131.5, 131.2, 129.7, 129.2, 128.7, 128.6, 128.3, 127.7, 127.4, 127.2, 125.6, 118.6; ESI-MS: 370.13 $[M–H]^-$. Anal. Calcd. for $C_{22}H_{17}N_3O_3$ (MW: 371.39 g/mol): C, 71.15%; H, 4.61%; N, 11.31%; Found: C, 71.16%; H, 4.60%; N, 11.32%.

3.3.7. *N*'-(4-Hydroxy-3-methoxybenzylidene)-2-((*E*)-styryl)benzohydrazide (5h)

Compound **5h** was synthesized from *E*-2-styrylbenzohydrazide (**4**) (0.5 g, 0.002 mol) and 4-hydroxy-3-methoxybenzaldehyde (0.36 g, 0.0024 mol) by following GP-1. Yield: 64%; dark brown solid; mp: 310 °C; λ_{max} (nm): 387; FT-IR (υ_{max}, KBr, cm^{-1}): 3380 (N-H), 3320 (O-H, broad), 3010 (C-H aromatic), 2900 (C-H aliphatic), 1660 (C=O), 1615 (C=N), 1605 (C=C); 1150 (-OCH_3); 1110 (C-N), 970 (CH=CH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.20, (s, 1H, C-H), 7.21–7.70 (m, 12H, Ar-H), 7.31 (d, 1H, HC=CH, J = 16 Hz), 6.97 (d, 1H, HC=CH, J = 16 Hz), 5.97 (s, 1H, N-H), 4.22 (s, 1H, Ar-OH), 3.85 (s, 3H, Ar-OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.8, 159.2, 144.9, 142.5, 141.7, 139.1, 135.5, 134.1, 133.5, 132.6, 131.7, 129.5, 128.8, 128.5, 128.2, 127.5, 127.4, 127.2, 126.7, 118.3, 55.5; ESI-MS: 371.13 $[M–H]^-$. Anal. Calcd. for $C_{23}H_{20}N_2O_3$ (MW: 372.42 g/mol): C, 74.18%; H, 5.41%; N, 7.52%; Found: C, 74.16%; H, 5.43%; N, 7.52%.

3.4. Determination of antioxidant potential of compounds 5a–5h

3.4.1. DPPH free radical scavenging activity

DPPH free radical scavenging activity of compounds **5a**–**5h** was determined using BHT as a standard antioxidant according to a reported method.[41] Different concentrations (20, 40, 60, 80, and 100 µ g/mL) of compounds **5a**–**5h** were prepared. To 1 mL of 0.1 mM DPPH ethanolic solution was added 3 mL of each compound concentration. The solutions were stirred at room temperature for 10 min and then permitted to stand for 1 h in the dark. The absorbance was determined at 517 nm beside ethanol as a blank. Antioxidant activity (D) was calculated by the equation given below:

$$D(\%) = \{(A_C - A_S)A_C\} \times 100, \quad (1)$$

where A_C and A_S are the absorbance of the control and sample, respectively.

3.4.2. Ferric thiocyanate (FTC) assay

Inhibitory effects of compounds **5a**–**5h** were assessed by using the FTC assay.[16] To 2.5 mL of linoleic acid emulsion (0.02 M, pH 7) and 2.0 mL of phosphate buffer (0.02 M, pH 7) was added 0.2 mL (100 µ g/mL) of each compound solutions **5a**–**5h**. Each resultant mixture was incubated at 40 °C for 5 days. The mixture without sample was taken as a blank. The incubated mixture (0.5 mL) was mixed with 75% ethanol (5 mL), 30% ammonium thiocyanate (0.5 mL), and 20 mM ferrous chloride in 3.5% HCl (0.1 mL). Then absorbance was taken at 500 nm after 3 min. The % inhibition of peroxidation (IP %) was measured by the equation given below:

$$IP(\%) = [1 - (abs.of sample)/(abs.of control)] \times 100. \quad (2)$$

3.5. Antibacterial activity

Antibacterial activity of synthesized stilbene hybrid azomethines **5a**–**5h** was determined by measuring their ZOIs against different bacterial strains by using the agar well diffusion method.[42] The MICs were determined by the serial dilution technique.[21] Bacterial strains *Bacillus subtilis* (ATCC 9637), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumoniae* (ATCC 10031) were obtained from the PCSIR laboratories complex, Lahore, Pakistan. Bacterial cultures were grown in potato dextrose agar. Cefradine served as the standard antibacterial drug (positive control).

3.6. Antifungal activity

Antifungal potential of synthesized stilbene azomethines **5a**–**5h** in terms of ZOIs was evaluated against different fungal strains by using the reported method.[42] The MICs were determined by the serial dilution technique.[21] The used fungal strains, i.e. *Aspergillus niger* (ATCC 16404), *Trichoderma harzianum* (ATCC 20846), and *Aspergillus flavus* (ATCC 9643), were already cultured in the microbiology laboratory of the Industrial Biotechnology Division of NIBGE Faisalabad, Pakistan. Terbinafine hydrochloride was used as the standard drug (positive control).

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