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Experimental validation of syringic Schiff bases with pyridine moiety as antibacterial and antioxidant agents along with *in silico* studies

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Abstract
A series of novel Schiff bases were synthesized from syringaldehyde by reacting with 2-aminopyridine, 3-aminopyridine, and 4-amino pyridine and evaluated for their antioxidant potential (DPPH assay, FRAP assay, and Phosphomolybdate assay) using ascorbic acid and BHT as standard. The antibacterial potential of these compounds against one Gram-positive (*Bacillus subtilis*) and four Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* sp., and *Enterobacter* sp.) revealed that compound III was more effective than standard ampicillin against *P. aeruginosa*. *In silico* studies were performed to predict the toxicity and bioactivity of compounds.

Keywords: Syringaldehyde, Schiff bases, anti-bacterial, antioxidant, *in silico* studies

Introduction
Reactive oxygen species (ROS) formed during respiration (1-4) in an organism escalates several processes like aging (Calabrese, V), Cancer (Mantovani *et al*. 2003) [6], diabetes (Rahimi *et al*. 2005) [7], inflammatory disorders (Geronikaki and gavalas 2006) [8], neurodegeneration (droge and schipper 2007) [9], rheumatoid arthritis (firuzi *et al*. 2006) [10], stroke (spence 2006) [11], atherosclerosis (siekmejer, steffen and maerz 2007) [12]. To balance these species, there is a requirement for a large number of antioxidants (2, Tiwari 2001) which were isolated naturally (flavonoids, anthocyanins, beta carotenes, etc) as well as synthesized artificially (butylated hydroxyl aniline, butylated hydroxyanisole) with hydroxyl group as a substituent common in all. Nowadays antioxidants played a vital role in the food industry as a source of nutrition, preventing lipid oxidation (Thorat *et al*. 2013) [14] as well as enhancing the shelf life of food (Dudonne *et al*. 2009) [33] through antimicrobial potential. Thus, the synthesis of a compound having properties of both anti-bacterial and anti-oxidant is a challenge to chemists. Syringaldehyde is one of the naturally occurring aromatic aldehyde isolated successfully from agricultural waste like corn stem (3.2%), wheat straw (3.2-4.3%), grapes (Lawther *et al*. 1996) [15]., and plants lignin, etc. It also possesses both antioxidant and antimicrobial properties and is, therefore, used in textiles, pulp, pharmaceuticals, food, cosmetics, and paper industries (Ibrahim *et al*. 2012) [16]. Thus, attracting scientists to develop its novel synthetic derivatives as chemopreventive solutions. No doubt, Schiff bases are also one of promising moiety shown to exhibit a broad range of biological activities, including antifungal (Aggarwal *et al*. 2009) [17], antibacterial (Harohally *et al*. 2017) [18], herbicidal (Wang *et al*. 2021) [19], anti-malarial (Jarrahpour *et al*. 2015) [20], antiviral (Jarrahpour *et al*. 2007) [21], antiproliferative, anti-inflammatory, and antipyretic properties (Da Silva *et al*. 2011) [22]. Thus, the present study deals with the evaluation of synthesized pyridine Schiff bases of syringaldehyde as anti-oxidant and anti-bacterial agents which were procured from microbiology lab department of plant breeding and genetics with aim to develop potent antibacterial agent for crops in comparison to standard compounds.

Material and Methods
Chemicals
2-Aminopyridine, 3-amino pyridine, 4-amino pyridine, Syringaldehyde, Glacial acetic acid, phosphate buffer, 2,4,6-tri(2-pyridyl)-s-triazine (TPTz), 2,2-Diphenyl-1-picrylhydrazylphosphate buffer, ammonium molybdate, glacial acetic acid, sodium hydroxide, Butylated hydroxytoluene, ascorbic acid, methanol were purchased from Hi-Media

Laboratories Private Limited, Mumbai**,** Sisco Research Laboratories Private Limited, Mumbai and Otto biochemical Private Limited, Mumbai.

Preparation of synthesized compounds (I-III)

The continuation of our previous work (Sahni T 2021) includes the synthesis of azomethine derivatives of syringaldehyde by condensation reaction with 2-aminopyridine, 3-aminopyridine, and 4-aminopyridine (I-III) using an appropriate catalyst is designed in Figure 1. These compounds have been characterized by spectral UV, IR, ^{1}H-NMR, and ^{13}C-NMR. The ^{1}H-NMR analysis was performed in DMSO solvent which displayed deshielded hydroxyl (OH) group singlet in all compounds in the 9.19-9.48 ppm range. Schiff bases (I-III) were marked with an imino proton singlet (CH=N) in the range of δ 7.97-8.99 ppm. In ^{13}C NMR, aliphatic methoxy carbons of compounds (I-III) were found in the range δ55.32-55.97 ppm while the deshielded azomethine carbon of Schiff bases was found in the range δ160.04-168.01 ppm. The significant occurrence of CH=N {1672-1616 cm-1} in FTIR bands along with the absence of aldehyde carbonyl (C=O) stretching of aldehyde was significant for compounds (I-III). Compound I, which was syringaldehyde 2-amino pyridine imine, the CH=N band appeared in IR at 1616, a singlet was observed at 8.99 due to the CH=N proton followed by the appearance of a peak at 160.04 in ^{13}C NMR. Compound II, (3-amino pyridine imine of syringaldehyde) showed a singlet of imine proton at 8.59 in PMR. The aromatic carbon bound to NH2 of (2-aminopyridine and 3-aminopyridine) gave a significant change from δ 160.70 to 142.96 in ^{13}C NMR due to a change in position of another nitrogen atom in the pyridine ring. Compound III (4-amino pyridine) gave (CH=N) band at 1654 cm^{-1} while in PMR, the singlet was perceived at δ 7.97 for the same. On comparing the impact of the nitrogen atom of the pyridine rings on the aromatic carbon adjacent to the NH_2 group, it was found that the maximum value of δ was observed in 2-amino pyridine azomethine followed by 4-aminopyridine and 3-amino pyridine. In the present study, these synthesized pharmacological scaffolds were further investigated for their antioxidant and antimicrobial potential, as they were found to be active fractions in the literature, Chaban *et al*. 2013 [24].

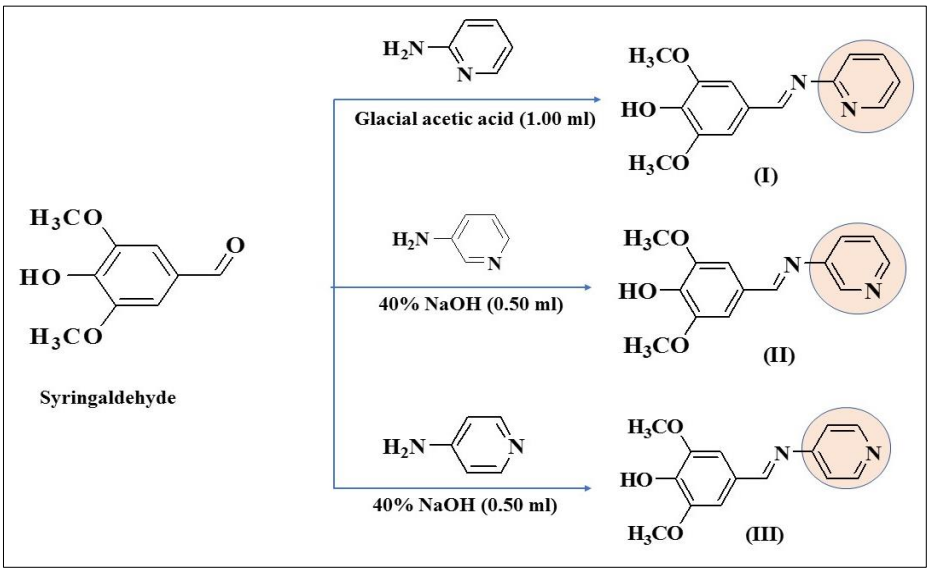

Fig 1: Preparation of synthesized compounds (I-III)

Computational study

Bioactivity score prediction for drug targets was done by *Molinspiration cheminformatics* and toxtree (Patlewicz *et al*. 2008) [54].

Spectrophotometric Measurements

The antioxidant potential of all tested compounds was estimated from absorbance measurements at a Visiscan 167 spectrophotometer.

Determination of antioxidant capacity

DPPH radical scavenging activity is a spectrophotometric analysis that was performed according to the method of Bloise 1958, Singh *et al*. 2019, McCue *et al*. 2004 [27], Kwon *et al*. 2007 [28]. Methanol was considered the best solvent for this determination (Om P Sharma 2009). Blank was prepared by dissolving DPPH in methanol (4.00 ml) and absorbance was taken at 515nm. Six different concentrations (25-1000 µg/ml) of each tested compound (0.04 ml) were mixed with 3.96 ml of above DPPH solution (Sahni *et al*. 2022). Solutions were incubated for 30 minutes in the dark at room temperature. The experiment was performed in triplicate and % radical scavenging capacity was calculated using the formula:

% Radical scavenging capacity= $[(A_B-A_E)/A_B] \times 100$

A_B =Absorbance of blank sample

A_E=Absorbance of tested compounds

IC_{50} µg/ml were calculated using SPSS 16.0

Ferric Reducing Antioxidant Potential (FRAP assay) modified version of FRAP assay (Benzie and Strain 1996) is based on the conversion of colorless ferric complex (Fe^{+3}-tripyridyltriazine) to a blue-colored ferric complex (Fe^{+2}-tripyridyltriazine) by donation of an electron from tested compounds. Absorbance was taken at 593 nm while ascorbic acid and BHT were used as standard and working FRAP reagent was prepared by method given in Dudonne S *et al* 2009 [33]. Two standard curves of ascorbic acid and BHT were prepared taking six different concentrations (25-1000 µg/ml) in consideration. Further, synthesized compounds were also tested at the above-mentioned concentrations by mixing (0.2 ml) to FRAP reagent (2.80 ml) which were incubated for 30

minutes in dark at ambient temperature. Tested compounds were expressed as Ascorbic acid and BHT equivalent (µg/ml) from the calibration curve equation. The experiment was performed in triplicate.

Total antioxidant capacity was done using Phosphomolybdate assay, based on reduction capacity of tested compounds to reduce Mo (+6) phosphomolybdate reagent yellow in color to Mo (+5) green color at acidic pH using method given in Prieto *et al.* 1999 [34]. It is spectrophotometric analysis and reduction was monitored by measuring absorbance at 695nm. Phosphomolybdate reagent was prepared following the procedure given in Sahni *et al.* 2022. Methanolic solutions of tested compounds at six different concentrations (25-1000 µg/ml) were prepared. Analysis was done by adding test solution (3.00 ml) to this reagent solution (1.00 ml) in a test tube which was covered with foil paper and boiled at 95°C for 90 minutes in the water bath. Blank was prepared by adding methanol (3.00 ml) to the reagent solution. The calibration curve of standard ascorbic acid was prepared at six different concentrations as mentioned above and ascorbic acid equivalents (AAE µg/ml) were calculated using the calibration curve equation.

Determination of Antibacterial activity

All the synthesized compounds were assessed *in-vitro* against an assortment of one Gram-positive bacteria *Bacillus subtilis* and four gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp. using disk dilution method (Kumar *et al* 2011, Mabkhot *et al* 2016, Arora *et al* 2018, Cauwelier *et al* 2004) [41]. All the bacterium were procured from Pulses Microbiology Laboratory, Department of Plant Breeding and Genetics, PAU, Ludhiana. DMSO was used as a solvent for the preparation of stock solutions of tested compounds and standard ampicillin. The experiment was conducted in triplicate. The antibacterial potential was expressed in terms of the zone of inhibition (mm) of each synthesized compound at six different concentrations and MIC (Minimum inhibitory concentration)

Results and Discussion

In silico studies

Molecular designing is one of the important steps in drug designing. In this study, synthesized compounds were screened for different physicochemical properties by two different software. Toxtree v2.6.6 is the open-source software of Ideaconsult Ltd. (Sofia, Bulgaria) under the terms of ECB contact. It is a computer-based estimation method for analyzing chemical toxicity. Molinspiration is used to estimate the Lipinski rule of five. By simply putting SMILE codes, all Lipinski characters along with bioactivity and 3D structure of molecules were generated. (Erti and Jelfs 2007, Ertl and Schuffenhauer 2009) [39]. This activity is based on score predictions like Kinase inhibitors, ion channel modulators, enzymes, nuclear receptors, GPCR ligands. Compounds with higher bioactive scores are predicted to be active. Hence, all the compounds found not much bioactive except ampicillin as given in Table 1.

Table 1: Physico-chemical properties, Drug likeness and Toxicity of compounds under study

	Physico-chemical properties				Drug likeness							Toxicity
Compound	**Log P**	**Molecular weight**	**n ON**	**n OHNH**	**TPSA**	**GPCRL**	**ICM**	**KI**	**NRL**	**PI**	**EN**	**Class**
Syringaldehdye	1.08	182.00	4	1	55.77	-0.94	-0.36	-0.80	-0.69	-1.27	-0.39	Low Class I
I	1.93	258.28	5	1	63.95	-0.60	-0.52	-0.18	-0.74	-0.65	-0.15	High-Class III
II	1.75	258.28	5	1	63.95	-0.59	-0.51	-0.02	-0.62	-0.67	-0.07	High-Class III
III	1.54	258.28	5	1	63.95	-0.56	-0.51	-0.10	-0.62	-0.68	-0.11	High-Class III
Ampicillin	-0.87	349.41	7	4	112.73	0.04	-0.47	-0.71	-0.61	0.87	0.25	High class III

***In vitro* antioxidant activity studies**

DPPH radical scavenging activity. DPPH (1,1'-diphenyl-2-picryl-hydrazyl) radical scavenging activity (RSA) of synthesized compounds was determined at six different concentrations (25-1000 µg/ml) along with standard ascorbic acid and Butylated Hydroxy Toluene (BHT) and presented in Fig 2. As it was already reported by several scientists that phenolic compounds must be evaluated for antioxidant activity due to their great potential (Kumar *et al*. 2011, Belkhiri *et al*. 2010) [41]. DPPH Scavenging capability of compounds was determined by a decrease in absorbance at 517nm. IC_{50} values of compound IV was found to be 42.50 µg/ml which is comparable to BHT (42.50 µg/ml) whereas IC_{50} values of other compounds (I, II, and III) were greater than 1000 µg/ml while that of syringaldehyde and ascorbic acid was 13.43 and 10 µg/ml respectively. Here, 4-amino-pyridine substituted azomethine of syringaldehyde. Henceforth, *p*-substituted nucleus or *p*-substituted products act as good scavengers by DPPH (Chaban *et al*. 2013) [24] and also pyridine moieties but of greater substitution with a hetero nucleus and enclosed rings led to an increase in scavenging activity. Another observation found was the presence of hydroxyl group on phenyl nucleus in syringaldehyde along with methoxy substituents possessed considerable DPPH activity (Belkhiri *et al*. 2010) while the addition of pyridine substituent decreased antioxidant potential remarkably. The higher potential of syringaldehyde was supported by findings that flavonoids with 3'-O-methoxy-4'-hydroxy substitution on aromatic nucleus bear good antioxidant activity (Pietta 2000). Also, Substituents like carbonyl (C=O) and hydroxyl (OH) bearing structures performed mild inhibition of lipid peroxidation by acting as free radicals (Rajkumar and Rao 1993, Nikolas *et al*. 2003) [44] which were analogous to our results with higher efficiency of syringaldehyde than pyridine moiety Schiff bases.

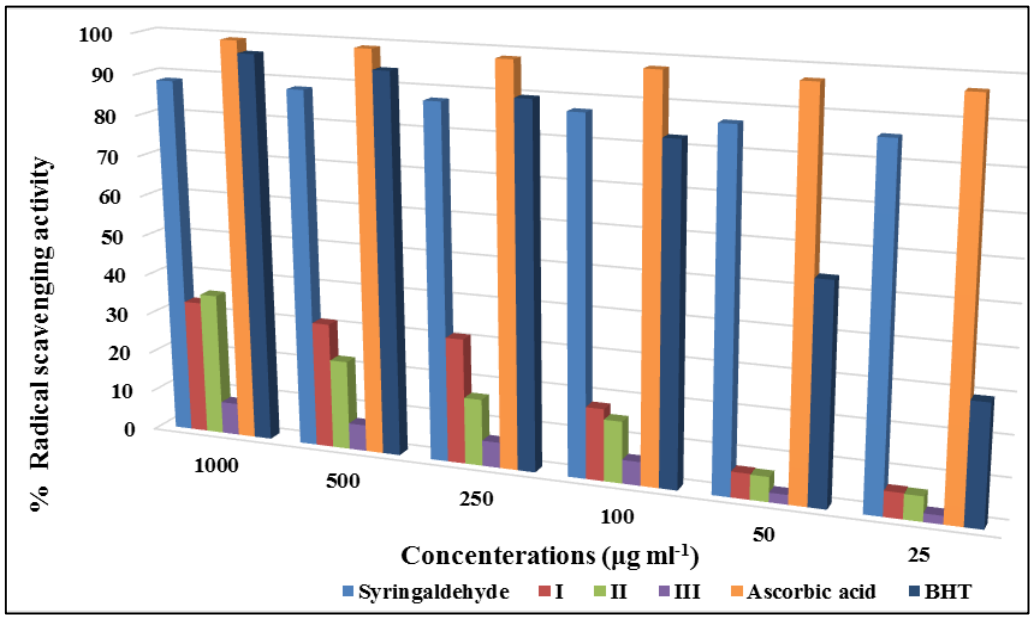

Fig 2: Ferric reducing antioxidant potential (FRAP) values of synthesized compounds at six concentrations in terms of ascorbic acid equivalent (AAE) (n=3)

Ferric Reducing Antioxidant Potential (FRAP assay). Reduction of TPTz Fe (III) to TPTz Fe (II) complex was estimated for synthesized compounds in terms of ascorbic acid equivalent (AAE µg/ml) and Butylated hydroxytoluene (BHTE µg/ml) as these were natural and artificial antioxidant standards. Values are presented in Figures 3 and 4. In this study, Syringaldehyde was found to be least effective with cumulative AAE and BHTE values of 113.77 and 27.02 µg/ml respectively. Conversion of an aldehydic group to azomethine moiety with pyridine nucleus insertion brings a positive impact by increasing cumulative AAE values which were found to be in order II (528.15)> III (413.43)~ II (413.22 µg/ml) and BHTE values in the order I (578.31)>II (425.35)>III (330.72) respectively. The presence of the hydroxyl aryl group always displayed a better antioxidant potential in Schiff bases (Kumar *et al*. 2011, Siddique *et al*. 2013) [41]. Furthermore, the presence of a higher electron density functional group always has more ability to transfer an electron to the Fe+3-TPTz complex (Nabatipour *et al* 2020). Therefore, the presence of nitrogen pyridine ring creates an electron-withdrawing impact which led to a lowering in the antioxidant potential of synthesized compounds as compared to aniline Schiff bases of syringaldehyde in our previous work (Sahni *et al*. 2022).

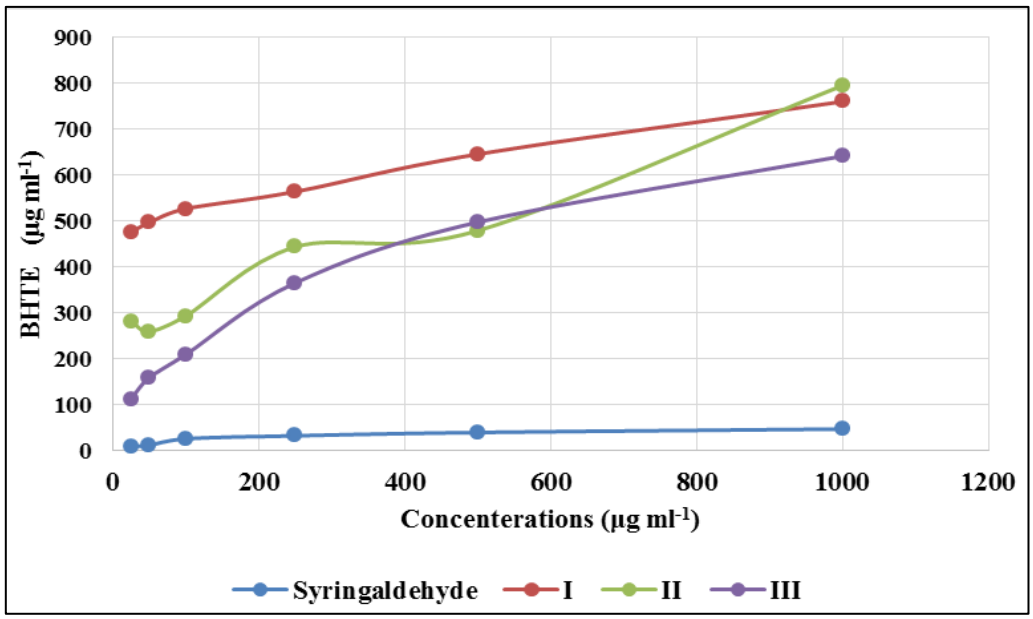

Fig 3: Ferric reducing antioxidant potential (FRAP) values of synthesized compounds at six concentrations in terms of Butylated hydroxy toluene equivalent (BHTE) (n=3)

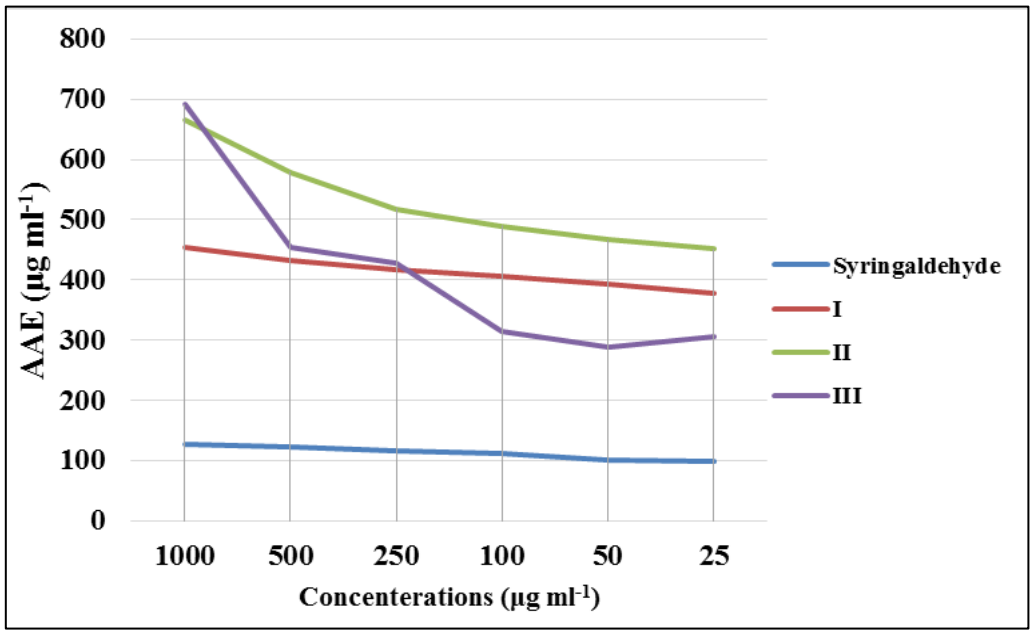

Fig 4: Ferric reducing antioxidant potential (FRAP) values of synthesized compounds at six concentrations in terms of Ascorbic acid equivalent (AAE) (n=3)

Phosphomolybdate assay. Antioxidant capacity of synthesized compounds showed potential in order which is as follows: Compound III>I>II>Syringaldehyde with cumulative Ascorbic acid Equivalent (AAE) values of 114.2±0.18, 60.5±0.03, 47.2±0.17, 28.2±0.10 respectively. Results were presented in Figure 5. The reduction ability of compounds was much less than ascorbic acid. It was found that conversion of the aldehydic group to azomethine moiety with insertion of pyridine nucleus increases molybdenum reduction potential of compounds with 4-amino pyridine exhibit greater potential than 2-aminopyridine and 3-aminopyridine. In general, Para substituted compounds were found more active molecules than ortho and meta substituted which was in concordance to literature (Ahmd and mir 2014, Arora *et al.* 2018) [37]. In some cases (Arora *et al*. 2018) [37], reported greater activity of indoles than benzimidazole was due to the presence of two nitrogen atoms. Hence, the presence of electron-withdrawing nitrogen nucleus increases the antioxidant potential of compounds thereby pyridine Schiff bases reflect greater antioxidant potential than precursor syringaldehyde molecules.

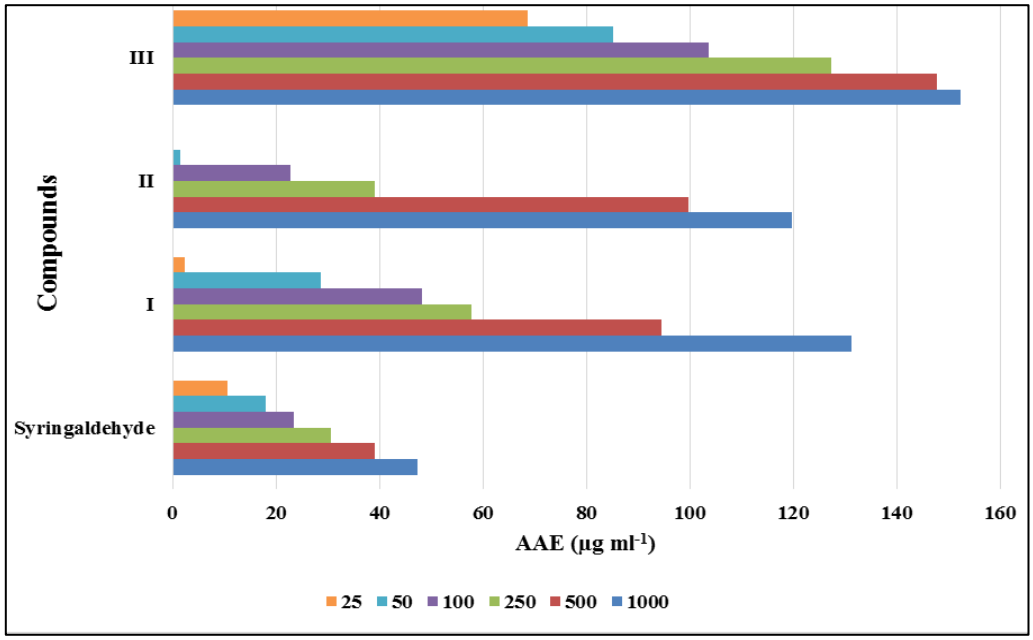

Fig 5: Total antioxidant capacity of different compounds at six concentrations in terms of ascorbic acid equivalent (AAE) (n=3)

Different compounds displayed differences in their activity as the mechanisms of the different assays were different. The substandard antioxidant potency of compounds was hypothesized due to their less electron transfer ability and radical scavenging ability (Huang *et al*. 2005) [50].

***In-vitro* Antimicrobial evaluation**

In vitro antimicrobial evaluation of syringic Schiff bases against one Gram-positive bacteria and four Gram-negative bacteria which also cause infections in humans. Zone of inhibition was taken as criteria for evaluation along with

evaluation of standard antimicrobial agent ampicillin for potency comparison of synthesized compounds. The results are presented in Figures 6-10. SAR studies revealed the effect of different substituents in pyridine benzylidenes of syringaldehyde on microbial stains.

Inhibition action of compounds (I-III) against *B. subtilis* was found to be equivalent among each other but less than syringaldehyde at all the six tested concentrations presented in figure 6. Thus, Syringaldehyde was most effective than derivatives which may be due to the presence of electron-withdrawing pyridine nucleus attributed to the presence of nitrogen atom. A similar assertion was given by Kumar *et al* 2011 [41]. Synthesized compounds exhibited good potential against *P. aeruginosa*. Compound III was the most effective displayed zone of inhibition of range 11.90±0.01-20.01±0.08 followed by syringaldehyde with the zone of inhibition in range 17.00±0.01-9.05±0.08 at six different concentrations (1000-25 µg/ml) respectively as presented in figure 7. Here p-substituted molecules were found more significant and better than standard ampicillin. Arora *et al*. 2018 [37] found p-isomer more effective than m-isomer of indoles against the same bacterium. A perusal of data is given in Figures 8, 9 and 10. It was found that the condensation of an aldehyde with amine gives a positive impact on microbial inhibition of *E.coli*. Compounds with the cumulative zone of inhibitions were found effective in the order I (17.11)>III (15.78)> II (12.32) > Syringaldehyde (10.38) which were less than ampicillin and results were in relevance with their MIC values of 15, 21 and 17 respectively. Compounds III and II showed maximum potential against *Klebsiella* sp. and *Enterobacter* sp. with MIC values of 15 and 19 respectively. Antimicrobial activity of pyridine Schiff bases was found significant only against *P. aeruginosa* which signifies the importance of syringaldehyde moiety as already reported pyridine Schiff bases were non-significant as an antibacterial agent against *E.coli*, *Klebsiella* sp. and *Enterobacter* spp. (Sobola and Watkins 2018, Mittal *et al*. 2009, Lv *et al*. 2006) [51, 52].

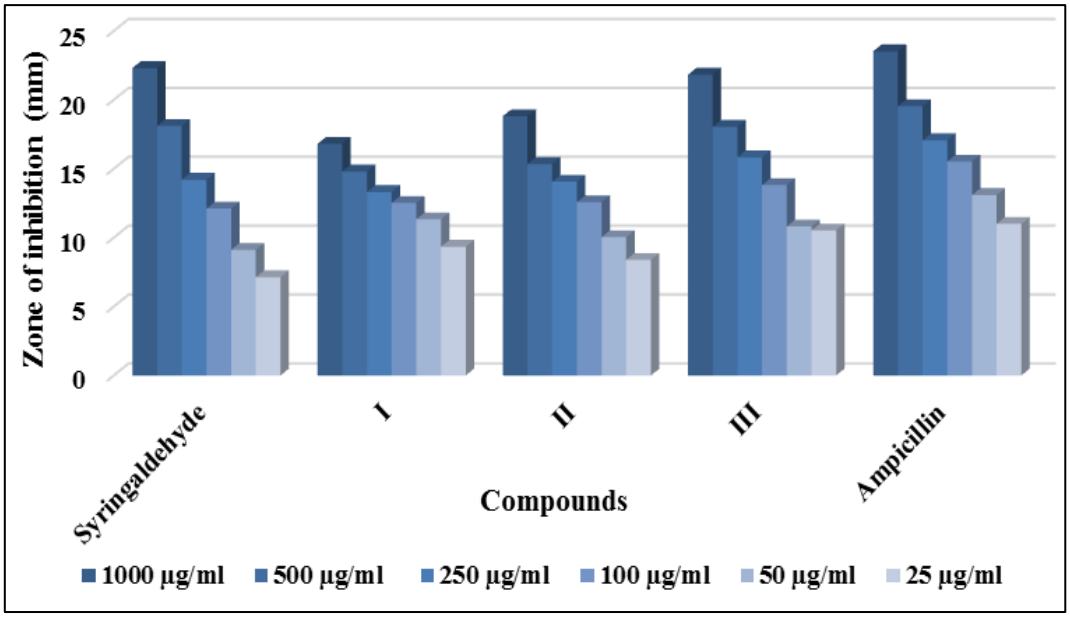

Fig 6: Zone of inhibition (mm) of different compounds against *Bacillus subtilis*

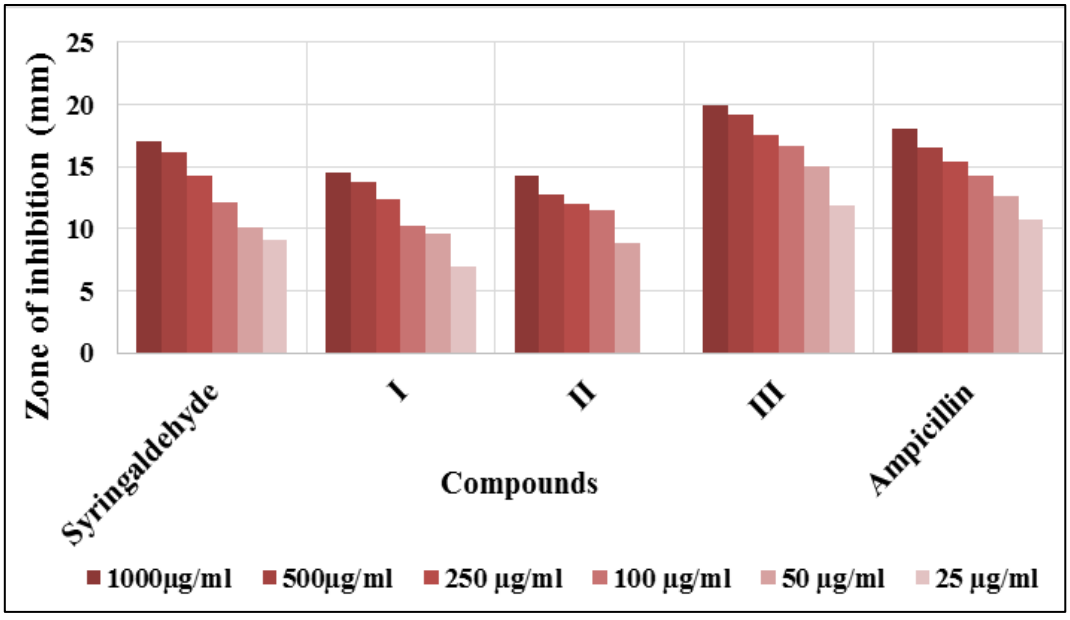

Fig 7: Zone of inhibition (mm) of different compounds against *Pseudomonas aeruginosa*

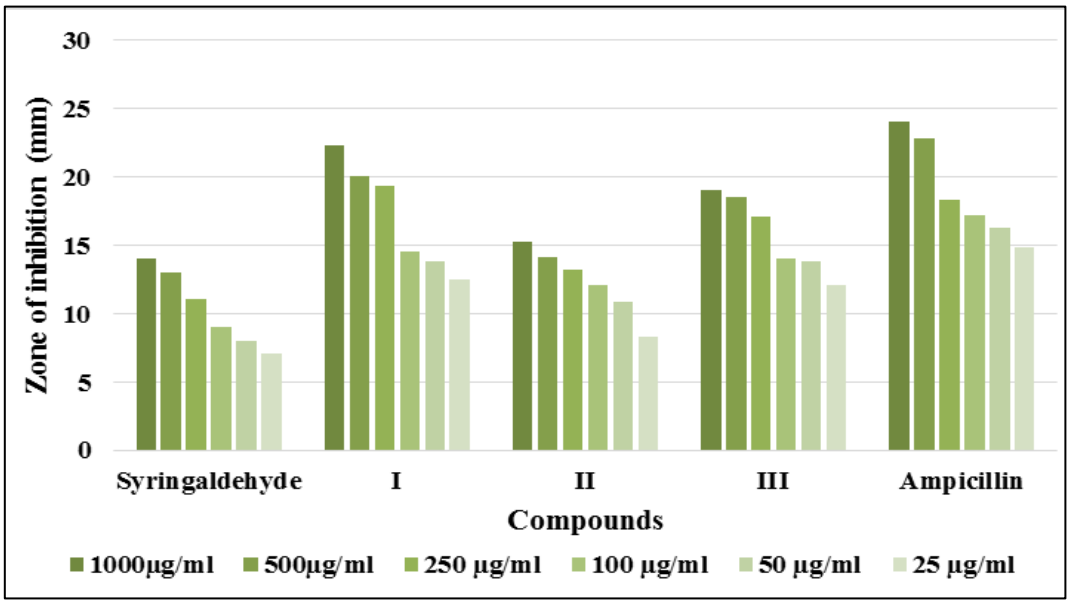

Fig 8: Zone of inhibition (mm) of different compounds against *Escherichia Coli*

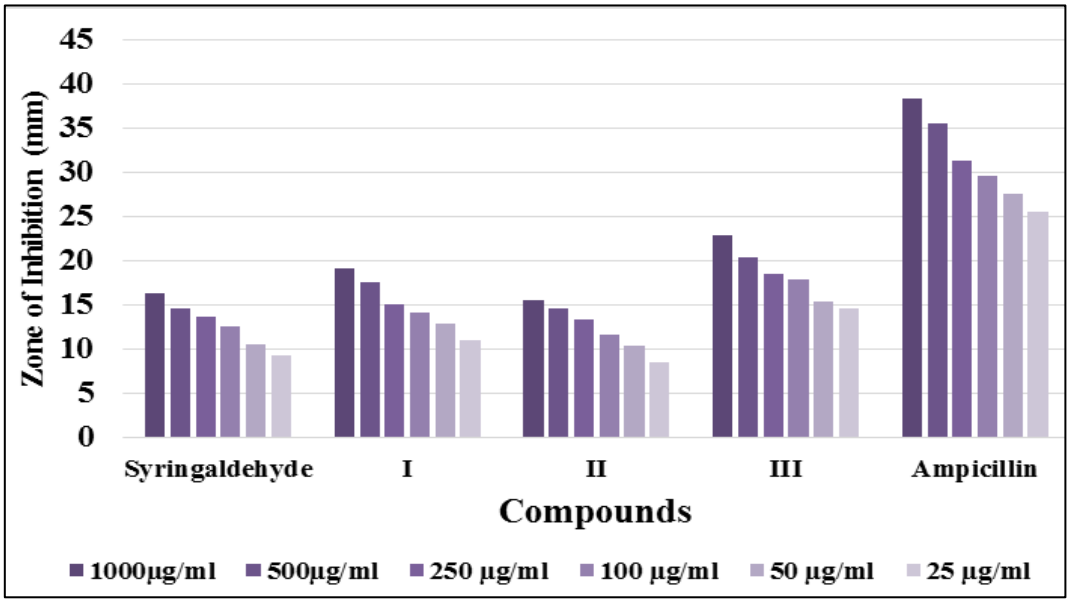

Fig 9: Zone of inhibition (mm) of different compounds against *Klebsiella* sp.

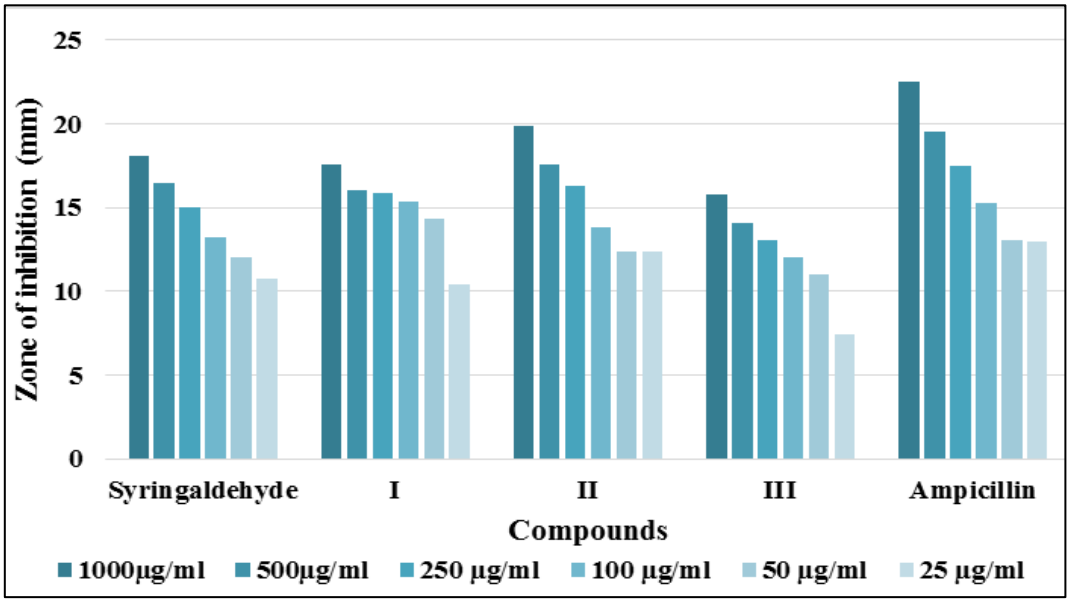

Fig 10: Zone of inhibition (mm) of different compounds against *Enterobacter* sp.

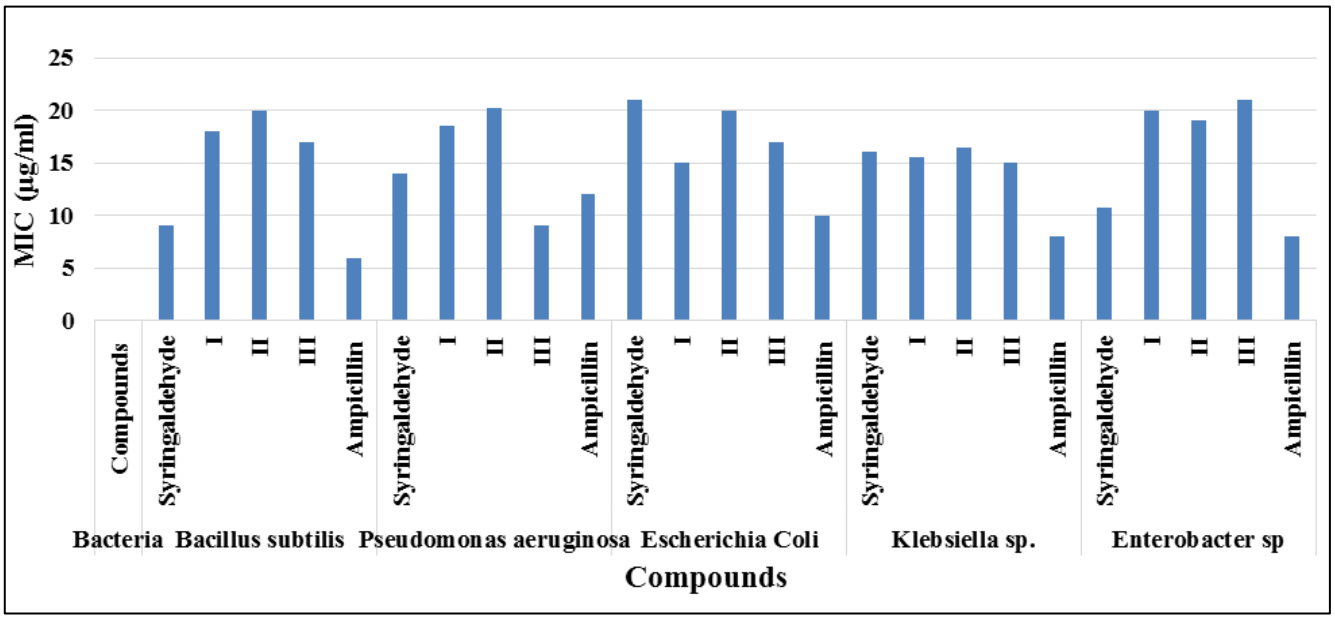

Fig 11: MIC values of compounds against five bacteria

The correlation between inhibition of Different bacteria by compounds

To correlate the inhibition caused by different compounds on bacterial growth. It was found that all the correlation coefficients (R) were positive which means there is a positive correlation between all the bacterium inhibition but found significant between *Klebsiella* and *E. coli* species (r = 0.890, p<0.01) only and presented in Table 2.

Table 2: Statistical analysis of data under study

		B. subtilis	***P. aeruginosa***	***E.coli***	***Klebsiella* sp.**
P. aeruginosa	Pearson Correlation	.498			
	Sig. (2-tailed)	.393			
	N	5			
E.coli	Pearson Correlation	.427	.310		
	Sig. (2-tailed)	.473	.612		
	N	5	5		
Klebsiella sp.	Pearson Correlation	.721	.441	.890*	
	Sig. (2-tailed)	.169	.458	.043	
	N	5	5	5	
Enterobacter sp.	Pearson Correlation	.957*	.260	.340	.675
	Sig. (2-tailed)	.011	.672	.575	.212
	N	5	5	5	5

*Correlation is significant at the 0.05 level (2-tailed).

Credit statements

Tanvi Sahni: conceived, designed, performed the experiment, Sunita Sharma: guides in designing, analyzing results throughout experiments; Diksha Verma: performing experiment; Sachin Kumar: helps in statistical analysis; Poonam Sharma; providing bacterial culture; Surekha Bhatia: analysis and interpreting antioxidant results.

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Summary

4-amino pyridine benzylidene of syringaldehyde was found to be a potent antibacterial agent than ampicillin against *Pseudomonas aeruginosa* during its assessment against one Gram-positive (*Bacillus subtilis*) and four Gram-negative bacteria (*P. aeruginosa*, *Escherichia Coli*, *Klebsiella* sp., and *Enterobacter* sp.). Toxicity and bioactivity of compounds were estimated using *Insilico* studies. The biological evaluation was further extended by its antioxidant estimation using three assays (DPPH, FRAP, and Phosphomolybdate)

Competing interest statement

The authors declare no conflict of interest

Additional information

No additional and supplementary material available for this paper

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