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Antioxidant and Antibacterial Evaluation of 2,6-dimethoxy-4-((phenylamino) methyl) Phenol and 2,6-Dimethoxy-4-((4′-nitrophenylimino)methyl) Phenol

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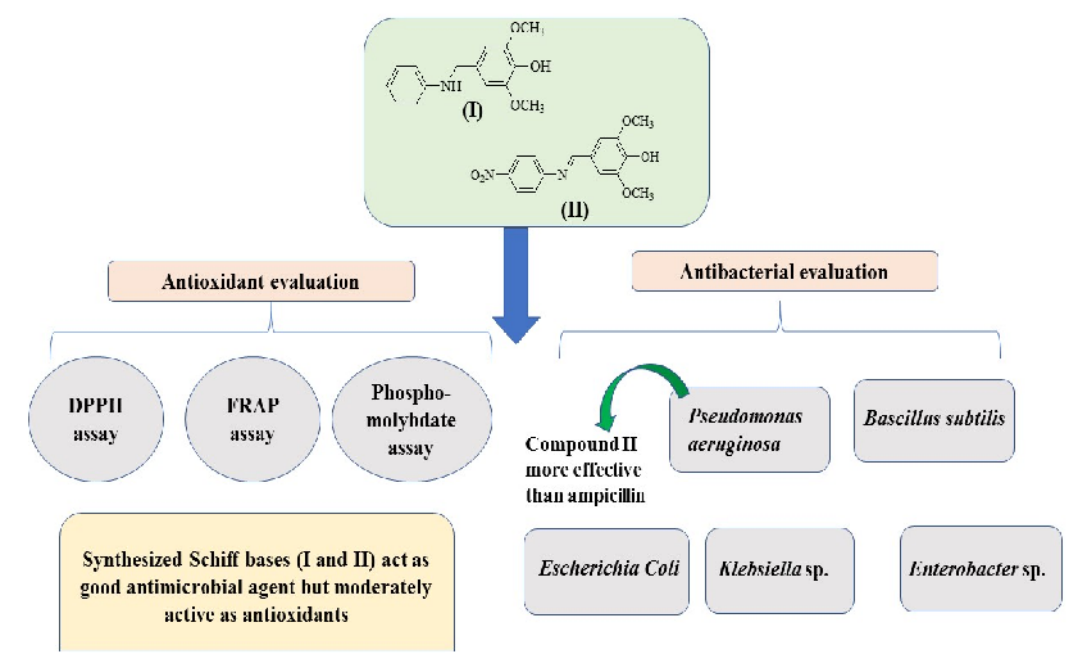
ABSTRACT: Two noval Shiff bases *i.e.* 2,6-dimethoxy-4-((phenylamino) methyl)phenol (I) and 2,6-Dimethoxy-4-((4′-nitrophenylimino)methyl)phenol (II) of syringaldehyde containing aniline as the scaffold were developed in the the lab and evaluated for their property as antioxidant using DPPH assay, FRAP assay, and phosphomolybdate assay. It was found that syringaldehyde the precursor of Schiff bases I and II had better DPPH reducing capacity while compound I was effective in reducing ferric Fe(+3) ions and 4-nitro aniline schiff base compound (II) was most effective in reducing Mo(+5) ions. The anti-microbial evaluation has proceeded for syringaldehyde and their Schiff bases I and II along with standard ampicillin against five bacteria *viz Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* spp., and *Enterobacter* spp. Compound II was more effective than standard ampicillin against *P. aeruginosa* only.



Keywords: Syringaldehyde, aniline, 4-nitroaniline, DPPH, FRAP**,** Phosphomolybdate, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp.

INTRODUCTION

In the advancement of various fields like agriculture, pharmaceuticals, and many more sectors, chemicals play their vital role among which aniline is considered as one of the most important chemicals. Aniline is an organic compound in which an amino group (NH_2) is attached to a phenyl ring (C_6H_5). It has been utilized as

a primary source for the product development of various industrial fields like in the automobile industry for manufacturing of rubbers and other materials, in the chemical industry for the synthesis of various bioactive molecules like paracetamol which is taken by the majority of human population for domestic purpose and has achieved its valuable position in the world market. Schiff bases are the compounds having (CH=N) moiety first discovered by Hugo Schiff and later on has been designed by various scientists owing to the presence of biological active nuclei nitrogen, that has antifungal (Kaur *et al.,* 2020; Sen and Srivastava 2009), antibacterial (Verma *et al.,* 2020; Pradhan and Vishwakarma 2018; Hania 2009), antioxidant (Bala *et al.,* 2012), antidyslipidemic (Sashidhara *et al.,* 2009), anthelmintic (Revanasiddappa *et al.,* 2010), antitubercular (Hearn *et al.,* 2009), antidepressants (Thomas *et al.,* 2016) properties. Some of the aniline derivatives are also reported to have biological activity on heart failure (Alejandra *et al.,* 2021), antifungal (Mann *et al.,* 2017), antibacterial (Kulkarni *et al.,* 2009), and antioxidant (Gizdavic-Nikolaidis *et al.,* 2004; Botteselle *et al.,* 2021) potential. Syringaldehyde on the other hand is an extraordinary plant isolate that shows tremendously effective antioxidant properties (Belkheiri *et al.,* 2010). Since spoilage of harvested vegetables was done by a variety of microorganisms, bacterial and fungal agentslike *Erwin carotovora*, *Pseudomonas* spp., *Corynebacterium*, *Xanthomonas campestris,*etc (Tournas 2005). Hence, there is a dire need to to synthesize antibacterial agents to compensate for this loss. On the other hand, the Quality of food and all farm ingredients had a dire urge of antioxidants for maintaining their quality during processing and distribution to consumers (Finley and Given 1986). Owing to have the tremendous biological potential of aniline and syringaldehyde derivatives, the present study is designed to evaluate aniline Schiff bases of syringaldehyde as antioxidant and antibacterial agents.

MATERIALS AND METHODS

All the chemicals were purchased from HiMedia Laboratories Private Limited, Mumbai, and Thermo Fisher Scientific India Private Limited, Mumbai. The chemicals and reagents were of anal. grade.

A. Synthesis of Schiff bases

Two Schiff base 6-dimethoxy-4-((phenylamino) methyl) phenol (I) and 2,6-Dimethoxy-4-((4′-nitrophenylimino)methyl)phenol (II) was synthesized by reaction of syringaldehyde with aniline using glacial acetic acid as solvent. The compound was characterized physically and through spectroscopic techniques (UV. FT-IT, ^{1}H-NMR, and ^{13}C-NMR) by Sahni T 2021, and further biological evaluation was given below:

B. Biological evaluation

(i) **DPPH radical scavenging activity (Blois, 1958).** DPPH radical scavenging activity was evaluated by method Blois (1958); Singh *et al.,* (2019). Six different concentrations (1000-25 µg ml^{-1}) of each compound along with syringaldehyde and two standards were prepared in methanol. 0.04 ml of test solution along with 3.96 ml of DPPH solution were mixed and shaken well. Each concentration was tested in triplicate and incubated for 30 minutes in dark. The scavenging capacity of the test compounds was estimated from the discoloration of the DPPH solution and estimated by absorbance at 517 nm. Ascorbic acid and BHT were used as standards. % Radical scavenging activity was estimated with the formula:

$$\text{Radical Scavenging Activity (\%)} = \frac{\text{O. D.}_{\text{control}} - \text{O.D.}_{\text{sample}}}{\text{O.D.}_{\text{control}}} \times 100$$

Where O.D.$_{control}$ is the absorbance of blank
O.D.$_{sample}$ is the absorbance of test compounds.
Kinetic parameter IC_{50} was also calculated using SPSS 16.0

(ii) **Ferric Reducing Antioxidants Potential (Benzie and Strain 1999).** In a test tube, test solution (0.2 ml) was added to FRAP reagent (2.80 ml) and made a total volume of 3.00 ml. Absorbance was taken at 593 nm after incubation for 30 minutes in dark. Solvent (0.2 ml) was taken in place of test solution along with frap reagent in the blank. Compounds were tested at six concentrations (1000-25 µg ml^{-1}) while ascorbic acid and BHT were used as standard. Test compounds were expressed as ascorbic acid and BHT equivalents (µg ml^{-1}) using an equation obtained from its calibration curve.

(iii) **Total antioxidant capacity by phosphomolybdate assay (Prieto *et al.,* 1999).** Total antioxidant capacity was determined using phosphomolybdate assay (Prieto *et al.,* 1999). Compounds were tested at six different concentrations (1000-25 µg ml^{-1}) and ascorbic acid was used as standard. Phosphomolybdate reagent was prepared from three solutions of 0.60 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. In a test tube, 3ml of the test compound which was prepared in methanol along with 1ml of reagent was added. Blank was prepared by adding 3.00 ml of methanol with 1ml of reagent. All the test tubes were covered with foil papers and boiled at 95°C for 90 min in a water bath. Test tubes were taken out of the water bath and cooled at room temperature for taking absorbance at 695nm in the spectrophotometer. Test compounds were expressed as ascorbic acid equivalents (µg ml^{-1}) using an equation obtained from its calibration curve.

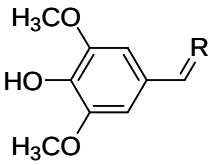
(iv) **Antibacterial activity.** Antibacterial activity of Schiff bases was tested against Gram-positive (*Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp.) in nutrient agar media. DMSO was used as a solvent for the preparation of different concentrations as well as control using the Disk plate method. This method involves the occurrence of the zone of inhibition by compounds on bacterial diffused plates after incubation for 24 hours incubation at 37 ±

2°C in the incubator (Verma *et al.*, 2020). The experiment was carried out in triplicate and ampicillin was used as standard. Minimum inhibitory concentrations (MIC) were used for comparing different compounds, syringaldehyde and standard.

C. Statistical analysis

Statistical analysis of data was done using Genstat for split-split plot design. Analysis was done to determine effect of compounds on bacteria at different concenterations. The fixed factors were compounds and bacteria while replication was random factor.

Table 1: Structure of Test compounds.



Compound No.	R
Syringaldehyde	O
I	Aniline
II	4-Nitro-aniline

RESULTS AND DISCUSSION

Presence of aromatic ring along with free hydroxyl group, imine (CH=N) moiety as well as nitro group were esponsible for respective biological activity of synthesized compounds.

A. Antioxidant activity

(i) **DPPH Radical scavenging activity.** DPPH radical scavenging activity was assessed in terms of % RSA and IC_{50} µg ml^{-1} which were presented in Fig. 1 and 2. Lone pairs present on nitrogen and oxygen plays an important role in scavenging DPPH radicals and making them better antioxidant. Syringaldehyde was found to be more effective than compounds I and II due to the presence of the hydroxy group at the para position due to the (+R) resonance effect. The decreasing trend of % RSA in compounds I and II was maybe due to the introduction of the benzene ring in compound I and benzene ring having a nitro group at the para position in compound II which leads to delocalization of lone pair of oxygen electrons and thus reduces their radical scavenging potential. IC_{50} (µg ml^{-1}) values were greater than 1000 µg ml^{-1} for compounds I and II, while a gradual decrease in antioxidant potential was observed with a decrease in concentration Fig. 1 Syringaldehyde, possessed an IC_{50} value of 13.43 which means more effective than standard BHT but less than ascorbic acid.

(ii) **Ferric Reducing Antioxidant Potential (FRAP).** In FRAP assay, the antioxidant potential was assessed by reduction of Fe (+3) to Fe (+2) in terms of Asc equivalent and BHT equivalent (Fig. 3, 4) due to formation of blue colored-tripyridyl triazine complex(Fe^{+2}-TPTZ) at pH 3.6.

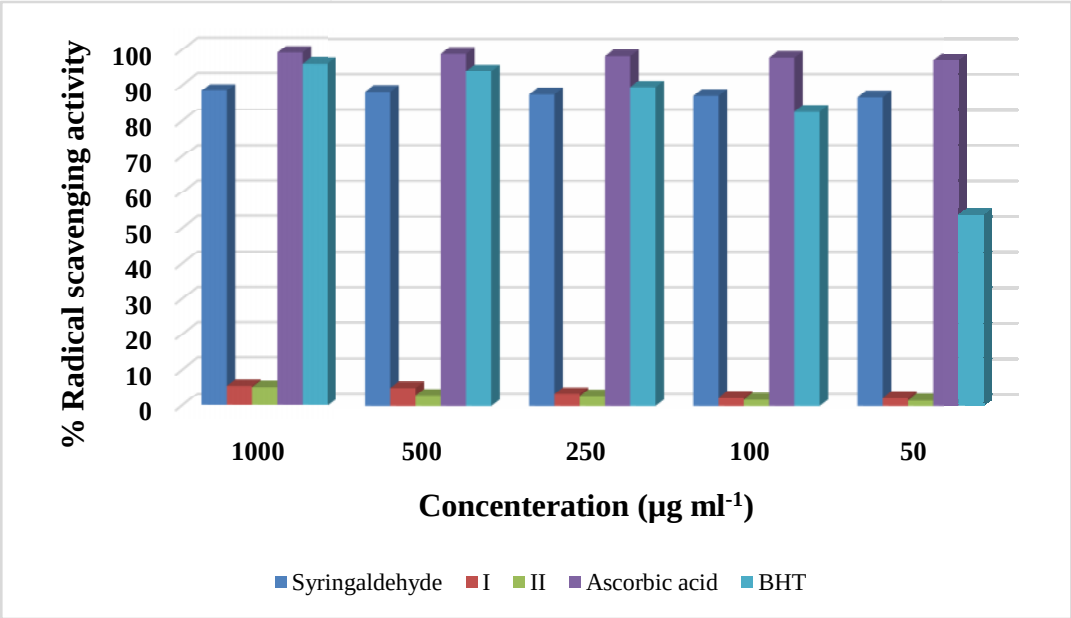


Fig. 1. % Radical scavenging of tested compounds at different concentrations.

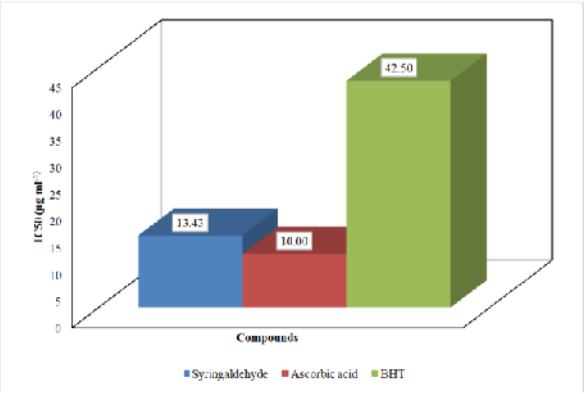


Fig. 2. IC_{50} values of compounds against DPPH assay.

All the tested compounds showed increase in antioxidant potential with increase in concenteration also reported in Ahmed *et al.,* (2015). Schiff base I was most effective followed by II with the nitro group at the para position. This can be due to the electron-withdrawing (-M) mesomeric effect of the nitro group which leads to the delocalization of conjugated electrons. Thus, the addition of electron-withdrawing nitro group prevents antioxidant potential. Schiff bases cause a greater reduction of ferric ions than syringaldehyde and it was found that the antioxidant potential of both Schiff bases was greater than standard ascorbic acid and BHT from 25-500 (µg ml^{-1}) concentrations but less effective at 1000 µg ml^{-1}. Thus, the substitution of the aldehydic group showed an increase in antioxidant potential against ferric ions.

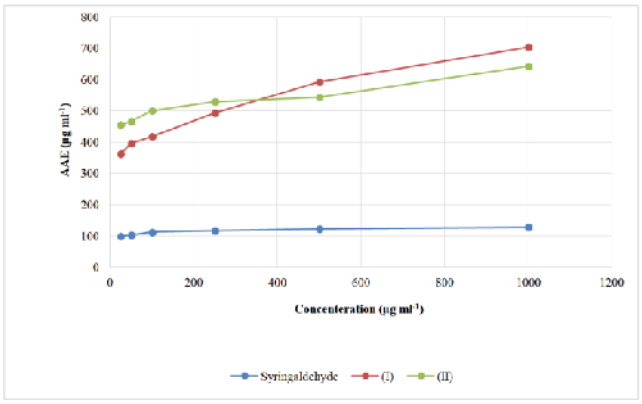


Fig. 3. Ferric reducing antioxidant potential (FRAP) valus of of different compounds at six concenterations in terms of ascorbic acid equivalent (AAE) (n=3).

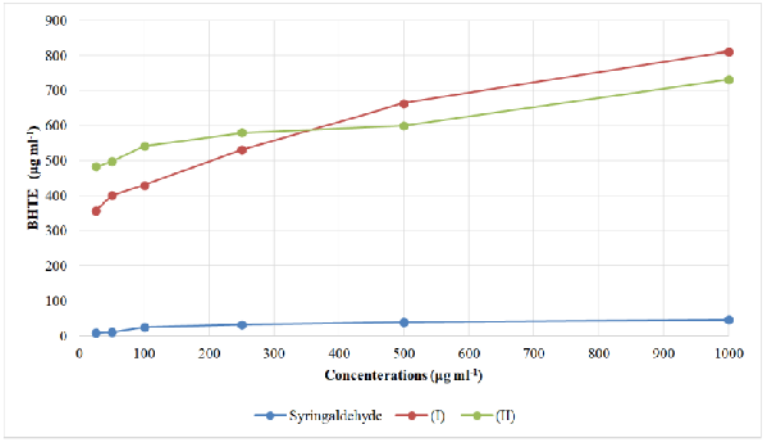


Fig. 4. Ferric reducing antioxidant potential (FRAP) valus of of different compounds at six concenterations in terms of Butylatedhydroxytoluene equivalent (BHTE) (n=3).

(iii) **Total Antioxidant Capacity as per Phosphomolybdate Assay.** Total antioxidant capacity was assessed in phosphomolybdate assay as reduction of Mo (V) to Mo (IV) as Asc equivalent since BHT is not a good antioxidant for reduction of molybdenum. It was found that 4-nitroaniline Schiff base (II) of syringaldehyde was a better antioxidant agent followed by aniline Schiff base (I) and syringaldehyde. Compound II was also more effective than standard ascorbic acid at concentrations 25-500 (µg ml^{-1}) as shown in Fig. 5. Compound I and syringaldehyde were found less effective than standard ascorbic acid than all other concentrations.

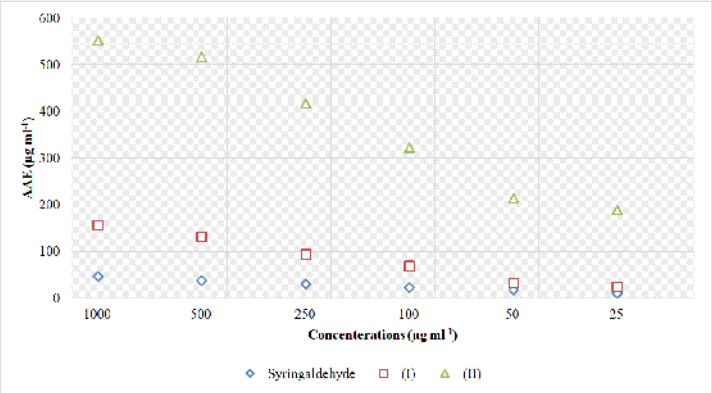


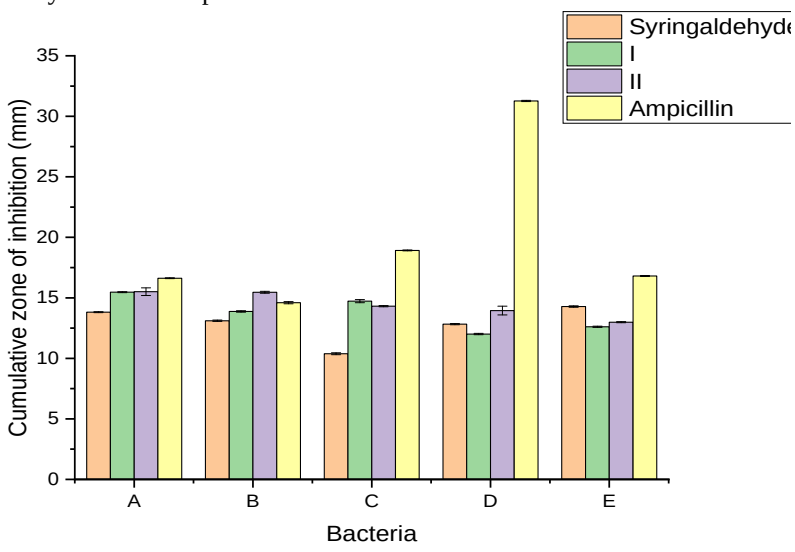
Fig. 5. Total antioxidant capacity of different compounds at six concenterations in terms of ascorbic acid equivalent (AAE) (n=3).

B. Antibacterial activity

Analysis of data presented in Fig. 6, 7 against one gram positive bacteria and four gram negative bacteria revealed that compound **II** (4-nitro aniline Schiff base of syringaldehyde) was more effective than ampicillin at all six tested concentrations against *P. aeruginosa* followed by compound I which was found comparable to standard ampicillin. Syringaldehyde was more effective than aniline Schiff bases of syringaldehyde and was found to be comparable with ampicillin against *B. subtilis* and *Enterobacter* sp. followed by compound II showed a greater zone of inhibition than compound I in both bacterium. In *Klebsiella* sp. none of the compounds showed a higher zone of inhibition than ampicillin while compound II was most effective followed by syringaldehyde and compound I at all concentrations except 50 µg ml^{-1}. In *E. coli* compound I was most effective followed by compound II and syringaldehyde at 1000, 50, and 25 µg ml^{-1} while reverse trend i.e. compound II was more effective than compound I at all other concentrations.

Thus in general due to presence of electron withdrawing Nitro group at para position increases the antimicrobial potential of tested compounds as compaired to unsubstituted precursors. This fact was also supported by Sheikh and Hadda (2013) and also schiff were known to be biologically active as antibacterial and atifungal agent (Singh *et al.*, 2012).

Analysis of antibacterial potential was further extended to find out Minimum Inhibitory Concentrations (MIC) values Fig. 6,7.



LSD (p<0.05): Compounds: 0.33, Bacteria: 0.37, Concenteration: 0.41, Bacteria*compounds : 0.74, Compounds*concenteration :0.81, Bacteria*concenteration: 0.91, Bacteria*Compounds*Concenteration: 1.82 A= *Bascillus subtilis*, B= *Pseudomonas aeruginosa* C= *Escherichia coli*, D= *Klebsiella* sp., E= *Enterobacter* sp

Fig. 6. Cumulative zone of inhibition (mm) of different compounds against five bacterial strains in comparison with ampicillin.

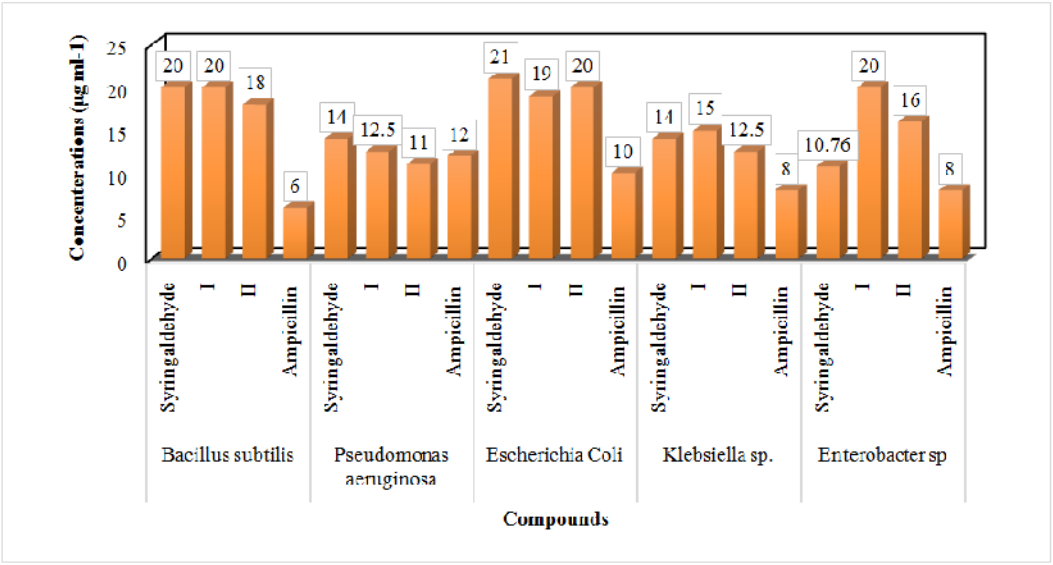


Fig. 7. MIC values of compounds against five bacterium.

Data revealed that all compounds had MIC values less than 22 µg ml^{-1} against all bacterium. Against *Klebsiella* sp. and *Enterobacter* sp. compounds followed the trend of I> Syringaldehyde>II. Compound II exhibited less MIC value of 11 µg ml^{-1} which was than ampicillin. Thus, considered a better antibacterial agent than ampicillin. Compound II also exhibited the least MIC value against *B. subtilis* and *P. aeruginosa* as well. None of the compounds was more effective than standard ampicillin against all bacterium except *P. aeruginosa.*

Statistically, it was found that interactions of bacteria with different concenteration of compounds was found maximum followed by compounds with concenteration and bacteria with compounds. All significant interations indicated that synthesized compounds had positive effect on inhibiting bacterial growth at different concenterations.

CONCLUSION

It may be concluded that aniline Schiff bases of syringaldehyde possessed better antibacterial activity than antioxidants. They were more effective in controlling Gram-negative bacterium than Gram-positive bacterium and were found to be more effective than standard ampicillin against *P. aeruginosa*. There is positive interactions between compounds and bacteria at different concenterations. Syringaldehyde had better radical scavenging activity than Schiff bases in terms of DPPH assay while the reverse trend was found in the reduction of ferric Fe (+3) and Mo (+5). None of the compounds was a better antioxidant than standard ascorbic acid and BHT. Thus, possessed moderate antioxidant potential.

LIST OF ABBREVIATIONS

Asc: Ascorbic acid
BHT: Butylated hydroxytoluene
DMSO- Dimethylsulfoxide
Anal.- Analytical
RSA- Radical scavenging activity.

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Conflict of Interest. None.

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