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## Evaluation and studies on the structural impact of 3-aryl-5-(4-methoxyphenyl)-4,5-dihydroisoxazole-4-carbonitriles on their biological activities

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### ABSTRACT

*In present study, a series of isoxazole derivatives was synthesized and evaluated for antibacterial and antifungal activities by disc diffusion method using different bacterial and fungal strains. Some compounds of the series exhibited promising antibacterial and antifungal activity compared to standard drugs. The minimum inhibitory concentration (MIC's) was determined against each organism. The compounds were tested for their antioxidant activity and reducing power ability. Free radicals play an important role in various pathological and xenotoxic effects so antioxidant may have protective role in these pathological conditions. Based on the results of an antimicrobial, anti-oxidant study, the effect of substitution on the activity and possible structure activity relationship of the compounds for their antioxidant activity is presented.*

**Key words:** Isoxazoles, antibacterial, antifungal, antioxidant, reducing power.

### INTRODUCTION

Resistance of pathogenic bacteria to available antibiotics is quickly becoming a major problem in the community and hospital based healthcare settings. Antimicrobials are one of the very important categories of drug. So it is quite clear from the spectrum of use that these categories of drugs are very important from medical point of view. But microbial resistance towards the drug creates a very serious problem; because of development of resistance, many drugs are now useless which were very effective before. Moreover, the toxic effects produced by these antibiotics are also reducing their significance [1]. So there is need for new antimicrobial agents for resistant microbial infections.

Reactive oxygen species [ROS], sometimes called as active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions ( $O_2^{\cdot -}$ ) and hydroxyl radicals ( $\cdot OH$ ) as well as non-free radical species such as hydrogen peroxide ( $H_2O_2$ ) [2]. These ROS play an important role in degenerative or pathological processes, such as aging, cancers, coronary heart diseases, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts and inflammations [3]. Living organisms have antioxidant defense systems that protects against oxidative damage by removal or repair of damaged molecules [4]. The term 'antioxidant' refers to the activity of numerous vitamins, minerals and phytochemicals which provide protection against the damage caused by ROS [5]. Antioxidants interfere with the oxidative processes by scavenging free radicals, chelating free catalytic metals and by acting as electron donors [6]. The natural antioxidant mechanisms maybe insufficient in variety of conditions and hence dietary intake of antioxidant compounds are important [7].

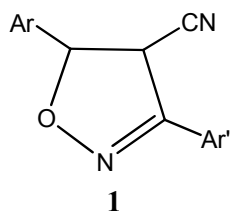
The wide occurrence of the heterocycles in bioactive natural products and pharmaceuticals has made them as important synthetic targets. Isoxazolines are very useful heterocycles in organic synthesis and medicinal chemistry. For instance, compounds possessing isoxazole moiety have revealed antimicrobial [8-10], anticancer [11], antitubercular [12], antioxidant [13], anti-inflammatory [14] properties. This paper describes the *in vitro* screening and results of the antibacterial, antifungal activity, minimum inhibitory concentrations, antioxidant activity and reducing power ability of the synthesized new title compounds [15]. The mechanistic path of structure activity relationship of antioxidant activity of the compounds presented.

## MATERIALS AND METHODS

### Source of chemicals

All chemicals used were of analytical grade. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. Methanol, DMF, ferric chloride, potassium ferricyanide, phosphate buffer, BHT (butylated hydroxyl toluene) and trichloroacetic acid (TCA) and solvents were purchased from Merck India Ltd. Absorbance was noted using UV/Visible Spectrophotometer (Elico).

In view of the enormous biological potency associated with isoxazole derivatives, a series of new synthesized 3-Aryl-5-(4-methoxyphenyl)-4,5-dihydroisoxazole-4-carbonitriles **1a-i** (Scheme-1) [15] were selected in the present work for the study of their biological activities.



- 1 a) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 4-FC<sub>6</sub>H<sub>4</sub>;
- b) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 4-ClC<sub>6</sub>H<sub>4</sub>;
- c) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 2-ClC<sub>6</sub>H<sub>4</sub>;
- d) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 4-BrC<sub>6</sub>H<sub>4</sub>;
- e) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 4-CNC<sub>6</sub>H<sub>4</sub>;
- f) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 4-Cl-2-NO<sub>2</sub>C<sub>6</sub>H<sub>3</sub>;
- g) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = C<sub>6</sub>H<sub>5</sub>;
- h) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>;
- i) Ar = 4-OCH<sub>3</sub>C<sub>6</sub>H<sub>4</sub>, Ar' = 2,4-(OCH<sub>3</sub>)<sub>2</sub>C<sub>6</sub>H<sub>3</sub>;

**Scheme 1: Isoxazole derivatives used in the present study**

**ANTIMICROBIAL ACTIVITY:** Antimicrobial activity of the synthesized compounds (3a-i) was done by paper disc diffusion method [16, 17].

### Antibacterial activity:

Gram-negative bacteria species such as *Escherichia coli*, *Salmonella typhimurium*, Gram-positive bacteria species such as *Bacillus subtilis*, *Staphylococcus aureus* were used as antibacterial test strains. The representative compounds **1a-i** was screened at the concentration (50 µg/mL) in methanol on the nutrient agar media. The antibiotic ciprofloxacin was used as standard drug against bacteria. The screening tests were performed in triplicate and the results were taken as a mean of three determinations. Minimum inhibitory concentrations (MICs) were determined by broth dilution technique. The nutrient broth, which contain logarithmic serially two-fold diluted amount of test compound and controls were inoculated with approximately  $5 \times 10^5$  c.f.u of actively dividing bacteria cells. The cultures were incubated for 24 hrs at 37°C and the growth was monitored visually and spectrophotometrically. The lowest concentration required to arrest the growth of bacteria was regarded as minimum inhibitory concentration (MIC). The experiments were carried out in triplicate and the results were taken as a mean of three determinations.

### Antifungal activity:

The synthesized compounds **1a-i** were tested for their antifungal activity against the fungi species *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* strains at a concentration of 25 µg/ml in DMF in the potato dextrose agar media. The antibiotic Griseofulvin was used as standard drug against fungi. The screening tests were performed in triplicate and the results were taken as a mean of three determinations. Minimum inhibitory concentrations (MICs) were determined by broth dilution technique. The cultures were incubated for 72 hrs at 37°C and the growth was monitored visually and spectrophotometrically. All the experiments were carried out in triplicate and the results were taken as a mean of three determinations.

## ANTIOXIDANT ACTIVITY

### DPPH free radical scavenging assay:

The effect of the samples **1a-i** in addition to the standard antioxidant butylated hydroxyl toluene (BHT) on DPPH radical was estimated according to the method [18,19]. Samples dissolved in methanol (0-50 µg/mL for samples **1a-i**; 0-5 µg/mL for BHT) in 200 µL aliquot was mixed with 100 mM tris-HCl buffer (800 µL, pH 7.4) and then added 1 mL of 500 µM DPPH in ethanol (final concentration of 250 µM). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The results of all experiments performed were expressed as mean of the three determinations.

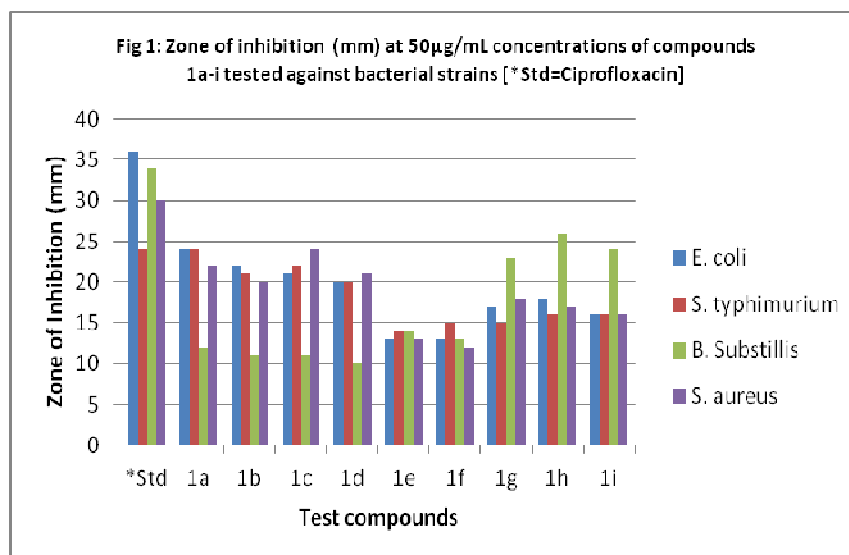
### Measurement of reducing power:

The reducing power of samples **1a-i** was determined according to the method [20]. The samples **1a-i** (0-50 µg/mL) was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then an equal volume of 10% trichloroacetic acid was added to the mixture and then centrifuged at 5000 rpm for 10 min. the upper layer of solution was mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:2 and the absorbance were measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The experiments were carried out in triplicates (n=3) and the results are expressed as mean of the three determinations.

## RESULTS AND DISCUSSION

### Antibacterial activity:

The results of antibacterial activity were depicted in Fig-1. The investigation of the antibacterial screening of the test samples **1a-i** revealed that all these compounds showed moderate to good antibacterial activity against all the organisms. The compounds **1a-d** showed lesser activity against *Bacillus subtilis* and remarkable activity against the bacterium *E. coli*, *S. typhimurium* and *S. aureus*, which is attributed to the presence of fluoro, chloro, bromo and cyano substituents at C<sub>3</sub>-substituted benzene ring. The compounds **1e**, **1f** found less active against all the organisms tested, this may be due to the presence of strong electron withdrawing –CN, –NO<sub>2</sub> substituents on the benzene ring. The compounds **1g-i** have exhibited moderate activity against *E. coli*, *S. typhimurium* and *S. aureus* and good activity against *Bacillus subtilis*, which is attributed to the presence of electron donating –OCH<sub>3</sub> groups or no substitution on the aromatic ring. The results thus obtained reveal that nature of substituents present on the benzene ring has a considerable impact particularly at *ortho* and *para* positions. The results indicate that the compounds **1a-d** may be used as control measures against *E. coli*, *S. typhimurium* and *S. aureus*, **1g-i** against *Bacillus subtilis* and different bacteria.

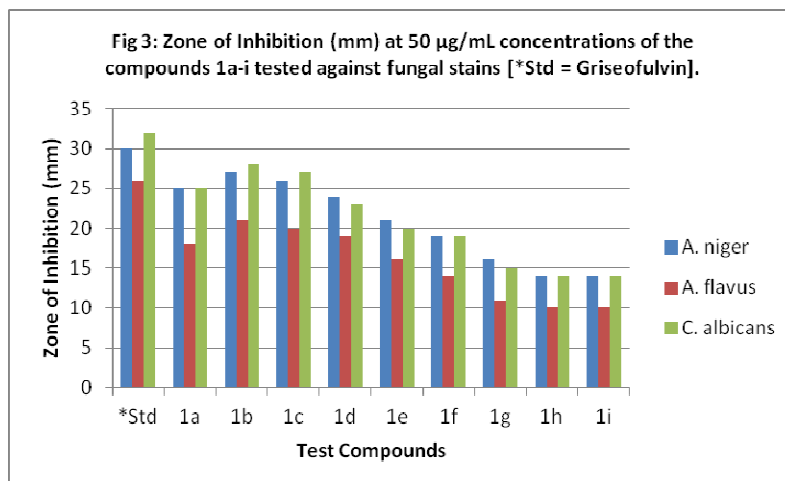
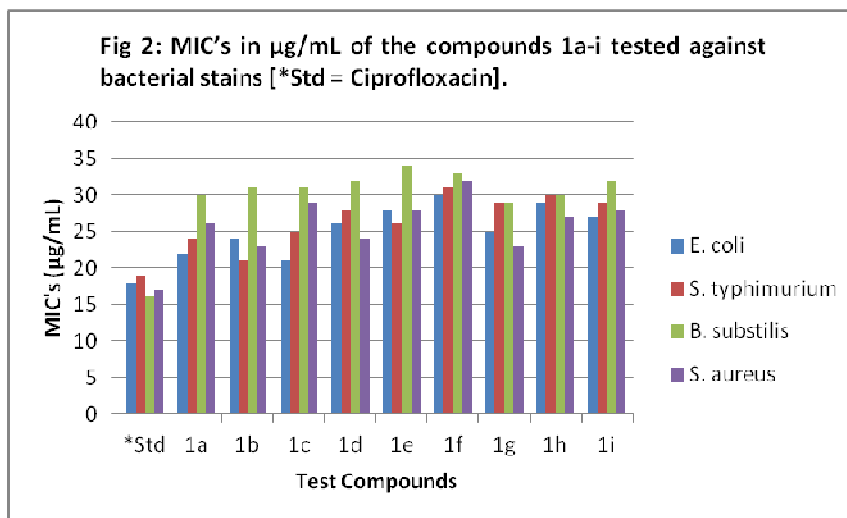


The results of MIC's determined against different bacterial were depicted in Fig-2. The results of MIC's indicate that some of these compounds can be used as reference antibacterial agents even at lower concentrations.

### Antifungal activity:

The results of antifungal activity were depicted in Fig-3. The experimental results of **1a-i** revealed that all these compounds showed promising antifungal activity against *A. niger* and *C. albicans*, moderate activity against *A.*

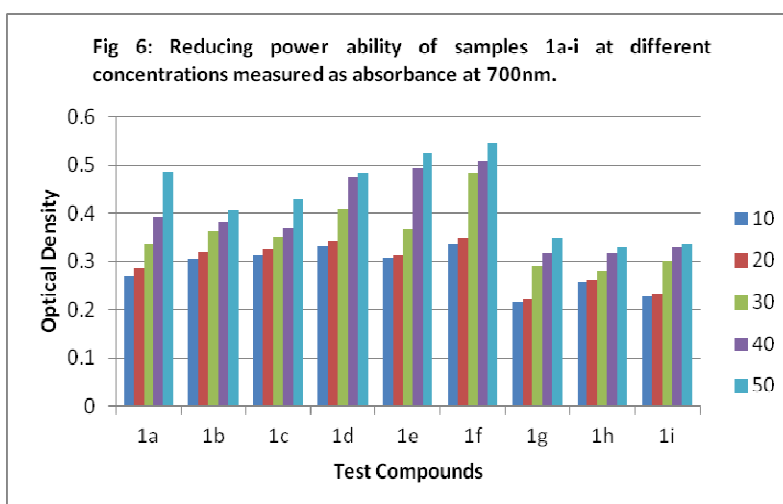
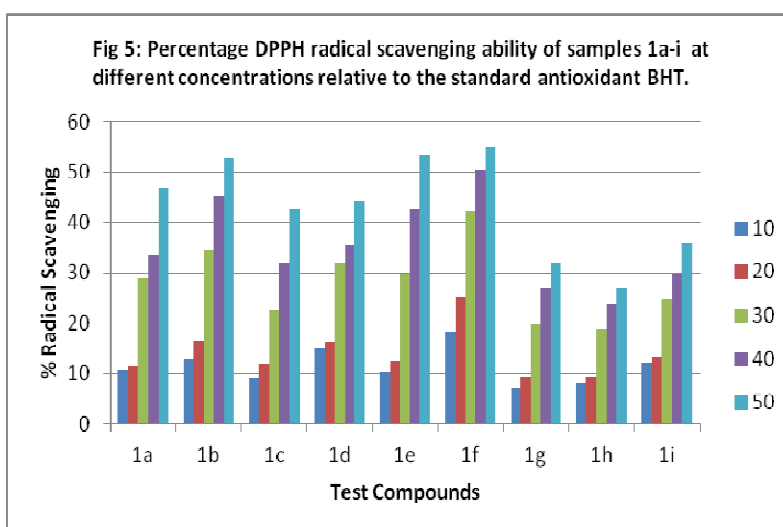
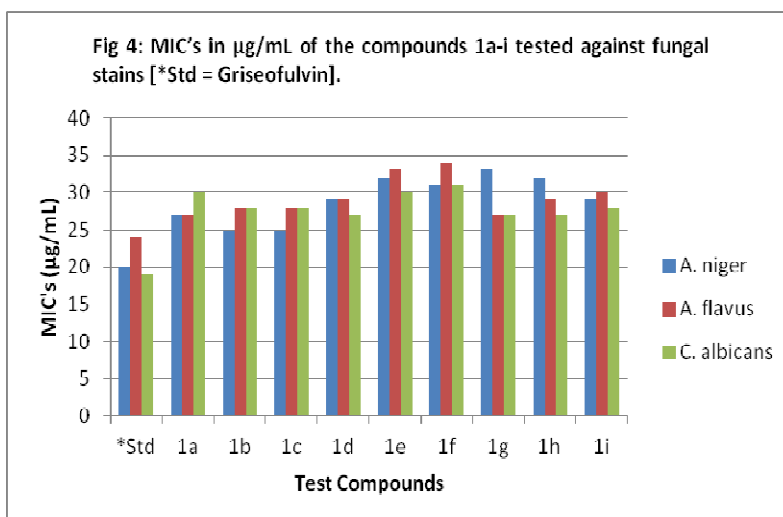
*flavus*. The compounds **1a-d** was highly active against *A. niger* and *C. albicans*, moderately active against *C. albicans*. Test samples **1e, 1f** have shown moderate activity against all the organisms tested, which may be due to the presence of electron withdrawing -CN and -NO<sub>2</sub> groups on the aromatic ring, while **1g-i** shown marked lesser activity against all the organisms, which may be attributed to the presence of electron donating -OCH<sub>3</sub> groups or no substituents on the C<sub>3</sub>-substituted benzene ring. The results thus obtained reveal that halogen substituents present on the C<sub>3</sub>-substituted benzene ring have a considerable impact particularly at *ortho* and *para* positions, and therefore they may be used as control measures against different fungi species.



The results of MIC's determined against different fungal species were depicted in Fig-4. The results indicate that some of these compounds can be used as reference antifungal agents even at lower concentrations.

#### Antioxidant activity:

The results of *in vitro* antioxidant activity of the title compounds were depicted in Fig-5. DPPH radical scavenging is considered a good *in vitro* model and is widely used to conveniently assess antioxidant efficacy. From the results it could be seen that most of the compounds showed significant antioxidant activity. At the initial concentrations of (10-20 µg/mL), not much significant variations in the free radical scavenging ability of samples 1a-g was observed. However, when the concentration was increased (30-50 µg/mL) all showed a promising radical scavenging ability. The compounds **1a-d** showed radical scavenging ability up to 50%, the samples **1e, 1f** showed radical scavenging ability up to 62% and **1g-i** showed up to 40% with reference to the standard antioxidant. Results indicate that the compounds **1e, 1f** containing electron withdrawing groups on the aromatic ring shows potential electron donating ability.



#### Reducing power:

The samples **1a-i** was evaluated for their reducing power ability to reduce ferric chloride and potassium ferricyanide complex. The results were depicted in Fig-6. It was observed that at the initial concentrations of (10-20  $\mu\text{g/mL}$ ), there was not much significant variations in the activity. However, when the concentration was increased (30-50  $\mu\text{g/mL}$ ), all showed remarkable reducing power. The compounds **1e,1f** containing electron withdrawing substituents on the aromatic ring showed higher reducing power, and **3a-d** having halogen substituents on the aromatic ring showed moderate reducing power, where as the samples **1g-i** with electron donating substituents on the aromatic

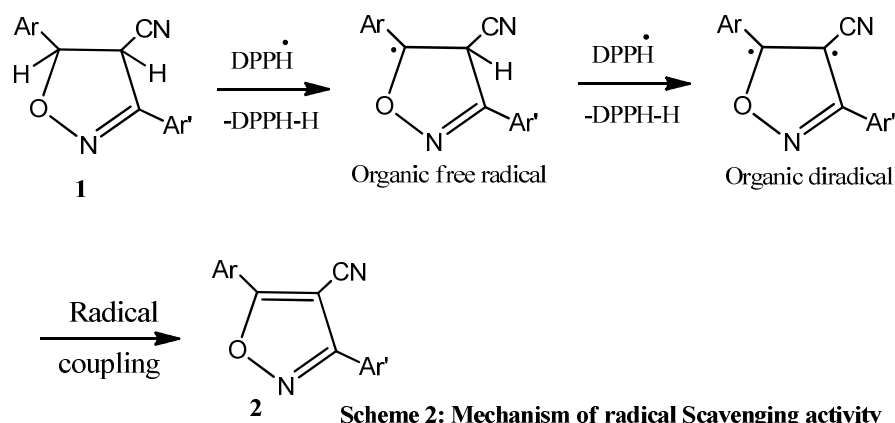
ring exhibited lesser reducing power ability compared to **1a-f**. The increased absorbance at 700 nm indicated the presence of reducing power ability of the test samples considered for the study.

**Mechanistic considerations of antioxidant activity:** DPPH is a stable organic nitrogen radical used as a scavenger for other radicals. DPPH radical scavenging test evaluates *in vitro* antioxidant capacity. In the presence of hydrogen/electron donor, DPPH radical scavenges the hydrogen radical from a donor molecule and it gets reduced as  $\text{DPPH}^\bullet + \text{H}^\bullet \rightarrow \text{DPPH-H}$ .

As and when DPPH radical scavenges the hydrogen radical, the absorption intensity is decreased and the radical solution is decolorized to pale yellow color depends upon the number of electrons captured.

The instability of the non-aromatic 3-Aryl-5-(4-methoxyphenyl)-4,5-dihydroisoxazole-4-carbonitriles **1** was expected to be the driving force for their antioxidant activity. The non-aromatic compounds have a tendency to become more stable aromatic compounds **2** with the loss of two hydrogen atoms and two electrons.

From the experimental results, the stoichiometry of the reaction was found to be 1:2 for test compounds: DPPH free radical, which suggests that each molecule (**1**) has a tendency to donate two hydrogen atom and two electrons to the acceptor molecules. In the presence of hydrogen donor organic compound (**1**) the DPPH free radical abstract the hydrogen atom bonded to C<sub>4</sub> and/or C<sub>5</sub>-atom along with one of its bonded electron to give organic free radical and it becomes reduced (DPPH-H). The second molecule of DPPH free radical abstracts the hydrogen atom of C<sub>5</sub> and/or C<sub>4</sub>-atom with one of its bonded electron to give organic diradical and it becomes reduced (DPPH-H). The organic diradical expected to undergo intramolecular coupling to form stable organic compound (**2**) (Scheme 2).



On the basis of this speculation, the C<sub>4</sub> and/or C<sub>5</sub> positions of the isoxzoline ring may be the active site responsible for antioxidant activity of the screened pyrazole derivatives.

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