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Evaluation and Studies on the Structural Impact of Substituted 4, 5-Dihydroisoxazoles on Their Biological Activities

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ABSTRACT
In present study, a series of isoxazole derivatives synthesized were evaluated for antimicrobial activities by disc diffusion method. 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 *in-vitro* 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.

Keywords: 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-}) 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] anti-tubercular, [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).

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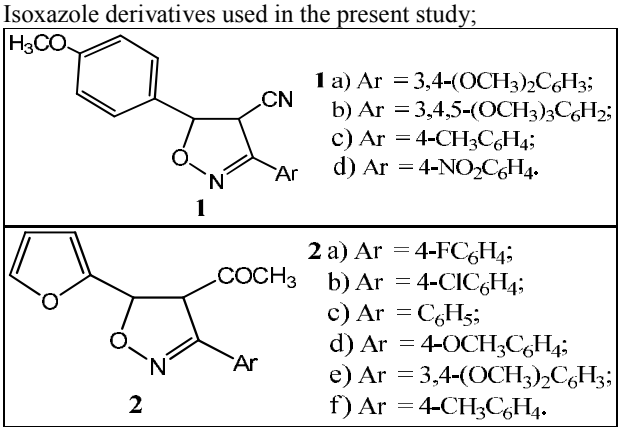
In view of the enormous biological potency associated with isoxazole derivatives, a series of new synthesized 3-Aryl-5-(4-methoxyphenyl)-4,5-dihydroisoxazole-4-carbonitriles 1a-d [15] and 4-Acetyl-3-aryl-5-(furan-2-yl)-4,5-dihydroisoxazoles 2a-f were selected in the present work for the study of their biological activities.

Isoxazole derivatives used in the present study;



ANTIMICROBIAL ACTIVITY: Antimicrobial activity of the synthesized compounds (1, 2) was done by paper disc diffusion method. [16-17]

Antibacterial activity

Gram-negative bacteria species such as *E. coli, S. typhimurium,* Gram-positive bacteria species such as *B. substilis*, *S. aureus* were used as antibacterial test strains against 1a-d; *E. coli,* and *B. substilis* were used against 2a-f. The representative compounds 1a-d, 2a-f was screened at the concentration (50µg/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 5×10^5 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-d were tested for their antifungal activity against the fungi species *A. niger, A. flavus* and *C. albicans* strains; 2a-f were tested against *A. niger, A. flavus* 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. MIC's were determined by broth dilution technique. The cultures were incubated for 72 hours 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-d and 2a-f in addition to the standard antioxidant butylated hydroxyl toluene (BHT) on DPPH radical was estimated using the known method. [17-18] Samples dissolved in methanol (0-50 µg/ml for samples 1a-d, 2a-f; 0-5µg/ml for BHT) in 200µl aliquot was mixed with 100 mM tris-HCl buffer (800µl, pH 7.4) and then added 1 ml of 500µM DPPH in ethanol (final concentration of 250µM). 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-d, 2a-f was determined using to the method. [19] The samples 1a-d, 2a-f (0-50µg/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 of the test compounds 1a-d, 2a-f was depicted in Fig. 1 and MIC's were given in Fig. 2. The investigation of the antibacterial screening of the test samples revealed that all these compounds showed moderate to good antibacterial activity against all the organisms. . The compounds 1a-c showed remarkable activity; 1d exhibited moderate activity against all organisms tested. The compounds 2a, 2b showed greater activity against all the organisms tested; which is attributed to the presence of electronegative fluorine and chlorine atom substituents. The compounds 2c-f showed moderate activity against the entire organism tested; which may be due to the presence of electron donating substituents on the benzene ring. The experimental results revealed that the compounds 1d, found less active against the bacterium *S. typhimurium*.

Antifungal activity

The results of antifungal activity and MIC's of the test compounds 1a-d, 2a-f were depicted in Fig. 3 and Fig. 4 respectively. The test compounds 1a-c has exhibited promising activity against *A. niger* and *C. albicans,* moderate activity against *A. flavus*. The compounds 2a, 2b found highly active against *A. niger* and *C. albicans*; which may be attributed to the presence of electronegative fluorine and chlorine substituents. The compounds 2d-f showed moderate activity; this might be due to the presence of electron donating substituents. It has been observed that the compound 2c which do not have substituents in the benzene ring exhibited lesser activity against the organisms tested. Therefore the results of the experiment draw the conclusion that, the nature of the groups, the number of substituents present in the aromatic substituent on the isoxazole ring has greater impact on the antifungal activity.

Antioxidant activity

The results of *in-vitro* antioxidant activity of the test compounds were depicted in Fig. 5. DPPH radical scavenging is considered a good *in-vitro* model and is widely

Fig 1: Zone of Inhibition (mm) at 50 µg/mL concentration of the test samples against bacterial strains [*Std= Ciprofloxacin].

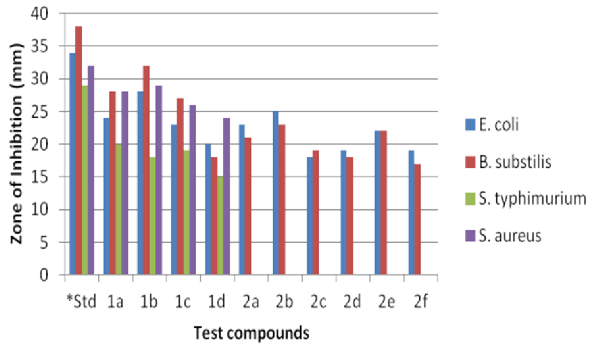


Fig 2: MIC's in µg/mL concentrations of the samples tested against bacterial strains [*Std= Ciprofloxacin].

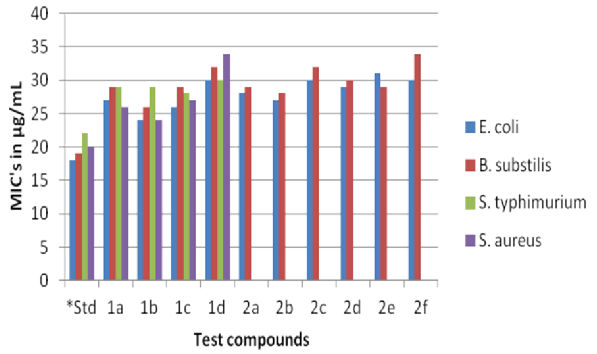


Fig 3: Zone of Inhibition (mm) at 50 µg/mL concentration of the test samples against fungal strains [*Std= Griseofulvin].

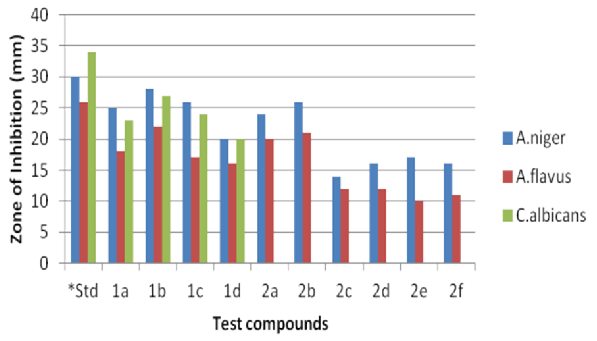


Fig 4: MIC's in µg/mL concentrations of the samples tested against fungal strains [*Std= Griseofulvin].

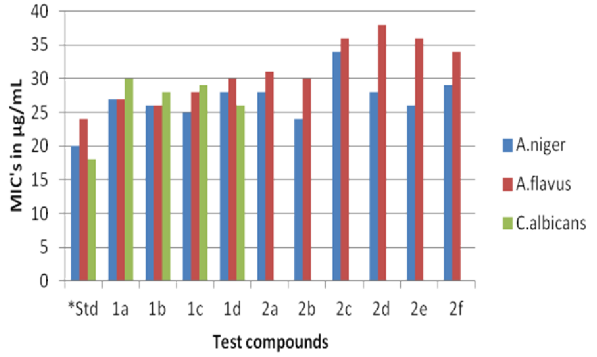


Fig 5: Percentage DPPH radical scavenging ability of samples at different concentrations relative to the standard antioxidant BHT

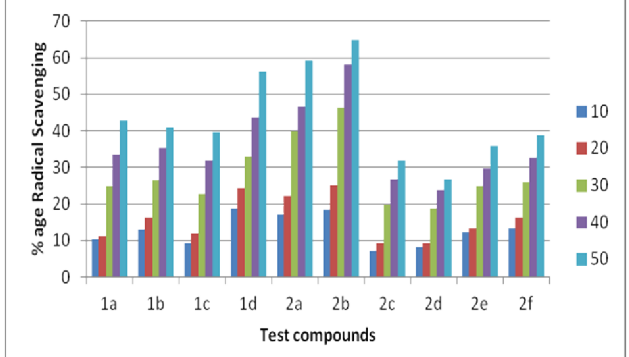
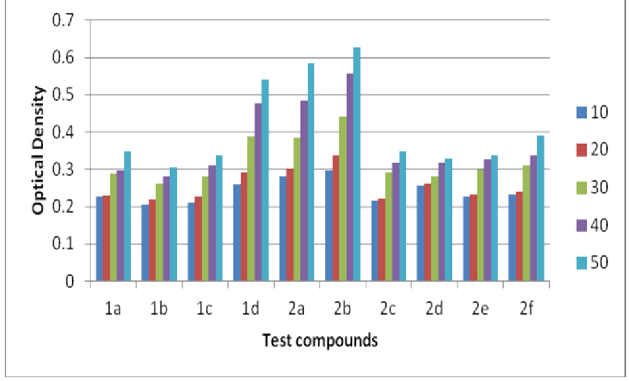


Fig 6: Reducing power ability of the samples at different concentrations measured as absorbance at 700 nm



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 (10-20µg/ml), not much significant variations in the free radical scavenging ability of samples 1a-g were observed. However, when the concentration was increased (30-50µg/ml) all showed a promising radical scavenging ability. The compounds 1a-c showed radical scavenging ability up to 47%, the samples 1d, 2a, 2b showed radical scavenging ability up to 65%; this was expected due to the presence of electronegative and electron with drawing substituents; which facilitates the release of hydrogen atom along with an electron bonded to C_4- and C_5- atom of isoxazole ring. The compounds 2c-f showed lesser radical scavenging ability; this might be due to un-substitution or presence of electron donating substituents in the benzene ring which retards the release of hydrogen atoms. Results indicate that the compounds 1d, 2a and 2b containing electron withdrawing groups on the aromatic ring shows potential electron donating ability.

Reducing power

The test compounds 1a-d, 2a-f 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 (10-20µg/ml), there were not much significant variations in the activity. However, when the concentration was increased (30-50µg/ml), all showed noticeable reducing power ability. The compounds 1d, 2a, 2b containing electronegative atoms and electron withdrawing substituents on the aromatic ring showed higher reducing power. While the compounds 1a-c,

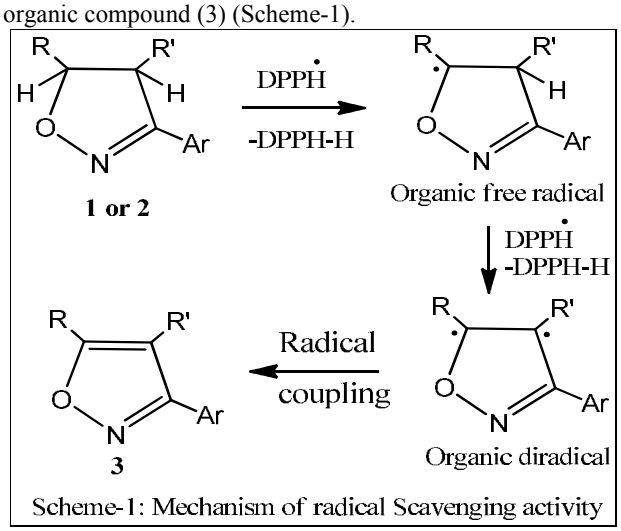
2-c-f showed moderate ability to reduce ferric chloride and potassium ferricyanide complex. The increased absorbance at 700 nm indicated the presence of reducing power ability of the test samples considered for the study.

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 $DPPH^{\cdot} + H^{\cdot} \rightarrow DPPH\text{-}H$.

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 and 4-Acetyl-3-aryl-5-(furan-2-yl)-4,5-dihydroisoxazoles 2 was expected to be the driving force for their antioxidant activity. These non-aromatic compounds have a tendency to become more stable aromatic compounds 3 with the loss of two hydrogen atoms and two electrons.

From the experimental results, the stiochiometry of the reaction was found to be 1:2 for test compounds: DPPH free radical, which suggests that each molecule (1, 2) has a tendency to donate two hydrogen atom and two electrons to the acceptor molecules. In the presence of hydrogen donor organic compound (1, 2) the DPPH free radical abstract the hydrogen atom bonded to C_4 and/or C_5-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_5 and/or C_4-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 (3) (Scheme-1).



Scheme-1: Mechanism of radical Scavenging activity

On the basis of this speculation, the C_4 and/or C_5 positions of the isoxazole ring may be the active site responsible for antioxidant activity of the screened isoxazole derivatives.

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