CHAPTER VIII

ANTIMICROBIAL ACTIVITY

8. 1 INTRODUCTION

The discovery of antimicrobials like penicillin and tetracycline provide the noble way for better health for millions of people around the world. Before its invention in the early 1940's, there was no true cure for gonorrhea, strep throat or pneumonia like diseases. Now, most of these infections can be cured easily with a short course of antimicrobials.

However, the effectiveness of antimicrobial drugs now days available in market are somewhat in doubt in future because microorganisms, especially bacteria, are becoming resistant to more and more antimicrobial agents. It leads to the discovery of new antimicrobial agents. However, microorganisms are becoming resistant more quickly than new drugs are being made available. So, today’s need is to overcome resistance to antimicrobials or to treat infections with alternative means. To fulfill this demand, various compounds are synthesized and screened for its antimicrobial activity. QSAR (Quantitative structure-activity relationship) is one of the best way for the prediction of biological activity of the structure prior to its synthesis and finally to syntheses more active compounds by structure modification of known antimicrobial agents.

In the recent years much attention has been focused on the synthesis of heterocycles containing nitrogen atom because of their biological and medicinal importance including ontological research. They are widely distributed in nature and are essential for life.

Nitrogen Heterocycles play a major part in the biochemical processes in living cells, DNA and RNA containing pyrimidine [cytosine and uracil] and thymine and purine [adenine and guanine ] bases are aromatic heterocycles. Most of enzymes have aromatic heterocycles as major constituents while coenzymes incorporate non-amino acids moieties, most
of them are aromatic nitrogen heterocycles. Some important vitamins are constructed on aromatic heterocyclic scaffold.

Observations of life in nature by primitive communities led humans to the discovery of many healing materials. Majority pharmaceutical products are mimics of natural products with good biological activity which includes many heterocycles. The efforts are being made to synthesize heteroaromatic bioactive molecules which is of through need. The routinely used antibiotics like penicillin and cephalosporin’s, alkaloids such as vinblastine, elliptine, morphine, reserpine and cardiac glucoside such as the class of digitalis are heterocyclic natural products of significance for human being and animal health. Modern life and civilization opened the way to other important practical applications of heterocycles for example dyestuffs, copolymers, solvent extraction, photographic sensitizers, vulcanization accelerators and antioxidants in the rubber industry. Many compounds bearing pyrazoles and their reduced forms pyrazolines constitute an interesting class of heterocycles due to their synthetic versatility and effective biological activities. Pyrazole derivatives have a long history of application in agrochemicals and pharmaceutical industry as herbicides and active pharmaceuticals. The recent success of pyrazole COX-2 inhibitor has further highlighted the importance of these heterocycles in medicinal chemistry. The emergence of drug - resistant pathogenic strains in recent years, e.g. Staphylococcus aureus, Entrococcus faecium, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Salmonella typhi, has been of major concern. Number of pyrazoline derivatives has been found to posses considerable biological activities, which stimulated the research activity in this field. 2-Pyrazolines seem to be the most frequently studied pyrazoline type compounds. Due to its wide range of biological activity, pyrazoles ring constitutes a relevant synthetic route in pharmaceutical industry. In fact, such a heterocyclic moiety represents the core structure for number of drugs. Some of the marketed products of pyrazole nucleus are listed below.
**Phenylbutazone:** Chemically, it is 4-butyl-1,2-diphenylpyrazolidine -3,5-dione. It is known for its analgesic anti-inflammatory and antipyretic actions and finds use for the treatment of rheumatic disorders. Due to its toxicity phenylbutazone(1) should not be employed routinely as an analgesic and antipyretic.

![Phenylbutazone molecule](image)

**Muzolimine:** Muzolimine(2) 1-substituted-2-pyrazolin-5-one derivative is a highly active diuretic, differing from the structures of other diuretics. Since it contains neither a sulfonamide nor a carboxyl group. It has a saluretic effect similar to furosemide and acts in the proximal tubule and in the medullary portion of the ascending limb of the loop of Henle. Pharmacokinetic studies in dogs, healthy volunteers and in patients with renal insufficiency show that the compound is readily absorbed after oral administration.

![Muzolimine molecule](image)

**Forbisen:** Forbisen(3)2,2’,3,3’-tetramethyl-1,1’-diphenyl-4,4’-bi-3,3’-pyrazoline-5,5’-Dione a by-product obtained in the manufacture of antipyrine, has been used in bovine anaplasmosis.
**Oxyphenbutazone:** It is a compound having \( p \)-hydroxyphenyl group instead of phenyl at position 1 of phenylbutazone. Its uses have been found to be similar to these of phenylbutazone. It also finds use in the treatment of inflammation of the eyes. Oxyphenbutazone(4) has been one of the active metabolite of phenylbutazone.

**Sulphinpyrazone:** An analogue of phenylbutazone is sulphinpyrazone(5) which is having a 2-phenylsulphinylethyl group in place of \( n \)-butyl group at position 4. It is having a better therapeutic index as a uricosuric agent; it promotes excretion of uric acid and urate by inhibiting their tubular reabsorption.
**Feprazone:** Structurally, it is similar to phenylbutazone except that the former is having a 3-methylbutenyl substituent at position-4 of pyrazoline-2,5-dione skeleton in place of a butyl substituent. Feprazone (6) also finds use in the treatment of rheumatic disorders.

![Chemical Structure of Feprazone](image)

**Phenazone:** It is a pyrazoline derivative which is chemically 2,3-dimethyl-1-phenyl 3-pyrazolin-5-one. It forms white crystals or white crystalline powder. It is freely soluble in water. Phenazone (7) is well known for its analgesic and antipyretic actions. Topically, it is known for its local anaesthetic and styptic actions and solutions having 5% are used locally as ear drops. Phenazone has been found to affect the metabolism of some other drugs. However, its own metabolism gets affected by drugs that are able to increase or reduce the activity of liver enzymes.

![Chemical Structure of Phenazone](image)

**Propylphenazone:** Propylphenazone (8) is a derivative of phenazone with an isopropyl group attached at position 4. Chemically, it is 4-isopropyl-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one is having analgesic properties.
**Anlagen:** Anlagen(9) is a derivative of phenazone which is having \( N-(CH_{3})CH_{2}SO_{3}Na \) substituent at position-4. It occurs as an almost white crystalline powder having a scarcely perceptible yellowish tinge. It is freely soluble in water and has a bitter taste. The drug is kept in tightly-closed containers. It is known for its analgesic and antipyretic actions.

Based on these observations, a literature work is carried out on heterocyclic compounds particularly therapeutic activity of pyrazoline derivatives and we now report the some pharmacological activity of pyrazoline derivatives.

### 8.2 ANTI-INFLAMMATORY ACTIVITY

**Navidpour L et al** designed and synthesized a new type of 1-aryl-5-(4 methyl sulfonyl phenyl) imidazoles. The compounds are evaluated for selective cyclooxygenase-2(COX-2) inhibitory activity with *in vivo* anti-inflammatory activity. The structures of the compounds have been established on the basis of their elemental analysis and spectral (IR, \(^{1}\text{H}-\text{NMR}, \text{and MS}\) data.  

![Chemical Structure of Anlagen](image-url)
S. Arunkumar, et al\textsuperscript{2}, synthesized a series of pyrazole derivatives. The compounds were evaluated for in vivo anti-inflammatory activity by carrageenan induced paw edema test. In general all compounds were found to exhibit good anti-inflammatory activity.

Sarangan, S et al\textsuperscript{3} have synthesized number of derivatives of pyrazole-(3,4-d) pyrimidine-4-6-diones (17) and reported the screening for C.N.S depression properties and anti-inflammatory activity. It was also reported that some derivatives showed anti-inflammatory properties equivalent to aspirin.
V. H. Bhaskar et al, synthesized a series of pyrazole derivatives and examined for their anti-inflammatory activity. All the compounds exhibited weak to potent anti-inflammatory activity. Some derivatives bearing a methoxy group exhibited very good anti-inflammatory activity.

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Valarmathy et al proposed an efficient synthetic method has been established for the synthesis of new 2-Pyrazoline derivative. The synthesized compounds were evaluated for anti tubercular, anticonvulsant and anti-inflammatory activity. All the compounds showed a significant decrease in inflammation.

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Barsoum et al reported a novel bis (1-acyl-2-pyrazolines) derivatives were synthesized and screened for anti-inflammatory as well as ulcerogenic activities, and found some of the compounds showed remarkable anti-inflammatory properties with low ulcerogenic liability than standard drug used.
8.3 ANTIOXIDANT ACTIVITY

Mondal et al\textsuperscript{7}, Synthesized a series of “novel 3[(substituted phenyl) imino] 1-3(substituted phenyl) 4,4dihydro pyrazol-3-yl] 1- 3 di hydro 2H indole-2-one and reducing power of the synthetic drug was determined by the method of Oyaizu. Substances which have reduction potential reacts with potassium ferricyanide to form potassium ferrocyanide which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700nm. Increase absorbance of the reaction mixture was indicated the increased reducing power Compound exhibit more potent antioxidant activity.

Tatiana Emanuelli et al\textsuperscript{8} synthesized six novel pyrazoline derivatives and the antioxidant capacity of a series of was evaluated as the capacity of compounds to transfer a hydrogen atom (protection against brain lipid peroxidation and glutathione oxidation) and their capacity to transfer a single electron [ferric-reducing antioxidant power (FRAP) and 1,1-
diphenyl-2-picrylhydrazyl radical scavenging (DPPH) assays]. Most of the exhibit highest free radical scavenging capacity in the DPPH assay and the highest FRAP value.

K.Geetha et al\(^9\) synthesized various 2-Aryl pyrazoline derivatives by reflux condensation of Chalcone with Hydrazine hydrate in ethanolic solution. The structures of the synthesized Pyrazoline derivatives have been established on the basis of their spectral data. The synthesized compounds were screened for antioxidant, anti-inflammatory & antimicrobial activities. The antioxidant activity of compounds were evaluated by following methods 1,1-diphenyl-2-picryl hydrazyl free radical scavenging activity, Phosphomolybdenum, scavenging of nitric oxide. Synthesized compounds exhibited significant antioxidant, antimicrobial and anti-inflammatory activities.

Rajeev K. Singla et al\(^{10}\) synthesized various 2-pyrazolines by the condensation of various substituted chalcones and hydrazine hydrate in the presence of ethanol. The structure of the synthesized molecules was confirmed on the basis of physical data and extensive spectral studies. All the 13 compounds have been screened for antioxidant activity using DPPH radical scavenging method, NO scavenging assay, superoxide radical scavenging assay and hydrogen
peroxide radical scavenging assay. The results indicated that 2-pyrazolines could be the potential candidates eliciting antioxidant activity.

Kiran Dasary et al\textsuperscript{11} synthesized a series of substituted pyrazoline derivatives by the reaction of substituted chalcones with isatinhydrazide. The starting materials, chalcones were prepared by clasien schimidt condensation of appropriate 1-hydroxy-2-acetonaphthone with substituted aldehydes in the presence of sodium hydroxide and in poly ethylene glycol (PEG-400). The structures of the synthesized compounds were confirmed by IR, \textsuperscript{1}H-NMR & Mass spectral data. The synthesized compounds were screened for Antioxidant Activity by DPPH method. most of the compounds show good antioxidant activity.
8.4 ANTICANCER ACTIVITY

Irfan Koca et al\textsuperscript{12} synthesized a new series of acyl thiourea derivatives containing pyrazole ring through one pot reaction of 4-benzoyl-1, 5-diphenyl-1H-pyrazole-3- carbonyl chloride with ammonium thiocyanate and various amines. The structures of the newly synthesized compounds were confirmed by IR, \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and elemental analysis. Anticancer activities of synthesized compounds were evaluated on human colon, liver and leukemia cancer cell lines. Cell culture studies have demonstrated significant toxicity of the compounds on the cell lines, and the levels of toxicity have altered in the presence of various side groups. These results confirm that novel pyrazolyl acyl thioureas derived compounds may be utilized for cancer treatment.

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Wei et al\textsuperscript{13} synthesized a series of novel small molecules, ethyl 1-(20-hydroxy-30-aroypropyl)-3-aryl-1H-pyrazole-5-carboxylate derivative which has its potency to suppress A549 lung cancer cell growth.

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Fan et al\textsuperscript{14} synthesized a series of novel 1-(20-hydroxy-30-aroxypropyl)-3-aryl-1H-pyrazole-5-carbohydrazide derivatives could inhibit the growth of A549 cells.

![Chemical structure of Fan et al's compound](image)

Lv et al\textsuperscript{15} developed and synthesized two series of pyrazole derivatives designing for potential EGFR kinase inhibitors as well as antiproliferative activity against MCF-7 with potent inhibitory activity in tumor growth inhibition would be a potential anticancer agent\textsuperscript{25}.

![Chemical structure of Lv et al's compound](image)

**8.5 MICROBIAL ACTIVITY**

Suvarna kini et al\textsuperscript{16} synthesized 1,3,5-trisubstituted-2-pyrazolines by refluxing isoniazid with various substituted diarylchalcones in N,N-dimethylformamide at 120-140°. The physical and spectral data such as M.P., Rf, elemental analysis, IR, NMR and Mass was obtained for the synthesized compounds and the structures were confirmed. The screening of the synthesized compounds for antimicrobial activity was performed against \textit{Staphylococcus aureus}, \textit{Bacillus subtilis}, \textit{Pseudomonas aeruginosa}, \textit{Escherichia coli} and \textit{Aspergillus niger}.

Seham Y. Hassan\textsuperscript{17} reported a series of 2-pyrazoline derivatives bearing benzenesulfonamide moieties by condensing the appropriate chalcones with 4-hydrazinyl benzenesulfonamide hydrochloride. All the newly synthesized compounds have been characterized on the basis of IR
and $^1$H-NMR spectral data as well as physical data. Antimicrobial activity against the organisms
*E. coli* and *P. aeruginosa* as examples of Gram-negative bacteria, *S. aureus* as an example of
Gram-positive bacteria and *C. albicans* as an example of a yeast-like fungus have been studied
using the Nutrient Agar and Sabouraud Dextrose Agar diffusion methods.

**Ahmet Özdemir**\(^{18}\) synthesized a series of pyrazoline derivatives. The chemical structures of the
compounds were elucidated by IR, $^1$H-NMR, $^{13}$C-NMR and FAB+- MS spectral data and
elemental analyses. The synthesized compounds were screened for their antimicrobial activities.
All compounds exhibited the highest antibacterial activity against *P. aeruginosa*.

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**Solanki NS et al**\(^{19}\) synthesized a series of nine pyrazoline compound, synthesized compounds
characterized by IR, $^1$H-NMR. Standard cup-plate technique was done for Antimicrobial activity
using Ciprofloxacin, Fluconazole and Amphotericin B as standard for comparison of Zone of
inhibition for the prepared compound. The results show that synthesized compound possesses
mild to moderate antibacterial activity and antifungal activity.
B. Dipankar et al\textsuperscript{20} reported two varieties of acetophenones were condensed with three varieties of substituted benzimidazole derivatives to get six chalcone derivatives which undergo condensation followed by cyclisation with isoniazid and 1-(2-naphthoxy acetate) hydrazine two get the final 2-pyrazoline derivatives. The synthesized compounds were characterized by IR, \textsuperscript{1}H-NMR and Mass spectral studies. The synthesized compounds were found to have good antimicrobial activity in the range of 20-70 \(\mu\)g/ml.

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N.V Kavitha et al\textsuperscript{21} synthesized pyrazoline derivatives by the condensation of various chalcones with hydrazine hydrate and synthesized pyrazoline derivatives reacted with chloroacetyl chloride and glacial acetic acid respectively. The structures of new compounds were established on the basis of elemental IR and \textsuperscript{1}H-NMR data, the compounds were evaluated for their antibacterial activities. Newly synthesized pyrazoline derivatives do possess considerable Antibacterial activity.

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Kendre M. M et al\textsuperscript{22} reported a new series of acetyl pyrazoline derivatives by conventional method in excellent yields and in less reaction time using ethanol via cyclization reaction of chalcones, hydrazine hydrate and few drops of glacial acetic acid. These newly synthesized compounds were screened for their antimicrobial activities which reflects moderate to good activity against different strains of bacteria and fungi employed. All the synthesized compounds were confirmed by IR, $^1$H-NMR and Mass spectral data.

Pravinkumar M. Patel et al\textsuperscript{23} synthesized the Series of N-(5-(Substituted phenyl)-4,5-dihydro-$1H$-pyrazol-3-yl)-$4H$-1,2,4-triazol-4-amine compounds by reaction of 4- amino-$1H$-1,2,4-Triazole with Acetyl Chloride followed by different aromatic aldehydes and cyclization with hydrazine hydrate. The structures of new compounds were confirmed by IR and $^1$H-NMR spectral data. Anti-bacterial and Anti-fungal activities were evaluated and compared with the standard drugs, some compounds of the series exhibited promising anti-microbial and anti-fungal activity compared to standard drugs.
Baluja S et al\textsuperscript{24} synthesized ten pyrazoline derivatives and their antibacterial activity was studied against four Gram positive \textit{Bacillus cereus}, \textit{Staphylococcus aureus}, \textit{Staphylococcus epidermidis} and \textit{Micrococcus luteus}, and three Gram negative bacteria viz. \textit{Proteus mirabilis}, \textit{Escherichia coli} and \textit{Klebsiella aerogenes} bacteria. The antibacterial activity was done using Agar well diffusion method. The pyrazoline derivatives showed different activity against different bacterial strains depending on their structural formula. Gram positive bacteria were more susceptible than Gram negative bacteria. \textit{E. coli} was the most resistant bacteria and \textit{B. cereus} was the most susceptible bacteria. The pyrazoline derivatives which had nitro group at para position showed best antibacterial activity.

![Pyrazoline Structure]

Nadia T. A. Dawood\textsuperscript{25} synthesized a series of nine 1-(4-aryl-2-thiazolyl)-3,5-diaryl and six of 1-(4-aryl-2-oxazolyl)-3,5-diaryl-2- pyrazoline derivatives by reacting 3,5-diaryl-1-thiocarbamoyl-or 3,5-diaryl-1- carbamoyl-D2-pyrazolines with substituted phenacyl bromide in ethanol. The structures of the synthesized derivatives were confirmed by IR, \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR as well as EIMS spectral data. Some of these derivatives were screened for their antimicrobial activity against Gram-positive, Gram- negative and pathogenic fungi, and showed a significant activity.
Zaman Ashraf et al\textsuperscript{26} synthesized Fourteen new N-acetylated and non-acetylated pyrazoline derivatives by reacting chalcones with hydrazine in the presence of absolute ethanol however reaction was carried out in the presence of glacial acetic acid to afford N-acetylated pyrazolines. The chemical structures of the synthesized pyrazolines were confirmed by FT-IR, \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and mass spectroscopic data. The pyrazolines were screened for antibacterial activity against ten bacterial strains using seven Gram-positive and three Gram-negative bacteria and antifungal activity against \textit{Aspergillus Flavus}, \textit{Aspergillus Niger} and \textit{Aspergillus pterus}. Pyrazolines found to exhibit good to excellent antimicrobial activities compared to the Levofloxacin and fluconazole used as standard drugs.

Navin B. P et al\textsuperscript{27} synthesized a series of pyrazolyl-quinazolin-4(3H)-ones from 2-[2-(phenylamino)phenyl]acetic acid by using efficient methods. An acid chloride of 2-[2-(phenylamino)phenyl]acetic acid on cyclization reaction with 5-iodo anthranilic acid yielded benzoaxazinone , which on condensation reaction with hydrazine hydrate afforded quinazolin-4(3H)-one. Acetylation of synthesized compound and then condensation with aromatic
aldehydes led to the formation of chalcones which on subjected to reaction with phenyl hydrazine yielded the desired compounds. All the synthesized compounds have been characterized by elemental analyses, IR and NMR spectra data. The title compounds have been screened against bacterial as well as fungal microorganisms. The potency of these compounds was calculated and compared with standard drugs, i.e. Penicillin-G and Fluconazole. Some of the compounds showed very good antimicrobial activity.
8.6 ANTIBACTERIAL ACTIVITY

8.6.1 INTRODUCTION

Man is closely influenced by the activities of microorganisms. Microorganisms are a part of our lives in more ways than most of us understand. They have shaped our present environment and their activities will greatly influence our future. Microorganisms should not be considered separate from human beings, but profound beneficial influence as a part of our life. They are employed in the manufacture of dairy products, certain foods, min processing of certain medicines and therapeutic agents, in manufacture of certain chemicals and in numerous other ways. Despite the established useful functions and potentially valuable activities of microorganism, these microscopic doors of life may be best known as agents of food spoilage and causal agents of human beings viz. Acquired immune deficiency syndrome, herpes, legionnaires disease, influenza, jaundice, tuberculosis, typhoid, dermatomycoses, dysentery, malaria etc. In human being, Animals and plants have also been known to be victims of microbial pathogens. So far as is known, all primitive and civilized societies have experienced diseases caused by microbes, frequently with disastrous results. Moreover, microorganisms have played profound roles in warfare, religion and the migration of populations. Control of microbial population is necessary to prevent transmission of disease, infection, decomposition contamination and spoilage caused by them, man’s personal comforts and convenience depend to a large extent on the control of microbial population.

8.6.2 Bacteria

In 1928, a German scientist C.E. Chrenberg first used the term “bacterium” to denote small microscopic organism with a relatively simple and primitive form of the cellular organization known as “prokaryotic”. Danish physician, Gram in peculiarly, bacteria are generally unicellular e.g. cocci, bacilli, etc filamentous, eg. actinomycetes, some being sheathed having certain cells specialized for reproduction. The microorganisms are capable of producing
diseases in host are known as ‘pathogenic’. Most of the microorganisms present on the skin and mucous membrane are non pathogenic and are often referred to as “commensals” or if they live on food residues as in intestine, they may be called “saprophytes”. Generally, the pathogenic cocci and bacilli are Gram positive and the pathogenic coco bacilli are Gram negative.

The following Gram-positive and Gram-negative strains have been used for the study.

1. Bacillus subtilis (Gram-positive)
2. Streptococcus pyogenes (Gram-positive)
3. Escherichia coli (Gram-negative)
4. Pseudomonas aeruginosa (Gram-negative)
5. Staphylococcus aureus (Gram-positive)

8.6.2.1 Bacillus subtilis

*Bacillus subtilis*, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium. A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct.

Although this species is commonly found in soil, more evidence suggests that *B. subtilis* is a normal gut commensal in humans. A 2009 study compared the density of spores found in soil (10^6 spores per gram) to that found in human feces (10^4 spores per gram). The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination. Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal.
They can contaminate food; however, they seldom result in food poisoning. They are used on plants as a fungicide. They are also used on agricultural seeds, such as vegetable and soybean seeds, as a fungicide. The bacteria, colonized on root systems, compete with disease causing fungal organisms. *Bacillus subtilis* use as a fungicide fortunately does not affect humans. Some strains of *Bacillus subtilis* cause rots in potatoes. It grows in food that is non-acidic, and can cause ropiness in bread that is spoiled (Todar).

**8.6.2.2 Escherichia coli**

*Escherichia coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K$_2$ and by preventing the establishment of pathogenic bacteria within the intestine.

*E. coli* and related bacteria constitute about 0.1% of gut flora, and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination. There is, however, a growing body of research that has examined environmentally persistent *E. coli* which can survive for extended periods outside of the host.

Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia.

UPEC (uropathogenic *E. coli*) is one of the main causes of urinary tract infections. It is part of the normal flora in the gut and can be introduced many ways. In particular for females,
the direction of wiping after defecation (wiping back to front) can lead to fecal contamination of the urogenital orifices. Anal sex can also introduce these bacteria into the male urethra, and in switching from anal to vaginal intercourse the male can also introduce UPEC to the female urogenital system.

8.6.2.3 Pseudomonas aeruginosa

*Pseudomonas aeruginosa* is a common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments. It uses a wide range of organic material for food in animals, the versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal. Because it thrives on most surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tarballs and oil from oil spills.

An opportunistic, nosocomial pathogen of immune compromised individuals, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections.

It is the most common cause of infections of burn injuries and of the outer ear, and is the most frequent colonizer of medical devices (e.g., catheters). *Pseudomonas* can, in rare circumstances, cause community-acquired pneumonias as well as ventilator associated pneumonias, being one of the most common agents isolated in several studies.
8.6.2.4 Staphylococcus aureus

*Staphylococcus aureus* is a bacterium that is a member of the Firmicutes, and is frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. Disease-associated strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g. MRSA) is a worldwide problem in clinical medicine.

*Staphylococcus* was first identified in Aberdeen, Scotland (1880) by the surgeon Sir Alexander Ogston in pus from a surgical abscess in a knee joint. This name was later appended to *Staphylococcus aureus* by Rosenbach who was credited by the official system of nomenclature at the time. It is estimated that 20 % of the human population are long-term carriers of *S. aureus* which can be found as part of the normal skin flora and in anterior nares of the nasal passages. *S. aureus* is the most common species of staphylococcus to cause *Staph* infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies. *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections. Each year, some 500,000 patients in American hospitals contract a staphylococcal infection.
8.6.2.5 *Streptococcus pyogenes*

*Streptococcus pyogenes* is a spherical, Gram-positive bacterium that is the cause of group A streptococcal infections. *S. pyogenes* displays streptococcal group A antigen on its cell wall. *S. pyogenes* typically produces large zones of beta-hemolysis (the complete disruption of erythrocytes and the release of hemoglobin) when cultured on blood agar plates, and are therefore also called Group A (beta-hemolytic) *Streptococcus*.

Streptococci are catalase-negative. In ideal conditions, *S. pyogenes* has an incubation period of approximately 1–3 days. It is an infrequent, but usually pathogenic, part of the skin flora. It is estimated that there are more than 700 million infections worldwide each year and over 650,000 cases of severe, invasive infections that have a mortality rate of 25%. Early recognition and treatment are critical; diagnostic failure can result in sepsis and death.

*S. pyogenes* is the cause of many important human diseases, ranging from mild superficial skin infections to life-threatening systemic diseases. Infections typically begin in the throat or skin. Examples of mild *S. pyogenes* infections include pharyngitis ("strep throat") and localized skin infection. Erysipelas and cellulitis are characterized by multiplication and lateral spread of *S. pyogenes* in deep layers of the skin. *S. pyogenes* invasion and multiplication in the fascia can lead to necrotizing fasciitis, a potentially life-threatening condition requiring surgical treatment.

Infections due to certain strains of *S. pyogenes* can be associated with the release of bacterial toxins. Throat infections associated with release of certain toxins lead to scarlet fever. Other toxigenic *S. pyogenes* infections may lead to streptococcal toxic shock syndrome, which can be life-threatening.

*S. pyogenes* can also cause disease in the form of postinfectious "nonpyogenic" (not associated with local bacterial multiplication and pus formation) syndromes. These autoimmune-
mediated complications follow a small percentage of infections and include rheumatic fever and acute postinfectious glomerulonephritis. Both conditions appear several weeks following the initial streptococcal infection. Rheumatic fever is characterised by inflammation of the joints and/or heart following an episode of streptococcal pharyngitis. Acute glomerulonephritis, inflammation of the renal glomerulus, can follow streptococcal pharyngitis or skin infection.

This bacterium remains acutely sensitive to penicillin. Failure of treatment with penicillin is generally attributed to other local commensal organisms producing β-lactamase, or failure to achieve adequate tissue levels in the pharynx. Certain strains have developed resistance to macrolides, tetracyclines, and clindamycin.

8.6.3 MATERIALS AND METHODS

The synthesized pyrazoline derivatives were screened for the antibacterial activity against three Gram-positive bacteria viz., *Streptococcus pyogenes*, *Bacillus subtilis* and *Staphylococcus aureus* and two Gram-negative bacteria viz., *Escherichia coli* and *Pseudomonas aeruginosa* by using the agar well diffusion method. Streptomycin was used as reference standard for comparing the results.

8.6.3.1 Culture medium:

Nutrient broth was used for the preparation of inoculum of the bacteria and nutrient agar was used for the screening method.

8.6.3.2 Composition of Nutrient agar medium:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5 gm</td>
</tr>
</tbody>
</table>
Yeast extracts  1.5 gm
Agar  15.0 gm
Distilled water  1000 ml
pH  7.4± 0.2

8.6.4 PROCEDURE

8.6.4.1 Determination of antibacterial activity by agar well diffusion method

The test organisms were subcultured using nutrient agar medium. The tubes containing sterilized medium were inoculated with the respective bacterial strain. After incubation at 37°C ±1°C for 18 hours, they were stored in a refrigerator. The nutrient agar medium was sterilized by autoclaving at 121°C for 15 min. The petriplates, tubes and flasks plugged with cotton were sterilized in hot-air oven at 160 °C, for an hour. Into each sterilized petriplate (20 cm diameter), was poured about 125 ml of molten nutrient agar medium which was already inoculated with the respective strain of bacteria (5 ml of inoculum to 250 ml of nutrient agar medium) aseptically. The plates were left at room temperature aseptically to allow the solidification. Once the agar was solidified, it was punched with a six millimeters diameter wells and filled with sample and blanks . The test was carried out by triplicate. Each test compound (5 mg) was dissolved in dimethyl sulfoxide (5 ml Analar grade) to give a concentration of 1000 µg/ml. Streptomycin solution was also prepared to give a concentration of 1000 µg/ml in sterilized distilled water. The pH of all the test solutions and control was maintained in between 2 to 3 by using conc.HCl. All the compounds were tested at dose levels of 1000 µg and DMSO used as a control. The solutions of each test compound, control and reference standard were added separately in the cups and the plates were kept undisturbed for at least 2 hours in a refrigerator to allow diffusion of the solution properly into nutrient agar medium. Petri dishes were subsequently incubated at 37±1 °C for 24 hours. After incubation, the diameter of zone of inhibition surrounding each of the cups was measured with the help of an antibiotic zone reader. The results are presented in Table 67.
Figure 4: Antibacterial activities by zone of inhibition of pyrazoline derivatives (P₁-P₅)
Figure 5: Antibacterial activities by zone of inhibition of pyrazoline derivatives (P$_6$-P$_{10}$)
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Bacteria</th>
<th>Streptomycin (standard)</th>
<th>Zone of inhibition( mm )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P₁</td>
</tr>
<tr>
<td>1</td>
<td>Bacillus subtilis</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Escherichia coli</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas aeruginosa</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Staphylococcus aureus</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Streptococcus pyogenes</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Chart 1: Antibacterial activities by zone of inhibition of pyrazoline derivatives \( (P_1-P_{10}) \)
8.6.5 RESULTS AND DISCUSSION

The synthesized pyrazoline derivatives were screened for the antibacterial activity against three Gram-positive bacteria viz., *Streptococcus pyogenes*, *Bacillus subtilis* and *Staphylococcus aureus* and two Gram-negative bacteria viz., *Escherichia coli* and *Pseudomonas aeruginosa* by using the agar well diffusion method. Streptomycin was used as reference standard for comparing the results. The antibacterial activity of the pyrazoline derivatives are shown in Fig: 4 & 5 for Plates 1 - 10, and the zone of inhibition values are given Table-67. The Clustered column Chart 1 showed that pyrazoline derivatives of (P1) to (P10) possess significant activity almost equipotent with the standard Streptomycin against both Gram +ve and Gram –ve pathogenic organism. Thus the substituents place a vital role in imparting enhanced antibacterial activity to the compounds. The screening results indicate that compounds (P6), (P8), (P9) and (P10) were found to be active against *S. aureus*. Compounds (P1), (P2) and (P3) were found to be moderately active be active against *S. aureus*, whereas compounds (P3), (P4) and (P7) were found to be inactive be active against *S. aureus*. Compounds (P4), (P6), (P7) and (P9) were found to be active against *B. subtilis*. Compounds (P8) and (P10) were found to be moderately active against *B. subtilis*. where as compound (P1) and (P2), (P3) and (P5) was found to be inactive against *B. subtilis*. Compound (P6) and (P10) was found to active against *E. coli*. Compounds (P4), (P5) and (P8) were found to be moderately active against *E. coli*, all other compounds were found to be inactive against *E. coli*. Compound (P6) was found to be active against S.pyogenes. Compounds (P4), (P7) and (P9) were found to be moderately active against S.pyogenes.where as (P1), (P2), (P3), (P5) and (P9) were found to be less active against S.pyogenes. Compounds (P9) and (P10) were found to be active against *P. aeruginosa*. Compounds (P4), (P5), (P6), (P7) and (P8) were found to moderately active be active against *P.aeruginosa*. whereas compounds (P1) and (P2) were found to be less active be active against *P.aeruginosa*. 
8.7 ANTIFUNGAL ACTIVITY

8.7.1 INTRODUCTION

It has been estimated that the life expectancy of humans has increased by at least 10 years since the discovery of antimicrobial agents for the treatment of microbial infections. A consequence of our success with antimicrobial agents and improved medical care is the number of fungal infections. The incidence of fungal infections has increased dramatically in the past 20 years partly because of the increase in the number of people whose immune systems are compromised by weather, aids, aging, organ transplantation or cancer therapy.

Accordingly, the increase in rates of morbidity and mortality because of fungal infections has been now recognized as a major problem. In response to the increased incidence of fungal infections, the pharmaceutical industry has developed a number of newer less toxic antifungal for clinical use. The increased use of antifungal, often for prolonged periods, has lead to recognition of the phenomenon of acquired antifungal resistance to one or more of the available antifungal. Fungi are nonphotosynthetic eukaryotes growing either as colonies of single cells (yeasts) or as filamentous multicellular aggregate [molds]. Most fungi live as saprophytes in soil or on dead plant material and are important in the mineralization of organic matter. A smaller number produce disease in human and animals. The in vitro methods used for detections of antifungal potency are similar to those used in antibacterial screening. As with bacteria, it is easy to discover several synthetic and natural compounds that, in small quantity, can retard or prevent growth of fungi in culture media.

The following fungal strains were used for the study

1. Aspergillus flavus

2. Aspergillus niger

3. Penicillium chryogenum
4. Trigoderma veride

5. Fusarium oxysporum

Sabouraud’s dextrose agar (SDA) medium was used for the growth of fungi and testing was done in sabouraud’s dextrose broth (SDB) medium.

8.7.1.1 *Aspergillus flavus*

*Aspergillus flavus* is a fungal pathogen, which causes post-harvest disease in cereal grains and legumes. Post-harvest rot typically develops during harvest, storage, and/or transit. *A. flavus* infections can occur while hosts are still in the field (pre-harvest), but often show no symptoms (dormancy) until post-harvest storage and/or transport. In addition to causing pre-harvest and post-harvest infections, many strains produce significant quantities of toxic compounds known as mycotoxins, which when consumed are toxic to mammals. *A. flavus* is also an opportunistic human and animal pathogen causing aspergillosis in immune compromised individuals.

The amount of aflatoxins produced by *A. flavus* are affected by environmental factors. If other competitive fungal organisms are present on host plants, aflatoxin production is low. However, if non-competitive fungal organisms are present on host plants, aflatoxin production can be quite high. The nature of the host is also an important factor in aflatoxin production. High *A. flavus* growth on soybean produces very little aflatoxin concentrations. High *A. flavus* growth aided by increased moisture content and warm temperatures on peanut, nutmeg, and peppers produces high concentrations of aflatoxins. *A. flavus* growth on spices produce low concentrations of aflatoxin as long as the spices remain dry.

Species sensitivity is highly variable when exposed to aflatoxins. Rainbow trout are highly sensitive at 20 parts-per billion, causing a liver tumor development in half the population. White rats develop liver cancer when exposed to 15 parts-per billion. Young piglets, ducklings, and turkeys exposed to high dosages of aflatoxin become sick and die. Pregnant cows, mature
pigs, cattle, and sheep exposed to low dosages of aflatoxin over long periods develop weakening, intestinal bleeding, debilitation, reduced growth, nausea, no appetite, and predisposition to other infections.

There are four major aflatoxins produced: B1, B2, G1, and G2. The production of the major toxins are a result of particular strains of *A. flavus*. Aflatoxin B1 is the most toxic and potent hepatocarcinogenic natural compound characterized. *A. flavus* also produces other toxic compounds including sterigmatocystin, cyclopiazonic acid, kojic acid, β-nitropropionic acid, aspertoxin, aflatrem, gliotoxin, and aspergillilic acid.

In humans, *A. flavus* aflatoxin production can lead to acute hepatitis, immunosuppression, hepatocellular carcinoma, and neutropenia. It is highly possible for *A. flavus* to invade arteries of the lung or brain and cause infarction. The absence of any regulation of screening for the fungus in countries that also have a high prevalence of viral hepatitis highly increases the risk of hepatocellular carcinoma. After *Aspergillus fumigatus*, *A. flavus* is the second leading cause of aspergillosis. Primary infection is caused by the inhalation of spores; bigger spores have a better chance of settling in the upper respiratory tract. The deposition of certain spore sizes could be a leading factor of why *A. flavus* is a common etiological cause of fungal sinusitis and cutaneous infections and non invasive fungal pneumonia. Countries with dry weather like Saudi Arabia, Sudan, and Africa are more prone to aspergillosis. Two allergens have been characterized in *A. flavus*: Asp fl 13 and Asp fl 18. In tropical and warm climates, *A. flavus* has been shown to cause keratitis in approximately 80 percent of infections.

8.7.1.2 *Aspergillus niger*

*Aspergillus niger* or *A. niger* is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is
commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mould").

Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins, but other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests some true *A. niger* strains do produce ochratoxin A. It also produces the isoflavone orobol.

*A. niger* causes black mold of onions. Infection of onion seedlings by *A. niger* can become systemic, manifesting only when conditions are conducive. *A. niger* causes a common postharvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in peanuts and in grapes.

*A. niger* is less likely to cause human disease than some other *Aspergillus* species, but, if large amounts of spores are inhaled, *A. niger* can be deadly. This is due to a serious lung disease, aspergillosis, that can occur. Aspergillosis is, in particular, frequent among horticultural workers that inhale peat dust, which can be rich in *Aspergillus* spores. It has been found on the walls of ancient Egyptian tombs and can be inhaled when the area is disturbed. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane.

**8.7.1.3 *Penicillium chrysogenum***

*Penicillium chrysogenum* is a fungus, common in temperate and subtropical regions and can be found on salted food products, but it is mostly found in indoor environments, especially in damp or waterdamaged buildings. It was previously known as *Penicillium notatum*. It has rarely been reported as a cause of human disease. It is the source of several β-lactam antibiotics, most significantly penicillin. Other secondary metabolites of *P. chrysogenum* include various
penicillins, roquefortine C, meleagrin, chrysogine, xanthocillins, secalonic acids, sorrentanone, sorbicillin, and PR-toxin.

Like the many other species of the genus *Penicillium*, *P. chrysogenum* usually reproduces by forming dry chains of spores (or conidia) from brush-shaped conidiophores. The conidia are typically carried by air currents to new colonisation sites. In *P. chrysogenum* the conidia are blue to blue-green, and the mold sometimes exudes a yellow pigment. However, *P. chrysogenum* cannot be identified based on colour alone. Observations of morphology and microscopic features are needed to confirm its identity and DNA sequencing is essential to distinguish it from closely related species such as *Penicillium rubens*. The sexual stage of *P. chrysogenum* was discovered in 2013 by mating cultures in the dark on oatmeal agar supplemented with biotin, after the mating types (MAT1-1 or MAT1-2) of the strains had been determined using PCR amplification.

**8.7.1.4 Trichoderma viride**

*Trichoderma viride* is a fungus and a biofungicide. It is used for seed and soil treatment for suppression of various diseases caused by fungal pathogens. It is also a pathogen in its own right, causing green mould rot of onion.

*T. viride* is a mold which produces spores asexually, by mitosis. It is the anamorph of *Hypocrea rufa*, its teleomorph, which is the sexual reproductive stage of the fungus and produces a typical fungal fruiting body. The mycelium of *T. viride* can produce a variety of enzymes, including cellulases and chitinases which can degrade cellulose and chitin respectively. The mould can grow directly on wood, which is mostly composed of cellulose, and on fungi, the cell walls of which are mainly composed of chitin. It parasitizes the mycelia and fruiting bodies of other fungi, including cultivated mushrooms, and it has been called the "green mould disease of mushrooms". The affected mushrooms are distorted and unattractive in appearance and the crop is reduced.
8.7.1.5 **Fusarium oxysporum**

The ascomycete fungus *Fusarium oxysporum* Schlecht. *F. oxysporum* strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes and degrade lignin and complex carbohydrates associated with soil debris. They are also pervasive plant endophytes that can colonize plant roots and may even protect plants or be the basis of disease suppression. Although the predominant role of these fungi in native soils may be as harmless or even beneficial plant endophytes or soil saprophytes, many strains within the *F. oxysporum* complex are pathogenic to plants, especially in agricultural settings.

Pathogenic strains of *F. oxysporum* have been studied for more than 100 years. The host range of these fungi is extremely broad, and includes animals, ranging from arthropods to humans, as well as plants, including a range of both gymnosperms and angiosperms. While collectively, plant pathogenic *F. oxysporum* strains have a broad host range; individual isolates usually cause disease only on a narrow range of plant species.

Healthy plants can become infected by *F. oxysporum* if the soil in which they are growing is contaminated with the fungus. The fungus can invade a plant either with its sporangial germ tube or mycelium by invading the plant's roots. The roots can be infected directly through the root tips, through wounds in the roots, or at the formation point of lateral roots. Once inside the plant, the mycelium grows through the root cortex intercellulary. When the mycelium reaches the xylem, it invades the vessels through the xylem's pits. At this point, the mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant. As it grows, the mycelium branches and produces microconidia, which are carried upward within the vessel by way of the plant's sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel. The fungus can also advance laterally as the mycelium penetrates the adjacent xylem vessels through the xylem.
Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaves' stomata to close, the leaves wilt, and the plant eventually dies. It is at this point that the fungus invades the plant's parenchymatous tissue, until it finally reaches the surface of the dead tissue, where it sporulates abundantly. The resulting spores can then be used as new inoculum for further spread of the fungus.

*F. oxysporum* is primarily spread over short distances by irrigation water and contaminated farm equipment. The fungus can also be spread over long distances either in infected transplants or in soil. Although the fungus can sometimes infect the fruit and contaminate its seed, the spread of the fungus by way of the seed is very rare. It is also possible that the spores are spread by wind.

### 8.7.2 Materials and methods

All those compounds screened earlier for antibacterial activity were also tested for their antifungal activity. The fungi employed for the screening were *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Trichoderma viride* and *Fusarium oxysporum*. Amphotericin -B was employed as standard to compare the results. The test organisms were sub-cultured using Potato-Dextrose-Agar (PDA) medium\textsuperscript{28-30}. The tubes containing sterilized medium were inoculated with test fungi and kept at room temperature for obtaining growth. After that, they were stored at 4 ºC in a refrigerator.

#### 8.7.2.1 Composition of Potato-Dextrose-Agar medium:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peeled potato</td>
<td>50.0 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>4.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>upto 200 ml</td>
</tr>
</tbody>
</table>
The test organisms were subcultured using PDA medium. The tubes containing sterilized medium were inoculated with respective fungal strain and kept aside at room temperature for growing the organism. After confirming the growth, they were stored in a refrigerator. The inoculum was prepared by aseptically transferring 10 ml of sterile water into freshly sub-cultured slants of the test fungi and making a suspension by scraping the growth with an inoculation medium.

The PDA medium was sterilized by autoclaving at 121 ºC for 15 min. The petri plates, tubes and flasks plugged with cotton, were sterilized in hot-air oven at 160 ºC, for an hour. Into each sterilized petri plate (20 cm diameter), poured about 125 ml of molten PDA medium which was already inoculated with the respective strain of fungi (5 ml of inoculum to 250 ml of nutrient agar medium) aseptically. The plates were left at room temperature aseptically to allow the solidification. After solidification, the cups of each of 7 mm diameter were made by scooping out medium with a sterilized cork borer from a petridish and labeled accordingly.

Each test compound (5 mg) was dissolved in dimethyl sulfoxide (5 ml, Analar grade) to give a concentration of 1000 µg/ml. Amphotericin -B solution was also prepared at a concentration of 1000 µg/ml in sterilized distilled water. The pH of all the test solutions and control was maintained at 2 to 3 by using conc. HCl. All the compounds were tested at dose levels of 200 µg (0.2 ml) and DMSO used as a control. The solutions of each test compound, control and reference standards were added separately in the cups and the plates were kept undisturbed for at least 2 hours in a refrigerator to allow diffusion of the solution properly into the PDA medium. Petri dishes were subsequently kept at room temperature for 48 hours. After that, the diameter of zone of inhibition in mm surrounding each of the cups was measured with the help of an antibiotic zone reader. The results are presented in Table 68.
Figure 6: Antifungal activities by zone of inhibition of pyrazoline derivatives (P₁-P₅)
Figure 7: Antifungal activities by zone of inhibition of pyrazoline derivatives (P6-P10)
Table 68: The antifungal activities of pyrazoline derivatives by disc diffusion method (P₁-P₁₀)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fungi</th>
<th>Amphotericin -B</th>
<th>Zone of inhibition in diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P₁</td>
</tr>
<tr>
<td>1</td>
<td><em>Aspergillus flavus</em></td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td><em>Aspergillus niger</em></td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td><em>Penicillium chrysogenum</em></td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>Trichoderma viride</em></td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td><em>Fusarium oxysporum</em></td>
<td>24</td>
<td>08</td>
</tr>
</tbody>
</table>
Chart 2: Antifungal activities by zone of inhibition of pyrazoline derivatives (P1-P10)
8.7.3 RESULTS AND DISCUSSION

A agar well diffusion method was employed for the in-vitro study of antifungal effects against Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum, Trichoderma viride and Fusarium oxysporum. The results of this evaluation were compared with Amphotericin –B as reference standard. The antifungal activity of the pyrazoline derivatives are shown in Fig: 6 & 7 for Plates 16 - 25, and the zone of inhibition values are given Table-68. The Clustered column Chart 2 Showed that pyrazoline derivatives of (P1) to (P10) posses significant activity almost equipotent with the standard Amphotericin –B. Thus the substituents place a vital role in imparting enhanced antifungal activity to the compounds. The screening results indicate that compounds (P6), (P7) and (P8) were found to be active against Aspergillus flavus. Compounds (P1), (P4), (P5) and (P9) were found to moderately active be active against Aspergillus flavus, whereas all other compounds were found to be inactive against Aspergillus flavus. Compounds (P1), (P2), (P5) and (P8) were found to be active against Aspergillus niger. Compounds (P3), (P4) and (P9) were found to be moderately active against Aspergillus niger, whereas all other compounds were found to be less active against Aspergillus niger. Compound (P3), (P6), (P7) and (P8) were found to active against Penicillium chrysogenum. Compounds (P5) and (P9) were found to be moderately active against Penicillium chrysogenum. Compound (P6), (P8) and (P9) was found to be active against Trichoderma viride. Compounds (P2), (P3) and (P5) were found to be moderately active against Trichoderma viride. Compound (P4), (P7) and (P10) was found to be active against Fusarium oxysporum. Compounds (P2), (P5) and (P6) were found to be moderately active against Fusarium oxysporum.
REFERENCE


12. Irfan Koca et al., Bioorg Med Chem., 21(13) 2013 3859


17. Y. Seham Hassan, Molecules. 18(3) (2013) 2683.


27. B.P. Navin et al., Orbital., 2 (2010)


8.8 ANTIOXIDANT ACTIVITY

8.8.1 INTRODUCTION

A free-radical is simply defined as any species capable of independent existence that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. They are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body’s normal use of oxygen such as respiration and some cell mediated immune functions. Also free radicals are generated through certain environmental pollutants. However continuous interaction of the animal physiological systems with these free radicals generated either indigenously or inhaled/ingested from exogenous sources therefore, lead to excess load of free radicals and cause cumulative damage of protein, lipid, DNA, carbohydrates and membrane, resulting in so-called oxidative stress\(^1\).

Therefore, living creatures have evolved a highly complicated defense system with antioxidants composed of enzymes and vitamins against oxidative stress in the course of their evolution. These defense systems are mainly classified as (i) suppression of generation of ROS, (ii) scavenging of ROS, (iii) clearance, repairing and reconstitution of damage and (iv) induction of antioxidant proteins and enzymes\(^2\). However, amounts of these protective devices present under normal physiological conditions are sufficient only to cope with the normal threshold of physiological rate of free-radical generation. Therefore, any additional burden of free radicals, either from an indigenous or exogenous source on the animal (human) physiological system can tip free radical (prooxidant) and anti-free radical (antioxidant) balance leading to oxidative stress\(^3\). The role of free radicals and reactive oxygen species (ROS) in the pathogenesis of human diseases such as cancer, aging, inflammatory response syndrome, respiratory diseases, liver diseases, and atherosclerosis, has been widely recognized\(^4\). The pyrazoline nucleus is a ubiquitous feature of
various compounds possessing many pharmacological and physiological activities and therefore they are useful materials in drug research. It was reported in the literature that different substituted pyrazolines possess antimicrobial, anti-inflammatory, analgesic, antipyretic, antidepressant, antitubercular, antiamoebic, anthelmintic, anticonvulsant, antihypertensive, antidiabetic, antitumor, anti-HIV, local anaesthetic, antioxidant, insecticidal and tranquilizing activities. Given below is an account of various modifications reported on pyrazoline nucleus, which showed a variety of biological and pharmacological activities. Pasin et al$^5$ synthesized a series of pyrazole derivatives and screened for their antioxidant activity. All compound showing good activity. Umesha et al$^6$ Synthesized of 5-methyl-2- (5- methyl- 1,3-diphenyl-1H-pyrazole-4- carbonyl)-2, 4- dihydro-pyrazol-3-one and evaluation of their antioxidant activity. Babu et al$^7$ synthesized a series of pyrazoline derivatives and evaluated antioxidant activity at various concentrations against standard drug ascorbic acid. Six of the synthesized compounds showed interesting antioxidant activity as compared with ascorbic acid.

8.9 Screening for Antioxidant activity by DPPH Method

DPPH is a common abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. The 1,1-diphenyl-2-picrylhydrazyl radical has been widely used to evaluate the free radical scavenging capacity of different antioxidants$^8$-$^10$. It is a crystalline powder composed of stable free-radical molecules. DPPH has two major applications, both in laboratory research: one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay.

8.9.1 Principle:

The scavenging reaction between DPPH and antioxidant (H-A) can be written as:

$$(\text{DPPH}) + (\text{H-A}) \rightarrow \text{DPPH-H} + (\text{A})$$

(Purple) \hspace{1cm} (Yellow)
Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPHH and as consequence the absorbance’s decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

8.9.2 Materials required:

1. Methanolic solution of DPPH (0.1 mM): 39.4 mg of DPPH was dissolved in one liter of analytical grade methanol.

2. Test sample

8.9.3 PROCEDURE

0.1mM solution of DPPH in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of test solution in methanol at different concentration (1-16 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. A blank was prepared without adding sample. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity (expressed as % inhibition). The capability to scavenge the DPPH radical was calculated using the following equation:

The formula used for %inhibition is as follows:

\[
% \text{ inhibition} = \left( \frac{\text{Blank OD} - \text{Sample OD}}{\text{Blank OD}} \right) \times 100
\]

Control is the absorbance of the methanol in DPPH alone.

Test means the absorbance in the presence of sample.
### Table - 69

**Antioxidant activity of pyrazoline derivatives**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compounds</th>
<th>Antioxidant activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>DPPH</strong></td>
</tr>
<tr>
<td>1</td>
<td>Ascorbic acid</td>
<td>97.67±0.58</td>
</tr>
<tr>
<td>2</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>51.00±1.00</td>
</tr>
<tr>
<td>3</td>
<td>P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>46.67±2.52</td>
</tr>
<tr>
<td>4</td>
<td>P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>57.00±2.65</td>
</tr>
<tr>
<td>5</td>
<td>P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>65.00±2.65</td>
</tr>
<tr>
<td>6</td>
<td>P&lt;sub&gt;5&lt;/sub&gt;</td>
<td>89.67±2.08</td>
</tr>
<tr>
<td>7</td>
<td>P&lt;sub&gt;6&lt;/sub&gt;</td>
<td>61.33±2.52</td>
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<tr>
<td>8</td>
<td>P&lt;sub&gt;7&lt;/sub&gt;</td>
<td>70.67±1.15</td>
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<tr>
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<td>43.00±1.73</td>
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<tr>
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<td>53.33±1.53</td>
</tr>
<tr>
<td>11</td>
<td>P&lt;sub&gt;10&lt;/sub&gt;</td>
<td>50.00±1.00</td>
</tr>
</tbody>
</table>

**Statistical analysis:** All the experiments were carried out in triplicates (n=3) and the results are expressed as mean of the three determinations.
Antioxidant activities of pyrazoline derivatives (P₁-P₁₀)

Chart 3: Screening results of DPPH radical scavenging activity of pyrazoline derivatives (P₁-P₁₀)
8.10 RESULTS AND DISCUSSIONS

All the synthesized compounds ($P_1$) to ($P_{10}$) were evaluated for their in-vitro Antioxidant activity by DPPH method. The result of this study is collected in Table 69. The following observations were made within the series, Compounds ($P_5$), ($P_7$), ($P_4$) and ($P_6$) showed maximum oxygen scavenging activity which is comparable to ascorbic acid. Compounds ($P_3$) and ($P_9$) exhibited moderate oxygen scavenging activity as compared to ascorbic acid, where as all other compounds were exhibited minimum antioxidant activity. However none of the compounds exhibited greater activity with respect to standard ascorbic acid.
REFERENCE


