6. IN-VITRO BIOLOGICAL STUDIES

6.1 ACUTE TOXICITY

Acute toxicity study was performed according to Organization for Economic Co-operation and Development OECD-423 guidelines. Albino rats of either three were used for study. The animals were fasted for 4 h, but were allowed free access to water ad libitum throughout. The animals were divided into 13 groups containing six animals each. Samples were dissolved in distilled water and administered orally as a single dose to mice at different dose levels viz. 500, 750, 1000, 1250, 1500 and 2000 mg/kg of body weight (b.w.). Mice were observed periodically for the symptoms of toxicity and death within 24 hours and then daily for next 14 days. All the chalcones and pyrazoline compounds employed in the pharmacological screening have been found to be free from toxicity as well as toxic symptoms even at a high dose of 1000 mg/kg body weight and hence they were considered safe.

6.2 ANTIBACTERIAL ACTIVITY

6.2.1 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 dorms 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.

6.2.2 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 peculiarity, 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)
6.2.2.1 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).

6.2.2.2 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 K₂ 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.

**6.2.2.3 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.

6.2.2.4 *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 join. 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. \textit{S. aureus} is the most common species of staphylococcus to cause \textit{Staph} infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies. \textit{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.

\textbf{6.2.2.5 \textit{Streptococcus pyogenes}}

\textit{Streptococcus pyogenes} is a spherical, Gram-positive bacterium that is the cause of group A streptococcal infections. \textit{S. pyogenes} displays streptococcal group A antigen on its cell wall. \textit{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) \textit{Streptococcus}.

Streptococci are catalase-negative. In ideal conditions, \textit{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.

\textit{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.

### 6.2.3 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 cup plate method \cite{146-148}. Streptomycin was used as reference standard for comparing the results.

### 6.2.3.1 Culture medium:

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

**Composition of Nutrient agar medium:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</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>
<tr>
<td>Yeast extracts</td>
<td>1.5 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.4± 0.2</td>
</tr>
</tbody>
</table>

### 6.2.4 PROCEDURE

#### 6.2.4.1 Determination of antibacterial activity by disk-diffusion method

The test organisms were sub cultured 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. 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. 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 0C 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 49.
Figure 3: Antibacterial activities by zone of inhibition of pyrazoline derivatives (81-86)

Plate 6

Plate 7

Plate 8

Plate 9

Plate 10

Figure 4: Antibacterial activities by zone of inhibition of pyrazoline derivatives (87-92)
Table 49: The antibacterial and antifungal activities of pyrazoline derivatives by disc diffusion method (81-92)

<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>81</td>
</tr>
<tr>
<td>1</td>
<td><em>Bacillus subtilis</em></td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td><em>Escherichia coli</em></td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td><em>Staphylococcus aureus</em></td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td><em>Streptococcus pyogenes</em></td>
<td>21</td>
<td>-</td>
</tr>
</tbody>
</table>
Chart 1: Antibacterial activities by zone of inhibition of pyrazoline derivatives (81-92)
6.2.5 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 cup plate method. Streptomycin was used as reference standard for comparing the results. The antibacterial activity of the pyrazoline derivatives are shown in Fig: 3 & 4 for Plates 1-10, and the zone of inhibition values are given Table-49. The Clustered column Chart 1 Showed that pyrazoline derivatives of (81) to (92) posses 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 (82), (90) and (92) were found to be active against *S. aureus*. Compounds (81), (84), (87) and (88) were found to moderately active be active against *S. aureus*, whereas compounds (83), (85), (86), (89) and (91) were found to be inactive be active against *S. aureus*. Compounds (81), (82), (84) and (85) were found to be active against *B. subtilis*. Compounds (83) and (86) were found to be moderately active against *B. subtilis*. Compounds (87), (88), (90) and (92) were found to be less active against *B. subtilis*, whereas as compound (89) and (91) was found to be inactive against *B. subtilis*. Compound (87), (88), (89), (90), (91) and (92) was found to active against *E. coli*. Compounds (84) and 85 were found to be moderately active against *E. coli*, whereas (81), (82) and (83) were found to be less active against *E. coli*. Compound (85) were found to be active against S.pyogones. Compounds (82), (87), (88) and (89) were found to be moderately active against S.pyogones.where as (84), (86), (91) and (92) were found to be less active against S.pyogones. Compounds (88) and (89) were found to be active against *P. aeruginosa*. Compounds (82), (83), (90) and (92) were found to moderately active be active against *P.aeruginosa*. whereas compounds (81), (85), (87) and (91) were found to be less active be active against *P.aeruginosa*. 

186
6.2.6 MINIMUM INHIBITION CONCENTRATION (MIC)

All the newly synthesized compounds were screened for their antibacterial activity. For this, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* *Streptococcus pyogenes* microorganisms were employed. Antimicrobial study was assessed by Minimum Inhibitory Concentration by serial dilution method. Several colonies of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* *Streptococcus pyogenes* were picked off a fresh isolation plate and inoculated in corresponding tubes containing 5 ml of trypticase soya broth. The broth was incubated for 6 h at 37°C until there was visible growth. McFarland No.5 standard was prepared by adding 0.05 ml of 1% w/v BaCl$_2$.2H$_2$O in Phosphate Buffered saline (PBS) to 9.95 ml of 1% v/v H$_2$SO$_4$ in PBS. The growth of all the four cultures was adjusted to Mc Farland No.5 turbidity standard using sterile PBS. This gives a 108 cfu/ml suspension. The working inoculums of aforementioned four different microorganisms containing 105 cfu/ml suspension was prepared by diluting the 108 cfu/ml suspension, 103 times in trypticase soya broth.

6.2.6.1 PROCEDURE

Testing was done in the seeded broth (105 cfu/ml). The test compounds were taken at different concentrations ranging from 200, 100, 50, 25, 12.5 and to 6.25 μg/ml for finding the minimum inhibitory concentration by using seeded broth as diluent. Similarly, the standard solution of Streptomycin drug prepared at the concentrations of 200, 100, 50, 25.5, 12.5 and 6.25 μg/ml of sterile distilled water and DMSO were maintained throughout the experiment simultaneously as control. The study involves a series of 7 assay tubes for the test compounds against each strain. In the first assay tube, 1.6 ml of seeded broth was transferred, and 0.4 ml of the test solution was added, followed by mixing it thoroughly to obtain a concentration of 200 μg/ml To the remaining nine assay tubes, 1 ml of seeded broth was transferred, and then, from the first assay tube, per
milliliter of the content was pipette out and added into the second assay tube, followed by mixing thoroughly. This type of dilution was repeated up to the 7th assay tube serially. The MIC values of standard drugs taken from literature and it compare with pyrazoline derivatives. Duplicates were also maintained; these were done under aseptic conditions. The racks of assay tubes were placed inside the incubator at $37 \pm 1^\circ\text{C}$ for 24 h. After the incubation period, the assay tube concentrations were again streaked into the nutrient agar plate due to turbidity of the drug microorganism mixture. The lowest concentration of the test compounds, which caused apparently a complete inhibition of growth of organisms, was taken as the MIC. The solvent control tube was also observed to find whether there was any inhibitory action. The sterile distilled water and DMSO did not show any inhibition. The details of results are furnished in Table-50.
Figure 5: The antibacterial activities of pyrazoline derivatives by serial dilution method

Plate 11: Bacillus subtilis (Compound 85)
Plate 12: Escherichia coli (Compound 87)
Plate 13: Pseudomonas aeruginosa (Compound 88)
Plate 14: Streptococcus pyogenes (Compound 85)
Plate 15: Staphylococcus aureus (Compound 82)
Table 50: The antibacterial activities of pyrazoline derivatives by serial dilution method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sample</th>
<th>Bacteria</th>
<th>Minimum Inhibitory Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.5 µg</td>
</tr>
<tr>
<td>1</td>
<td>85</td>
<td>Bacillus subtilis</td>
<td>58.33</td>
</tr>
<tr>
<td>2</td>
<td>87</td>
<td>Escherichia coli</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>88</td>
<td>Pseudomonas aeruginosa</td>
<td>42.72</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>Staphylococcus aureus</td>
<td>22.47</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
<td>Streptococcus pyogenes</td>
<td>38.33</td>
</tr>
</tbody>
</table>
6.2.6.2 RESULTS AND DISCUSSION

The minimal inhibitory concentrations of the strongly active compounds were also measured. The antimicrobial activity of the synthesized pyrazolines derivatives (82), (85), (87) and (88) were examined by two fold serial dilution methods. Bacterial strains, viz. *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes* species. In the present study, DMSO is used as control, while Streptomycin are used as standards for bacterial strain. The representative photographs of serial dilution methods are depicted in Figure 5. Antibacterial activity of all synthesized pyrazolines was measured by serial dilution method, and the MICs are presented in Table 50. From Table 50, compounds (82), (85), (87) and (88) showed the growth inhibitory concentration against the tested organism fall in the range of 12.5 to 200 μg/mL. However, compounds (85) and (87) showed the inhibition against *Bacillus subtilis*, *Escherichia coli* bacterial strains in the range from 12.5 to 50 μg/ml. compounds (82), (85) and (88) showed the inhibition against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* bacterial strains in the range from 12.5 to 100 μg/ml.
6.3 ANTIFUNGAL ACTIVITY

6.3.1 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 non photo synthetic eukaryotes growing either as colonies of single cells (yeasts) or as filamentous multi cellular 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. Penicillim 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.

6.3.1.1 *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, asper toxin, aflatrem, gliotoxin, and aspergillic 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.

### 6.3.1.2 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.

### 6.3.1.3 *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.

The airborne asexual spores of *P. chrysogenum* are important human allergens. Vacuolar and alkaline serine proteases have been implicated as the major allergenic proteins.

### 6.3.1.4 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.

6.3.1.5 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.

### 6.3.2 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 *Aspergillusflavus, Aspergillusniger, Penicillimchryogenum, Trigodermaveride* and *Fusariumoxysporum*.. Amphotericin -B was employed as standard to compare the results. The test organisms were sub-cultured using Potato-Dextrose-Agar (PDA) medium. 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 °C in a refrigerator.
6.3.2.1 Composition of Potato-Dextrose-Agar medium:

- Peeled potato: 50.0 gm
- Dextrose: 5.0 gm
- Agar: 4.0 gm
- Distilled water: upto 200 ml

The test organisms were sub cultured 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 °C for 15 min. The petri plates, tubes and flasks plugged with cotton, were sterilized in hot-air oven at 160 °C, 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 7.
Figure 6: Antifungal activities by zone of inhibition of pyrazoline derivatives

(81-86)
Figure 7: Antifungal activities by zone of inhibition of pyrazoline derivatives

(87-92)
Table 51: The antifungal activities of pyrazoline derivatives by disc diffusion method (81-92)

<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>81</td>
</tr>
<tr>
<td>1</td>
<td><em>Aspergillus flavus</em></td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td><em>Aspergillus niger</em></td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td><em>Penicillium chryogenum</em></td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td><em>Trigoderma veride</em></td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td><em>Fusarium oxysporum</em></td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>
Chart 2: Antifungal activities by zone of inhibition of pyrazoline derivatives 81-92
6.3.3 RESULTS AND DISCUSSION

A filter paper disc method was employed for the in-vitro study of antifungal effects against *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chryogenum*, *Trigoderma veride* 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-51. The Clustered column Chart 2 showed that pyrazoline derivatives of (81) to (92) 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. However the majority of the compounds like (81), (84) and (86) showed activity almost equal to that of the standard. The screening results indicate that compounds (81), (84) and (86) were found to be active against *Aspergillus flavus*. Compounds (83), (85), (90) and (91) were found to moderately active be active against *Aspergillus flavus*, whereas all other compounds were found to be inactive against *Aspergillus flavus*. Compounds (81), (89) and (90) were found to be active against *Aspergillus niger*. Compounds (82) and (83) were found to be moderately active against *Aspergillus niger*, where as all other compounds was found to be inactive against *Aspergillus niger*. Compound (86) was found to active against *Penicillium chryogenum*. Compounds (81), (83), (85), (87) and (88) were found to be moderately active against *Penicillium chryogenum*, where as (90) were found to be less active against *Penicillium chryogenum*. Compound (86) was found to be active against *Trigoderma veride*. Compounds (81), (82), (84), (89), (91) and (92) were found to be moderately active against *Trigoderma veride*. Compound 81 and 86 was found to be active against *Fusarium oxysporum*. Compounds (84), (85), (87), (89) and (91) were found to be moderately active against *Fusarium oxysporum*. 

205
6.3.4 MINIMUM INHIBITION CONCENTRATION (MIC)

All the newly synthesized compounds were screened for their antifungal activity. For this, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chryogenum Trigoderma veride* and *Fusarium oxysporum* microorganisms were employed. Antimicrobial study was assessed by Minimum Inhibitory Concentration by serial dilution method. Several colonies of *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chryogenum Trigoderma veride* and *Fusarium oxysporum* were picked off a fresh isolation plate and inoculated in corresponding tubes containing 5 ml of trypticase soya broth. The broth was incubated for 6 h at 37°C until there was visible growth. Mc Farland No.5 standard was prepared by adding 0.05 ml of 1% w/v BaCl$_2$.2H$_2$O in Phosphate Buffered saline (PBS) to 9.95 ml of 1% v/v H$_2$SO$_4$ in PBS. The growth of all the four cultures was adjusted to Mc Farland No.5 turbidity standard using sterile PBS. This gives a 108 cfu/ml suspension. The working inoculums of aforementioned four different microorganisms containing 105 cfu/ml suspension was prepared by diluting the 108 cfu/ml suspension, 103 times in trypticase soya broth.

6.3.4.1 Preparation of dilutions

Testing was done in the seeded broth (105 cfu/ml). The test compounds were taken at different concentrations ranging from 200, 100, 50, 25, 12.5 and to 6.25μg/ml for finding the minimum inhibitory concentration (MIC) by using seeded broth as diluent. Similarly, the standard solution of Streptomycin drug prepared at the concentrations of 200, 100, 50, 25.5, 12.5 and 6.25 μg/ml of sterile distilled water and DMSO were maintained throughout the experiment simultaneously as control. The study involves a series of 7 assay tubes for the test compounds against each strain. In the first assay tube, 1.6 ml of seeded broth was transferred, and 0.4 ml of the test solution was added, followed by mixing it thoroughly to obtain a concentration of 200 μg/ml To the remaining nine assay tubes, 1 ml of seeded broth was transferred, and then, from the first assay tube, per milliliter of the content was pipette out and added into the second assay tube, followed by
mixing thoroughly. This type of dilution was repeated up to the 7th assay tube serially. The same procedure was followed for standard drugs. Duplicates were also maintained; these were done under aseptic conditions. The racks of assay tubes were placed inside the incubator at 28 ± 1°C for 72 h. After the incubation period, the assay tube concentrations were again streaked into the nutrient agar plate due to turbidity of the drug microorganism mixture. The lowest concentration of the test compounds, which caused apparently a complete inhibition of growth of organisms, was taken as the MIC. The solvent control tube was also observed to find whether there was any inhibitory action. The sterile distilled water and DMSO did not show any inhibition. The details of results are furnished in Table-52.
Figure 8: The antifungal activities of pyrazoline derivatives by serial dilution method
Table 52: The antibacterial activities of pyrazoline derivatives by serial dilution method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>Fungi</th>
<th>Minimum Inhibitory Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.5 µg</td>
</tr>
<tr>
<td>1</td>
<td>86</td>
<td><em>Aspergillus flavus</em></td>
<td>32.00</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td><em>Aspergillus niger</em></td>
<td>28.32</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td><em>Penicillium chrysogenum</em></td>
<td>38.50</td>
</tr>
<tr>
<td>4</td>
<td>86</td>
<td><em>Trichoderma viride</em></td>
<td>33.33</td>
</tr>
<tr>
<td>5</td>
<td>86</td>
<td><em>Fusarium oxysporum</em></td>
<td>26.10</td>
</tr>
</tbody>
</table>
6.3.4.2 RESULTS AND DISCUSSION

The minimal inhibitory concentrations of the strongly active compounds were also measured. The MIC activity of the synthesized pyrazolines derivatives (81) and (86) were examined by two fold serial dilution methods. Fungal strains, viz. Aspergillus flavus, Aspergillus niger, Penicillium chryogenum Trigoderma veride and Fusarium oxysporum species. In the present study, DMSO is used as control, while Amphotericin -B are used as standards for fungal strain. The representative photographs of serial dilution methods are depicted in Figure 8. Antifungal activity of all synthesized pyrazolines was measured by serial dilution method, and the MICs are presented in Table 52. From Table 52, compounds (81) and (86) showed the growth inhibitory concentration against the tested organism fall in the range of 12.5 to 100 μg/ml. However, compounds (86) showed the inhibition against Penicillium chrysogenum fungal strains in the range from 12.5 to 50 μg/ml. compounds (81) and (86) showed the inhibition against Aspergillus flavus, Aspergillus niger, Trigoderma veride and Fusarium oxysporum fungal strains in the range from 12.5 to 100 μg/ml.
6.4 ANTIOXIDANT ACTIVITY

6.4.1 INTRODUCTION

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reaction can produce free radicals which start chain reaction that damage cells.

Antioxidant terminates these chain reactions by being oxidized themselves. Although oxidation reactions are crucial for life, they can also be damaging, hence plants and animals maintain a complex system of multiple steps of antioxidants such as glutathione, vitamin C, E as well as enzymes such as catalyse, superoxide dismutase and various peroxidases. Low levels of antioxidant or inhibition of the antioxidant enzymes causes oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human disease antioxidant are used in the treatment of stroke and neurodegenerative disease.

Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing disease such as cancer and coronary heart disease. In addition to uses in medicine antioxidants have industrial uses also like preservative in food and cosmetics and preventing the degradation of rubber and gasoline\(^\text{152}\).

6.4.1.1 Mechanism:

Although we know that vast majority of complex life requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS) which include oxygen ions, free radicals and peroxidases both organic and inorganic. These are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. These are formed as a natural byproduct of the normal metabolism of
oxygen and have important roles in cell signaling. However, during times of environment stress, ROS levels can increase dramatically which results in significant damage to cell structures. Consequently organism contains a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids. In general antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell\textsuperscript{153-154}.

### 6.4.1.2 Adverse effects:

1. Non polar constituents such as eugenol, a major component of oil of cloves have toxicity limits that can be exceeded with the misuse of undiluted essential oils.

2. Smokers if given supplements containing beta-carotene and vitamin A may have an increases rate of lung cancer.

3. Beta carotene, vitamin A or E supplements if taken in excessive amount are associated with increased mortality as these increased the risk of colon.

### 6.4.1.3 Screening of antioxidant activity\textsuperscript{155}

To evaluate the antioxidant potential of all the compounds in-vitro free radical scavenging activity using DPPH (2,2-diphenyl-1-picryl hydrazyl) reduction method.

### 6.4.1.4 Principle:

DPPH [2,2-diphenyl-1-picryl hydrazyl] is a stable free radical with purple color. Antioxidant reduces DPPH to 2,2-diphenyl-1-picryl hydrazine, a colorless compound which is measured at an absorbance of 516 nm.
6.4.2 MATERIALS AND METHOD

6.4.2.1 Preparation of Control (DPPH) Solution

10 mg of DPPH was dissolved in 10 ml of methanol. From this stock solution dilutions were made to obtain concentrations of 10, 20, 30, 40 μg/ml. The absorbance was recorded for these dilutions at 516 nm. The concentration of 30 μg/ml showed the absorbance of 0.946.

6.4.2.2 Preparation of standard solution (Ascorbic acid)

10 mg of ascorbic acid was dissolved in 10 ml of methanol. From this stock solution dilutions were made to obtain concentrations of 10, 20, 30, 40 μg/ml. 1 ml from each of these solutions was taken in different volumetric flasks to which 1 ml of DPPH solution (300 μg/ml concentration) was added and volume was made up to 10 ml. The absorbance was recorded for these dilutions at 516 nm after duration of 30 mins.

6.4.2.3 Preparation of test solutions:

The test solutions were prepared in similar manner as that of standard Ascorbic acid and the absorbance was recorded at 516 nm after duration of 30 mins.
Table 53.
Screening results of DPPH radical scavenging activity of pyrazoline derivatives
(81–92)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compounds</th>
<th>Antioxidant activity (%) DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>98.00±1.00</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>51.67±3.51</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>42.67±2.08</td>
</tr>
<tr>
<td>4</td>
<td>83</td>
<td>54.33±2.08</td>
</tr>
<tr>
<td>5</td>
<td>84</td>
<td>61.00±1.00</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>89.67±1.53</td>
</tr>
<tr>
<td>7</td>
<td>86</td>
<td>66.00±3.46</td>
</tr>
<tr>
<td>8</td>
<td>87</td>
<td>70.67±1.15</td>
</tr>
<tr>
<td>9</td>
<td>88</td>
<td>43.67±1.53</td>
</tr>
<tr>
<td>10</td>
<td>89</td>
<td>51.33±4.16</td>
</tr>
<tr>
<td>11</td>
<td>90</td>
<td>53.67±1.53</td>
</tr>
<tr>
<td>12</td>
<td>91</td>
<td>88.33±3.21</td>
</tr>
<tr>
<td>13</td>
<td>92</td>
<td>29.33±1.53</td>
</tr>
</tbody>
</table>
Chart 3: Screening results of DPPH radical scavenging activity of pyrazoline derivatives 81–92
6.4.3 RESULTS AND DISCUSSIONS

All the synthesized compounds (81) to (92) were evaluated for their in-vitro free radical scavenging activity by DPPH (2,2'-diphenyl-1-picryl hydrazyl) reduction method using ascorbic acid as the standard. The result of this study is collected in Table 53. The following observations were made within the series, Compounds (85), (87) and (91) showed maximum oxygen scavenging activity which is comparable to ascorbic acid. Compounds (84) and (86) exhibited moderate oxygen scavenging activity as compared to ascorbic acid, where as compounds (81), (83), (89) and (90) exhibited minimum antioxidant activity. However none of the compounds exhibited greater activity with respect to standard ascorbic acid.