3.1. MATERIALS:

**Plant material:** *Iris kashmiriana* is an angiosperm, monocot plant and generally grows on temperate region at the altitude of 1000-3000 m, while in other geographical region (Tropical region) they may be grown as bulb or corn. The rhizomes were collected from district Kullu (HP) in the month of Aug.-Sept. 2012 and duly authenticated by Dr. R Raina, Senior Scientist/Professor (Medicinal Plants), Department of Forest Products, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni 173230, Solan (HP) India, linked to UHF- Herbarium with Field Book No: 12566.

**Chemicals:** The chemicals used in the present project were of AR and LR grade and were purchased from Fischer scientific, Thomas bakers, Sigma Aldrich.

3.2. Experimental

3.2.1. Analytical Techniques

a) **Physical Data:** Melting points of the newly isolated natural compounds and their analogues were determined using Thiele’s melting point apparatus and were uncorrected.

b) **Thin layer chromatography (TLC):** Purity of the compounds was checked by Thin layer chromatography using aluminium coated plates and various combinations of solvents like ethyl acetate: chloroform, toluene: ethyl acetate, hexane: ethyl acetate, methanol: chloroform as mobile phases. In present work chloroform: methanol was used as solvent in ratio of 9:1 TLC silica gel 60 F_{254} is used which is aluminium coated and is of MERCK Company. The spots revealed were visualized using iodine chamber along with spray reagents.

c) **Instrumentation:** The techniques employed for the characterization of the isolated compounds were done by U.V. IR spectra, \(^1\)H-NMR, \(^{13}\)C- NMR along with COSY, (2-DNMR) techniques.

**Infrared spectra:** The IR of the synthesised compounds was recorded by using CARY 630 FTIR Spectroscope of Agilent Technologies.
d) \( ^1\text{H-NMR, } ^{13}\text{C- NMR and COSY (2-D NMR)} \) spectral data were recorded in Bruker Avance II 400MHz NMR spectrometer available at SAIF Panjab University Chandigarh and CDCl\(_3\)/DMSO was used as the solvent.

### 3.2.2. Plan of Work

- The extraction and isolation of the bioactive molecules were done from *Iris kashmiriana*.
- Phytochemical screening was performed to identify the presence of secondary metabolites.
- Purification and Characterization of the isolated compounds and their analogues were done by melting point determination and TLC, analysis. (Rf Value data)
- Purification and qualitative evaluation of isolated compounds and their analogues were done by reverse phase HPLC.
- Structures of the isolated molecules and their analogues were established by UV, IR, \(^1\text{H-NMR, } ^{13}\text{C-NMR, Mass spectrometry and 2-D NMR spectroscopy.} \)
- Structures were also established by U.V. shift by adding AlCl\(_3\) and HCl in methanol to locate the position of hydroxyl groups in the isoflavonoids nucleus.
- All isolated compounds and their analogues were subjected to following biological activities.
  - Antioxidants
  - Antimicrobials
    - a) Antibacterial
    - b) Antifungal.
  - Cytotoxic activity on different cell lines. Eg. MCF-7 (Esterogenic positive), MDA-MB-231, (Esterogenic negative), A-549 (Lung Cancer), PC-3 (Prostate cancer), Vero (Normal kidney epithelial cell line)
  - Cell cycle analysis was done using flow cytometer for selected compounds, showing marked cytotoxic activity.

### 3.2.3. Extraction and Isolation

Rhizomes of *Iris kashmiriana* were dried, chopped, and powdered (500g). The extraction of powdered drug was done with petroleum ether (60-80) using Soxhlet apparatus (24
hrs. run). The petroleum ether extract (gums and resins 2.13 g) was obtained and the marc was subjected for extraction with methanol using Soxhlet apparatus (24 hrs. run). The methanolic extract (5 g) was subjected for successive fractionations with toluene, chloroform and ethyl acetate and n-butanol. The phytochemical screening of each extract and fraction were done. On the basis of phytochemical screening results, the glycosides were present in n-butanol fraction. Column Chromatography was performed with n-butanol fraction in chloroform: methanol (9:1) as solvent system. Fractions (1-100) were collected and TLC was observed, three different single spots were found in range of 40-50, 55-60 and 85-100 test tube respectively. The identical TLC pattern ones were pooled. Three isolated compounds were obtained, one was yellow rosette shaped crystals, another one was yellow needle shaped and last one was amorphous in nature.

The extract of Petroleum ether, methanol and fractions were subjected to preliminary phytochemical screening for the detection of various phytoconstituents such as flavonoids, alkaloids, glycosides, tannins and phenolic compounds, steroids, saponins, proteins, amino acids, and triterpenoids by using different phytochemical tests (Kokate et al. 2006). The results were based on the characteristic test.

3.2.4. Phytochemical Screening

Table No: 3.2.4.1

<table>
<thead>
<tr>
<th>Test</th>
<th>Methanolic Extract</th>
<th>Toluene Fraction</th>
<th>Chloroform Fraction</th>
<th>Ethyl Acetate Fraction</th>
<th>n-Butanol Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Shows presence of secondary metabolites
- = Shows absence of secondary metabolites.

From above Table No: 3.2.4.1 it has clearly attributed that the flavonoids were found in n-butanol fraction in good quantity.
3.3. Methodology:

3.3.1. AJD-1

To detect the presence of various phytochemicals in methanol extracts, followed by fractionation by toluene, chloroform, ethyl acetate and n-butanol fractions of powdered *Iris kashmiriana*. Phytochemical tests were performed and following compounds were identified in n-butanol fraction.

1) White solid crystalline mass was obtained in n-butanol fraction and was further crystallized from methanol which was appeared to be a glycoside and named as AJD-1(1g), melting point 285-290°C.

![Chemical structure of AJD-1](image)

**Fig No: 3.3.1 (AJD-1)**

**Table No. 3.3.1 Physical data of AJD-1**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{22}H_{22}O_{11}.</td>
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<tr>
<td>Molecular weight</td>
<td>462.4</td>
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<tr>
<td>Melting Point</td>
<td>285-290°C</td>
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<tr>
<td>M^+1 Peak</td>
<td>m/z 463.4</td>
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<tr>
<td>Rf Value</td>
<td>0.63</td>
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<tr>
<td>Solvent System</td>
<td>Chloroform : Methanol (9:1)</td>
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<tr>
<td>Elemental Analysis Found (%)</td>
<td>C;57.14,H;4.80, O;38.06</td>
</tr>
<tr>
<td>Elemental Analysis Calculated (%)</td>
<td>C;57.02,H;4.46, O;37.96</td>
</tr>
</tbody>
</table>
3.3.2. Acetylation of AJD-1 to produce AJD-2

Procedure: The glycoside 500 mg (AJD-1) was subjected to acetylation reaction by the addition of acetic anhydride along with a few drops of Conc. H$_2$SO$_4$ for 4-5 hrs to yield AJD-2 (400 mg), an acetylated glycoside. The presence of six acetyl groups was confirmed by $^1$H-NMR showing the chemical shift from 2-2.65 ppm. The melting point of AJD-2 was observed at 310-315$^0$C and was uncorrected.

![Chemical Structure of AJD-2](image)

**Fig No: 3.3.2 (AJD-2)**

<table>
<thead>
<tr>
<th>Table No: 3.3.2 Physical data of AJD-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
</tr>
<tr>
<td>Molecular weight</td>
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<td>Melting Point</td>
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<tr>
<td>$M^+$+1 Peak</td>
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<tr>
<td>Rf Value</td>
</tr>
<tr>
<td>Solvent System</td>
</tr>
<tr>
<td>Elemental Analysis Found (%)</td>
</tr>
<tr>
<td>Elemental Analysis Calculated (%)</td>
</tr>
</tbody>
</table>
3.3.3. Hydrolysis of AJD-1 to produce AJD-4

**Procedure:** AJD-1 (500 mg) was subjected to acid hydrolysis in the presence 10% HCl in methanol under reflux condition for 7-8 hrs. Most of the methanol was distilled out and water was added along with chloroform. The reaction mixture was separated out by separating funnel after pouring more water. From organic layer solvent was removed by distillation and residue further dried. The compound was considered as aglycone (250mg) of the glycoside. The aglycones were subjected to column chromatography. The aqueous portion of the above subjected to paper chromatography, using butanol, acetone and water in the ratio 4:1:5, where it was clearly observed that the sugar attached was glucose, with Rf value 0.65.

**Description of column:**

**Sample:** Organic part (Aglycones)

**Adsorbent:** Silica G 60/120

**Mobile Phase:** Chloroform: Methanol (9: 1)

**Fraction Number:** 100-100 (TLC showing identical spot with same Rf value were pooled.)

Fraction volume each: 8 ml.

**Results:** Two single spots were found in the number and range, of 40-60 and 85-100 test tubes respectively. The identical TLC pattern ones were pooled. (40-60).

The yellow, rosette shaped crystals were observed and was named as AJD-4 (400mg). These compounds were subjected for characterization and purity by melting point determination, Rf value analysis along with the establishment of the structure by U.V. IR, $^1$H-NMR, $^{13}$C-NMR, 2-D NMR, and Mass spectrometry. On the basis of these observations the AJD-4 was found to be an isolated and considered to be a novel compound.
3.3.4. Acetylation of AJD-4 to produce AJD-6

Procedure: The AJD-4 (500 mg) was subjected to acetylation reaction by the addition of acetic anhydride along with a few drops of Conc. H$_2$SO$_4$ for 4-5 hrs to produce AJD-6 (350 mg). The presence of three acetyl groups was confirmed by $^1$H-NMR showing the chemical shift at around 2.65 ppm. The melting point of AJD-6 was observed at 270-275°C and was uncorrected.
3.3.5. Per methylation of AJD-1 to produce AJD-8

**Procedure:** The glycoside AJD-1 was subjected to per methylation by dimethyl sulphate in the presence of K₂CO₃ in dry acetone. After six hours of refluxing the product was processed and subjected to acid hydrolysis (HCl in methanol) under the reflux condition followed by usual method of processing. The aglycone was isolated, purified by column chromatography and recrystallized with methanol to yield a compound AJD-8 m.p. 243-245°C and analyzed for C₁₈H₁₆O₆. It was identified by ¹H NMR,(DMSO δ with TMS=O) and Mass Spectrometry as 7-hydroxy-5,8,4’-trimethoxy isoflavone. ¹H-NMR (DMSO δ with TMS=O) showed three methoxyls at 3.983, 3.886, 3.857 assigned to C-5, C-8 and C-4’ position respectively. A singlet at 6.485 could be assigned to C-6 proton beside two doublets (J= 8.1 Hz each) centered at 6.845 and 7.359 ppm for two protons each of 3’, 5’ and 2’, 6’ proton respectively. C-2 proton as singlet appeared at 8.157 ppm.
3.3.5 Physical data of AJD-8

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{18}H_{16}O_{6}</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>328.09</td>
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<tr>
<td>Melting Point</td>
<td>243-245°C</td>
</tr>
<tr>
<td>M^+1 Peak</td>
<td>m/z 329.10</td>
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<tr>
<td>Rf Value</td>
<td>0.70</td>
</tr>
<tr>
<td>Solvent System</td>
<td>Chloroform: Methanol(9:1)</td>
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<tr>
<td>Elemental Analysis</td>
<td></td>
</tr>
<tr>
<td>Found (%)</td>
<td>C: 59.40, H: 5.78, O: 34.82.</td>
</tr>
<tr>
<td>Calculated (%)</td>
<td>C: 60.00, H: 5.42, O: 33.92</td>
</tr>
</tbody>
</table>

3.3.6. Acetylation of AJD-8 to produce AJD-10

Procedure: The aglycone AJD-8 was subjected to acetylation reaction by the addition of acetic anhydride in the presence of few drops of H_2SO_4 for 4-5 hrs. The mixture was poured into cold water and white solid crystal mass was obtained and further recrystallized with methanol to yield a compound AJD-10 m.p.195-198 °C and analyzed for C_{20}H_{18}O_{7}.
Fig No: 3.3.6 AJD-10

Table No: 3.3.6 Physical data of AJD-10

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{20}H_{18}O_{7}</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>370.35</td>
</tr>
<tr>
<td>Melting Point</td>
<td>195-198 °C</td>
</tr>
<tr>
<td>M^{+}+1 Peak</td>
<td>m/z 370.35</td>
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<tr>
<td>Rf Value</td>
<td>0.71</td>
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<tr>
<td>Solvent System</td>
<td>Chloroform: Methanol(9:1)</td>
</tr>
<tr>
<td>Elemental Analysis Found (%)</td>
<td>C;64.86, H;4.90, O;30.24</td>
</tr>
<tr>
<td>Elemental Analysis Calculated (%)</td>
<td>C;64.70, H;4.72, O;30.92</td>
</tr>
</tbody>
</table>

3.3.7. Glycoside-2 (AJD-3)

The other fraction (85-100) were pooled to get solid crystalline mass and were crystallized from absolute alcohol to yield orange needle shaped crystals was marked as AJD-3 (250 mg). The compound was subjected to characterization and purity by melting point determination, Rf value analysis along with the establishment of the structure by U.V. IR, $^1$H-NMR, $^{13}$C-NMR, On the basis of these observation the AJD-3 was found to be an isolated single isoflavone glycoside molecule. Details given in table no. 3.3.7
3.3.8. Acetylation followed by acid hydrolysis of AJD-3 to produce AJD-5

**Procedure:** The AJD-3 (250 mg) was subjected to acetylation reaction by the addition of acetic anhydride and a few drops of Conc. H$_2$SO$_4$ for 4-5 hrs, followed by acid hydrolysis to produce AJD-5 (150 mg). The presence of one acetyl group was confirmed by $^1$H-NMR showing the chemical shift at around 2.65 ppm. The melting point of AJD-5 was 163-165$^0$C and is uncorrected. The IR shows the presence of major functional group and the probable structure was established by $^1$H-NMR and $^{13}$C-NMR. This reaction also confirms the position of glycosidic linkage which was appeared to be located at C-7 position.
Fig No: 3.3.8  AJD-5

Table No: 3.3.8 Physical data of AJD-5

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{18}H_{14}O_{6}</td>
</tr>
<tr>
<td>Molecular weight</td>
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</tr>
<tr>
<td>Melting Point</td>
<td>163-165°C</td>
</tr>
<tr>
<td>M^+ +1 Peak</td>
<td>m/z 327.08</td>
</tr>
<tr>
<td>Rf   Value</td>
<td>0.67</td>
</tr>
<tr>
<td>Solvent System</td>
<td>Chloroform : Methanol (9:1)</td>
</tr>
<tr>
<td>Elemental Analysis Found (%)</td>
<td>C: 65.22, H: 4.38, O: 30.41</td>
</tr>
<tr>
<td>Elemental Analysis Calculated (%)</td>
<td>C:65.00, H: 3.9, O: 30.03</td>
</tr>
</tbody>
</table>

3.3.9. Complete Acetylation of AJD-3 to produce AJD-7

**Procedure:** A mixture of AJD-3 (200 mg.), acetic anhydride (2ml), and few drops of conc. H_2SO_4 were added and kept for 3-4 hrs. after usual process of acid hydrolysis. The resulting mixture was poured into cold water; a white crystalline mass was obtained and further re-crystallized from absolute ethanol, named as AJD-7 (100mg). The melting point was found to be 178-180°C, (uncorrected).
3.3.10. Acid hydrolysis of AJD-3 to produce AJD-9

Procedure: The AJD-3 (250 mg) was subjected to complete acid hydrolysis by the addition of 5% HCl in methanol and refluxed for 4-5 hrs to produce yellow solid mass and recrystallized from absolute alcohol to produce AJD-9. The melting point of AJD-9 was 175-180°C and is uncorrected. The IR shows the presence of major functional group and the probable structure was established by $^1$H-NMR and $^{13}$C-NMR.
Fig No: 3.3.10 AJD-10

Table No: 3.3.10 Physical data of AJD-10

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Molecular Formula</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>284.26</td>
</tr>
<tr>
<td>Melting Point</td>
<td>175-180°C</td>
</tr>
<tr>
<td>M&lt;sup&gt;+&lt;/sup&gt;+1 Peak</td>
<td>m/z 285.26</td>
</tr>
<tr>
<td>Rf Value</td>
<td>0.62</td>
</tr>
<tr>
<td>Solvent System</td>
<td>Chloroform: Methanol(9:1)</td>
</tr>
<tr>
<td>Elemental Analysis Calculated (%)</td>
<td>C: 66.90, H: 4.19, O: 28.03</td>
</tr>
</tbody>
</table>
3.3.11. General reaction schemes of glycoside 1 and 2

**Glycoside-1**

1) Conversion of AJD-1 to AJD-2:

2) Conversion of AJD-1 to AJD-4 (Novel Isoflavone):
3) Conversion of AJD-4 to AJD-6 (Novel Isoflavone Analogue):

\[
\text{AJD-4} \xrightarrow{\text{Conc H}_2\text{SO}_4} \text{Acetic Anhydride} \rightarrow \text{AJD-6}
\]

Conversion of AJD-1 to AJD-8 (Novel Isoflavone Analogue):

\[
\text{AJD-1} \xrightarrow{\text{Dimethyl Sulphate}} \xrightarrow{\text{K}_2\text{CO}_3, \text{Dry Acetone}} \text{1. Permethylation} \xrightarrow{\text{2. Acid hydrolysis}} \text{AJD-8}
\]
4) Conversion of methylated AJD-1 to AJD-10 (Novel Isoflavone Analogue):

**Glycoside-2. Conversion of AJD-3 to AJD-5:**
5) Conversion of AJD-3 to AJD-7 (Novel Isoflavone Analogue):

6) Conversion of AJD-3 to AJD-9 (Novel Isoflavone Analogue).

Fig No: 3.3.11 General reaction scheme of glycoside 1 and 2
3.4. Biological Activity

3.4.1. Antioxidant Activity by DPPH Method: Belsare et al. (2010)

Oxygen metabolism although essential for life, imposes a potential threat to cells due to the formation of free radicals or partially reduced oxygen species (Reactive Oxygen Species- ROS). Free radicals are atoms or group of atoms possessing an odd (unpaired) electron, which are extremely reactive because of their tendency to gain an additional electron to complete the octet. During normal metabolism (cellular) and exposure to environmental threats like infectious agents, pollution, UV light, radiation etc, harmful free radicals are generated in the body.

When these radicals are not checked by body defense mechanism, they can cause damage to vital proteins, lipids and DNA. Free radicals have been implicated in the causation and progress of several diseases including cardiovascular diseases, cancer, inflammatory diseases, respiratory diseases, diabetes, cataract, infertility, ageing process, liver cirrhosis etc.

Principle:

DPPH (1, 1-diphenyl-2-picryl hydrazyl) is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorption band at 517nm. DPPH radical reacts with suitable reducing agents, resulting in the pairing of electron and subsequent loss of colour. The colour decreases stochiometrically with the number of electrons taken up and the decrease in the absorbance can be directly measured and compared with that of the standard (ascorbic acid) and the blank. This method is widely reported for screening of antioxidant activity.
Fig No: 3.4.1 Reaction of DPPH and antioxidant

**Procedure:**

**DPPH solution**
A working solution of methanolic DPPH having an absorbance of 0.9 at 517nm was used. This was prepared by taking 95µl of stock solution containing 12.9 mg of DPPH in 10 ml of methanol.

**Standard solution**
Ascorbic acid was used a standard free radical scavenger. This was prepared by dissolving 10.24 mg of ascorbic acid (stock solution) in 10 ml of methanol.

**Test solution**
The test solutions of the test compounds were prepared in DMSO, by dissolving 10.24 mg of test compound in 10 ml of DMSO (stock solution). Serial dilutions of test and standard solutions were made so as to get 200, 100, 50, 25, 12.5, 6.25 ppm each.

**Method:**
To 1 ml of various concentrations of test solution in DMSO, 1 ml of DPPH solution was added; control was prepared by adding 1 ml of DMSO and 1 ml of DPPH solution and kept 20 min. for incubation in dark. After 20 min., the decrease in the absorbance of the test solution (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition was calculated.

\[
\text{% inhibition} = \left( \frac{\text{control} - \text{test}}{\text{control}} \right) \times 100
\]
3.4.2. Antioxidant Activity by ABTS Method: Sithisarn et al. (2005)

ABTS [2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Method:

**Principle:**
The pre-formed radical mono cation of 2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) is generated by oxidation of ABTS with potassium per sulfate (a blue chromogen) and is reduced in the presence of such hydrogen donating antioxidants.

**Chemicals and Reagents used:**
**Preparation of ABTS solution**
**Solution I:** ABTS (2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (2 mM solution is prepared using distilled water).
**Solution II:** Potassium per sulfate (17 mM solution is prepared using distilled water) 0.3 mL of solution II was added to 50 mL of solution I. The reaction mixture was left to stand at room temperature overnight in dark before use.

**Preparation of Test Solution**
10 mg of each of the drug samples and the standard (Ascorbic acid) were accurately weighed separately and dissolved in 10 mL of DMSO to produce a stock solution of 1000 µg/ml. The stock solutions were serially diluted with DMSO to obtain 200, 100, 50, 25, 12.5 and 6.25 µg/ml of both drug sample and standard (Ascorbic acid).

**Method:**
1 mL of distilled DMSO was added to 0.2 mL of various concentrations of the drug samples or standard, and 0.16 mL of ABTS solution was added to make a final volume of 1.36 mL. Absorbance was measured spectrophotometrically, after 20 min at 734 nm using ELISA reader. Blank was maintained without ABTS. IC₅₀ value obtained is the concentration of the sample required to inhibit 50 % ABTS radical mono cation.
3.4.3 MTT Anticancer Screening Assay:

MTT full assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular growth. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials.

**Screening methods:**

1. *In vitro* screening for cytotoxicity

   A) Brine shrimp lethality: a rapid general bioassay for bioactive compounds.

   B) Tryphan Blue Exclusion Method (Cell Viability Test)

   C) MTT assay

There are various ways to determine the toxicity of any compound *in vitro*. These methods are basically dependent on uptake of some compound by live cells followed by their analysis using various techniques. Metabolic activity assays measure mitochondrial activity. The cells are incubated with a colorimetric substrate (MTT). An advantage being it is relatively sensitive, reproducible; miniaturization allows many samples to be analyzed rapidly and simultaneously. The micro plate format also reduces the amount of culture medium and cells required as well as the cost of plastic ware and the samples could be measured directly in the micro plate with an ELISA plate reader.

**Method followed in present work (MTT assay):**

**MTT Assay Protocol:**

MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular growth. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilisation solution (usually either dimethyl sulphoxide, an acidified ethanol solution, or a solution of the sodium dodecyl sulphate in dilute hydrochloric acid, 0.04N acidified isopropyl alcohol is added to dissolve the insoluble purple product into a colored solution. The absorbance of this
colored solution can be quantified by measuring at wavelength (540 nm) by ELISA reader. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells.

![Yellow MTT and Purple formazan](image)

**Fig No: 3.4.3 Structure of MTT**

**Experimental:**
Add 100μl of cells to a 96 well plate at a cell density of 5-10x 10^4 cells per well. Incubate (37°C, 5% CO₂) overnight to allow the cells to attach to the wells. Flick off old media. Add 100 μl of (drug+ media) solution in each well according to dilution. Same volume also keeps of blank, positive & negative control. Incubate (37 °C, 5% CO₂ for next 48-72 hr. to allow the cells to attach to the wells. Flick off old media and add 100 μl (1 mg/ ml) of MTT. Plates are incubate (37 °C, 5% CO₂) for next 4hr.Flick off MTT and insoluble formazan product is dissolved in100 μl of DMSO .Leave at room temperature for a few minutes to ensure all crystals are dissolved. Read plate using a wavelength of 540 nm. Be sure to read plates within one hour of adding the DMSO. Reagents MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (Sigma #M2128): dissolve at 1 mg/ml in PBS, filter and sterilize. PBS = Phosphate buffer saline pH 7.4.
3.4.4. Flow Cytometer:

Flow Cytometer is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle’s relative size, relative granularity or internal complexity, and relative fluorescence intensity.

**Cell cycle analysis protocol using propidium iodide**

**Reagents:** Propidium Iodide Staining Solution: 25 µg/ml PI + 40 µg/ml RNase A + NP-40 (0.03%) in PBS. 70% ethanol stored in the freezer at -20 °C. Wash buffer: PBS + 0.1% bovine albumin or serum.

**Protocol:**
- 1 X 10^6 cells were seeded in petri plates and incubated for 24 hrs.
- All the treatments were given and again the cells were incubated for 24 hrs. MCF-7 cells without any treatment served as control and standard drug used was doxorubicin at 2µg/ml.
- Trypsinized the cells and washed with phosphate buffer saline.
- Added 1 ml of ice cold (-20 °C) 70% ethanol into the cell pellet and kept for fixation for 2hrs at 4 °C.
- Washed the cells with PBS to remove the alcohol.
- Added 1 ml of propidium iodide staining solution to cell pellet, mixed well and incubated for 20 min at room temperature in dark.
- Samples were run in BD Accuri C6 flow cytometer and analysed using BD Accuri C6 software.
3.4.5. Antimicrobials: Chamber et al. (2001), Black et al. (2004)

The discovery of antibiotics is one of the greatest events in the history of medicine, which has a profound effect on human life and society as a whole. Modern era of antimicrobial chemotherapy dates back to 1936, with the introduction of sulfanilamide into clinical practice. Since then numerous classes of antimicrobial agents have been discovered, and literally hundreds of drugs are available for use today.

**Methods for studying antimicrobial activity**

Antimicrobial activity is determined based on their *in vitro* activity in pure cultures. *In vitro* susceptibility tests are done by the following methods:

1. Agar diffusion method
2. Tube dilution method
3. Micro-dilution method

**Method used in present study:** Micro dilution method

**Bacterial strains used:**

1. **Gram negative** - *Escherichia coli* (NCIM 2574) *Pseudomonas aeruginosa* (NCIM 2036) and *Klebsiella pneumonia* (NMIC 2578)
2. **Gram positive** - *Staphylococcus aureus* (NCIM 2079) *Bacillus subtilis* (NCIM 2063) and *Bacillus cereus* (NCIM 2051)

**Media used:** Mueller Hinton agar Mueller Hinton broth.

**Procedure:**

**Preparation of Inoculum:**

For preparation of inoculum, growth from the agar slant was scrapped by adding 3 ml of sterile saline solution. This saline cell suspension was then spread evenly on large sterile Petri plates containing solidified Nutrient Agar using a sterile glass spreader. These plates were incubated in bacteriological incubator at 37°C for 24 hours. After profuse growth of the organism in the Petri dish, it was scrapped using sterile spatula and adding small portion of sterile saline. This suspension was transferred to a sterile 100ml conical flask. The final volume of the suspension was made up to 50ml with sterile saline.
**Standardization of Inoculum:**

For determination of MIC, the inoculum density was adjusted to contain $5 \times 10^6$ CFU/ml which have turbidity equal to 0.5 McFarland standards. For this, 0.5 McFarland standard was prepared by adding 0.05ml of 0.048M BaCl$_2$ (1.17% w/v BaCl$_2$.2H$_2$O) to 9.95ml of 0.18M H$_2$SO$_4$ (1% w/v) with constant stirring. The standard was transferred to a glass screw capped bottle. Absorbance of the McFarland standard was checked at 625nm (absorbance at 625nm should range between 0.08-0.13).

**Method followed in present work: Micro dilution Assay**

Micro dilution assay was performed in 96 well plates to estimate % inhibition of the growth of the organism by the drug. Double strength and single strength Mueller Hinton medium was prepared and sterilized. In first row, alternatively 100µL of double strength medium was added and in remaining wells, 100µL of single strength medium was added. Concentrated solution of drug (1000µg/ml) was added in first row in triplicate and further diluted serially till fourth well of column. Then wells were inoculated with 10µL of standard inoculum of test organism. Similarly solvent controls were also tested in triplicate for each test organism. In remaining columns positive and negative control were prepared in triplicate.

After 24 hours, plate was read by the ELISA plate reader at 590 nm. Optical density of growth in each well was calculated by reducing the absorbance of sample blank. The IC$_{50}$ of the copper nano particles was calculated as compared to positive control of each test organisms.

$$\text{% inhibition of growth} = (\text{control} – \text{test/control}) \times 100$$

From above serial dilution process each test compound contain following concentration 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml,

The standard drug molecules were taken = Amikacine and Gatifloxacin