3.1 STUDY MATERIAL

The study material for the present experimentation included plants of *Brassica juncea* L. var. PBR 91. This cultivar shows stability for most of the important yield contributing characters under the existing conditions.

The certified and disease-free seeds (Fig. 3.1) of *Brassica juncea* L. variety PBR-91 were procured from Punjab Agricultural University, Ludhiana (India).

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Angiospermae</td>
</tr>
<tr>
<td>Class</td>
<td>Dicotyledons</td>
</tr>
<tr>
<td>Order</td>
<td>Brassicales</td>
</tr>
<tr>
<td>Family</td>
<td>Brassicaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Brassica</td>
</tr>
<tr>
<td>Species</td>
<td>juncea</td>
</tr>
<tr>
<td>Variety</td>
<td>PBR 91</td>
</tr>
</tbody>
</table>

Fig. 3.1. Seeds of *Brassica juncea* L. (PBR 91)

3.1.1 Origin, Distribution and Description

*B. juncea* is an amphidiploid species having *Brassica nigra* (L.) Koch (2n=16) and *Brassica rapa* L. (2n= 20) as parents. Regions of western and central Asia are assumed as the centre of origin. For its leaves and seeds, it has been cultivated for thousands of years in Asia and Europe. (Schippers and Mnzava, 2007). In India, it is grown across the Northern plains. The maximum area is centered in North-West agro-climatic zone. It is popularly known as rai, raya or laha and is one of the most important oil seed crops of the country which occupies considerably large acerage among the *Brassica* group of oil seed crops. *B. juncea* is an annual to biennial herb, often unbranched and up to 160cm tall. Leaves are alternate and pinnately lobed with upper ones often simple. The petiole is short. The inflorescence is an umbel like raceme which
gets elongated up to 60 cm. Flowers are bisexual, regular, 4-merous with obovate, 6-10 mm long bright yellow petals. Stamens are 6, ovary is superior with globose stigma. Fruit is a linear siliquae (2.5-7.5 cm long) having upto 20 seeds. Seeds are globose, finely reticulate having pale to dark brown colour.

3.1.2 Nutritional Aspects of *Brassica juncea*

The genus *Brassica*, one of 51 genera in the family *Cruciferae*, is the most economically important genus within the family, containing 37 different species. Many crop species are included in the genus *Brassica*, which yield edible roots, stems, leaves, buds, flowers and seeds condiment. (Weerakoon and Somaratne, 2011). Vegetables included in the *Brassica* genus are a good source of nutrients as well as health promoting phytochemicals (Liu, 2004). The risk of age-related chronic illnesses as well as risk of different types of cancer gets reduced with the high intake of *Brassica* vegetables (Kris-Etheron *et al.*, 2002; Bjorkman *et al.*, 2011).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Per 100 grams of leaf</th>
<th>Per 100 grams of seed</th>
<th>Per 100 grams of roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories</td>
<td>24</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td>Water</td>
<td>91.8g</td>
<td>6.2g</td>
<td>85.2</td>
</tr>
<tr>
<td>Protein</td>
<td>2.4g</td>
<td>24.6g</td>
<td>1.9g</td>
</tr>
<tr>
<td>Fat</td>
<td>0.4g</td>
<td>35.5g</td>
<td>0.3g</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>4.3g</td>
<td>28.4g</td>
<td>8.8g</td>
</tr>
<tr>
<td>Fiber</td>
<td>1g</td>
<td>8g</td>
<td>2.0g</td>
</tr>
<tr>
<td>Ash</td>
<td>1.1g</td>
<td>5.3g</td>
<td>3.8g</td>
</tr>
<tr>
<td>Calcium</td>
<td>160mg</td>
<td>-</td>
<td>111mg</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>48mg</td>
<td>-</td>
<td>65mg</td>
</tr>
<tr>
<td>Iron</td>
<td>2.7mg</td>
<td>-</td>
<td>1.6mg</td>
</tr>
</tbody>
</table>

3.1.3 Medicinal Aspects of *B. juncea*

Reported to be anodyne, apertif, diuretic, emetic, rubefacient, and stimulant, Indian mustard is a folk remedy for arthritis, footache, lumbago, and rheumatism (Duke and Wain 1981). Seed used for tumors in China. Seed used as a galactagogue in Africa.
Sun-dried leaf and flower are smoked in Tanganyika to "get in touch with the spirits." Ingestion may impart a body odor repellent to mosquitoes (Burkill, 1966). Believed to be aperient and tonic, the volatile oil is used as a counterirritant and stimulant. In Java the plant is used as an antisyphilitic emmenagogue. Leaves applied to the forehead are said to relieve headache (Burkill, 1966). In Korea, the seeds are used for abscesses, colds, lumbago, rheumatism, and stomach disorders. Chinese eat the leaves in soups for bladder, inflammation or hemorrhage. Mustard oil is used for skin eruptions and ulcers (Perry, 1980).

**Fig. 3.2.** Medicinal values of *Brassica juncea* L. plant

**3.1.4 Phenolic Compounds in *B. juncea***

Quercetin and kaempferol are the main flavonols in this species. The HPLC–ESI–MSn analysis of two Chinese leaf mustard cultivars grown under field conditions was studied for the phenolic compound variation. The study leads to the identification of free polyphenol content in the outer and inner leaves as well as in their leaf blades and leaf
stalks. It was concluded that hydroxycinnamic acids and flavonoids were higher in the leaf blade. Kaempferol derivatives (mono-, di-, triglucosides) are the main flavonoids, while Isorhamnetin and hydroxycinnamoyl gentiobiose were also detected, but no quercetin derivatives were detected. Further, the main hydroxycinnamic acids were malate derivatives of sinapic, ferulic, hydroxyferulic and caffeic acids. Ferulic acid content was significantly higher in the leaf blade than in the stalk (Harbaum et al., 2008 a, b).

Fang et al. (2008) determined the contents of the total free phenolic acids, the total phenolic acids, the total phenolics as well as the antioxidant activities in leaf mustard and the effects of pickling methods on these compounds. The study leads to the identification of several hydroxycinnamic acids such as caffeic, \textit{p}-coumaric, ferulic and sinapic alongside with benzoic acid derivatives such as gallic, protocatechuic, \textit{p}-hydroxybenzoic, and vanillic acids.

### 3.1.5 Uses

The leaves are eaten as vegetable in Africa and many parts of Asia. Mustard greens, which are the young tender leaves, are used in salads. Mustard oil is among the major edible oils in India. In Russia, it is used as a substitute for olive oil. The oil is used as hair oil as well as a lubricant. It is also used to retard the fermentation process while the making of cider from apples. It has also got medicinal importance as it has diuretic, anodyne, emetic and rubefacient properties. It is used as a folk remedy for rheumatism, lumbago, foot-ache and arthritis. The leaves and seeds are also important medicinally as seeds are used as medicine against tumors in China and leaves are used to treat head-ache and haemorrhage. Residual part of seeds is used as cattle feed and in fertilizers (Schippers and Mnzava, 2007).

### 3.2 HEAVY METAL TREATMENTS

Heavy metals used in the present study were:

1. Nickel (Ni II) is used in the form of NiSO$_4$.7H$_2$O (Central Drug House Pvt. Ltd., Mumbai, India)
2. Chromium (Cr VI) is used in form of K$_2$CrO$_4$ (Qualigens Fine Chemicals Pvt. Ltd., Mumbai, India)
3. Arsenic (As) is used in the form of Na$_2$HAsO$_4$.7H$_2$O (Qualikems Fine Chemical Pvt. Ltd., New Delhi, India)

3.3 CHEMICALS USED IN PRESENT INVESTIGATION

3.3.1 Standards

Standard of brassinosteroids like 24- Epibrassinolide (24-EBL) (Sigma-Aldrich, India Pvt. Ltd., New Delhi), 24-Epicastasterone (24-ECS), 28-Homocastasterone (28-HCS) was procured from Sci-Tech Prague, Czech Republic. Castasterone (CS), Typhasterone (TY), Dolicholide (DL) and Teasterone (TE) were procured from Chemical clones Pvt. Ltd., Canada.

3.3.2 Chemicals Used

Silica gel (60-120 mesh size) for column chromatography was procured from Qualigens Fine Chemicals, Glaxo India Limited, Mumbai. Sephadex LH-20 and Methane Boronic acid were procured from sigma Aldrich, St. Louis, USA. For TLC analysis pre-coated ALUGRAM SIL G/UV$_{254}$ plates were used and procured from Macherey-Nagel, Germany. All the solvents used in the extraction process were of HPLC grade.

3.4 RAISING OF B. juncea PLANTS FOR IC$_{50}$ CALCULATION UNDER LAB CONDITIONS

3.4.1 Growth Conditions

Each petriplate was supplied with 3ml of test solution on first day and 2 ml of test solution on alternate days, up to 7 days. Control seedlings were supplied with distilled water. The experiment was conducted under controlled conditions (25 ± 1°C, 16 h photoperiod, 175μmol m$^{-2}$ s$^{-1}$ light intensity). Mother stock solution i.e., 10mM of these three heavy metals was prepared and stored in pyrex glass reagent bottles at 4°C. To calculate IC$_{50}$ value (i.e., value of that concentration of HM at which 50% growth of the Brassica seedlings is inhibited) a range of various concentrations from 0mM to 2.0mM of each HM was prepared. After calculating the IC$_{50}$ value of each HM, then final concentrations of HM were decided. On the basis of their IC$_{50}$ values and so as to maintain homogeneous concentrations, only 3 concentrations of each HM were decided i.e., IC$_{50}$ value, one concentration below and one above IC$_{50}$ values. The concentrations of heavy metals chosen for present investigation were:
# for Nickel metal – CN, 0.2mM, 0.4mM and 0.6mM
# for Chromium metal – CN, 0.1mM, 0.3mM and 0.5mM.
# for Arsenic metal – CN, 0.1mM, 0.2mM and 0.3mM.

### 3.4.2 Soil Preparation and Filling of Pots:

Garden soil was prepared as mixture of soil and organic manure in the ratio of 3:1. Model pot experiments were established in 10×12 inches pots. The pots were filled with garden soil (5.0 kg), which was mixed with solutions of different concentrations of heavy metals (Ni, Cr and As). The pots were then marked for different HM with their respective concentration. Control plants were raised in normal tap water. The plants were kept in natural seasonal conditions in the botanical garden of the university. (Fig. 3.3)

**Fig.3.3.** Schematic representation of field experimental set up including soil preparation, labeling of pots, metal treatment, ploughing of pots, seed sowing and germinated plants.
3.4.3 Raising of Plants in Field

The seeds of *B. juncea* were surface sterilized with 0.01% sodium hypochlorite for 1 minute followed by five rinses in double distilled water (DDW). In order to study the effects of heavy metals on morphological parameters, metal uptake and expression of BRs in *B. juncea* plants, a seasonal field experiment was performed under above mentioned metals stress (Fig.3.3).

3.5 STUDIES ON MORPHOLOGICAL PARAMETERS

On 30\textsuperscript{th}, 45\textsuperscript{th} and 60\textsuperscript{th} days, the observations were made on the following morphological parameters (Figs. 3.4):

1) Shoot length
2) Number of leaves

15 (fifteen) plants from each of the three replicates (Seventy five plants from three replicate) were analyzed for shoot length and number of leaves per plant after 30, 45 and 60 days of sowing

3.6 BRASSINOSTEROIDS ANALYSIS IN *B. juncea* PLANTS GROWN UNDER METAL STRESS

3.6.1 Extraction of Brassinosteroids

The plant material was homogenized and extracted with 80% methanol. The extract was dried under vacuum at 40\textdegree C. The methanol extract was partitioned between chloroform and water. Chloroform extract was further partitioned between 80% methanol
and hexane. The resulting 80% methanol extract was further partitioned between water and ethyl acetate (Fig. 3.5).

3.6.2 Purification of Brassinosteroids

The schematic representations of various steps involved in purification are shown in Fig.3.5. Ethyl acetate fraction was subjected to silica gel (60-120 mesh) column chromatography with step-gradient from CHCL$_3$ to MeOH 0, 1, 2, 5, 7, 10, 15, 20, 50, 60, 100% (each fraction of 500-1000ml). All the fractions were then subjected to Radish hypocotyl bioassay with the aim to find the bioactive fraction. The active fractions were pooled and subjected to second silica gel column with same elution gradient described above. Fraction collected after second column chromatography was assessed for their biological activity by employing radish hypocotyls bioassay. After passing through two silica gel columns, the bioactive fractions were further purified on sephadex LH-20 column chromatography eluted with methanol: chloroform (4:1). Ten fractions of 50 ml each were collected and their biological activity was again checked by employing the radish hypocotyl bioassay.

3.6.3 Radish Hypocotyls Bioassay

The biological activity of different fractions obtained after chromatographic separations was determined by Radish Hypocotyl Bioassay (Takatsuto et al., 1983). Three days old seedlings of radish were transferred to test solutions. After incubation at 25°C in the darkness for 24 hrs, the elongation percentage of the hypocotyls with respect to the control, determined the biological activity.

3.6.4 Derivatization of Purified Fraction

Methaneboronic acid (100µg) and dry pyridine (60µL) were mixed and 20µL of this mixture was added to the active fractions. These were heated to 80°C for 25–30 min. Further trimethyl silylation of methane boronrates was conducted by reacting with N-methyl-N-trimethylsilyl-triflouroacetamide (MSTFA). Three microliter of this solution was injected into GC–MS. The standard BRs were also derivatized and subjected to GC–MS analysis.
Fig. 3.6 Schematic representation for extraction, purification and characterization of Brassinosteroids in *Brassica juncea* L. plants.

### 3.6.5 Characterization of BRs

#### 3.6.5.1 Thin layer chromatography:

The bioactive fractions obtained after ODS-HPLC were spotted along with the standard on TLC plates coated with 60 F_{254} silica gel, and developed with CHCl₃-CH₃OH (8:2) as the mobile phase. The spots were detected by spraying Liebermann–Burchard reagent. *Rf* values for the standard and samples were recorded.

#### 3.6.5.2 GC–MS analysis

The GC–MS analysis was carried out with gas chromatograph connected with mass spectrometer (Shimadzu, GC–MS, QP 2010) for the analysis of Brassinosteroids with the following conditions: EI (70eV), source temperature 250°C, column Rxt-1 (Length 30 m, Diameter 0.25 mm and 0.1 lm thickness), injection temperature 280°C,
column temperature programmed 200°C for 5 min, then raised to 280°C at rate of 20°C min⁻¹ and held on this temperature for 35 min; inter phase temperature 290°C, carrier gas He, flow rate 1.0mL min⁻¹ with split injection.

3.6.5.3 QTOF analysis

Electrospray ionization mass spectrometry (ESI-MS) of bioactive fractions was carried out by the addition of 10μl of concentrated aqueous formic acid solution to the sample mixture at a total volume of 1000μl (i.e., a 0.1% final concentration). ESI-QTOF-MS was performed in positive ionization mode in QTOF Mass Spectrometer (Micromass, Manchester, UK). ESI-MS was performed by direct infusion (source temperature of 2800 C, capillary voltage of 2.1kV and cone voltage of 23V) with a flow rate of 10μl min⁻¹ using a syringe pump and mass spectra were acquired and accumulated over 60 s. Mass Lynx 4.0 (Waters, Manchester, UK) was used for data analysis. Tandem mass spectrometry of single molecular ion in the mass spectra was performed by mass selecting the ion of interest, which was in turn submitted to 15–35eV collisions with argon in the collision quadruple.

3.7 METAL UPTAKE STUDIES

Metal uptake studies were carried out by using atomic absorption spectrophotometer (AAS).

3.7.1 Principle

The technique based on absorption spectrometry to assess the concentration of an analyte in particular sample. Therefore it requires standards with known analyte concentration to set up the relation between the measured absorbance and analyte concentration and follows Beer-Lambert Law. Basically, the electrons of the atoms in the atomizer can be excited to higher orbitals for a short duration of time by absorbing a defined quantity of energy. This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element

3.7.2 Procedure and Sample Preparation

The leaves and shoots of 30, 45 and 60 days old plants of *B. juncea* L. were harvested. The collected samples were oven dried at 80°C for 24 hours. The dried samples (1g) of *B. juncea* were digested in 50ml beakers with 15mL of nitric acid (HNO₃) at 120°C as per standard methods for the examination of water and wastewater
19th Edition, America (APHA, 1995) with minor modifications. The content was evaporated to dryness. The dried sample treated by 3ml of perchloric acid (HClO₄) for further oxidation from the sample solution for 30 min at 200°C. After digestion the content were cooled, filtered and made up to 100mL with distilled water. The heavy metal measurement was performed with a Shimadzu model AA-6300 Atomic Absorption Spectrophotometer (Japan). For arsenic measurements, an on-line hydride generation device was coupled to the AAS (HG-AAS).

Fig 3.7. Analysis of digested samples for metal uptake analysis by AAS.

3.7.3 Reagents
1) Perchloric acid
2) Nitric acid

3.7.4 Calculation

The metal content was determined by calibration with standard curve made with different concentration of metals. All the samples were analyzed with five replicates and whole experiment was repeated twice to obtain statistically significant data.

\[
\text{Metal concentration (mg g}^{-1} \text{ DW)} = \frac{A \times B}{g \text{ (samplletaken)}}
\]

Where A= concentration of metal in digested solution (mg/L)

B= final volume of the digested solution

\[ g = \text{amount of the sample taken.} \]
3.8 STUDIES ON BIOCHEMICAL PARAMETERS

Leaves were harvested from the plants given different treatments on 30th, 45th and 60th days after sowing (DAS) and analyzed for the following biochemical parameters:

1) Protein content

2) Antioxidative enzyme activities
   a) Superoxide dismutase (SOD)
   b) Catalase (CAT)
   c) Guaiacol peroxidase (POD)
   d) Ascorbate peroxidase (APOX)
   e) Glutathione reductase (GR)
   f) Monodehydroascorbate reductase (MDHAR)
   g) Dehydroascorbate reductase (DHAR)

3) Lipid peroxidation and Total osmolytes

3.8.1 Assessment of Biochemical Parameters

To scavenge reactive oxygen species, plants are equipped with very efficient antioxidative defence system which protects them from destructive oxidative burst. Oxidative burst under stress was assessed by studying protein content and antioxidative enzyme activities viz. SOD, CAT, POD, APOX, GR, MDHAR and DHAR in B. juncea leaves using UV-Visible PC Based Double Beam Spectrophotometer (Systronics 2202).

3.8.2 Principle

Spectrophotometric analysis is based upon Lambert–Beer’s law. It states that the quantity of the monochromatic light absorbed through a substance dissolved in a non-absorbing solvent is directly proportional to the concentration of the substance and the path length of the light through the solution. It is commonly written as

\[ A = \varepsilon CL \]

Where \( A \) is absorbance (no units)

\( \varepsilon \) is the molar absorbance coefficient with units of \( l/mol/cm \)

\( L \) is the path length of the sample—that is, the path length of the cuvette in which the sample is put, expressed in cm.

\( C \) is the concentration of the compound in solution, expressed in mol/l
3.8.3 Protein content and Antioxidative enzyme activities

3.8.3.1 Preparation of plant extract

For the estimation of protein and activities of antioxidative enzyme such as superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase, 0.5g of leaves were homogenized in mortar and pestle with 5.0ml of 100mM potassium phosphate buffer at pH 7.0 under ice cold conditions. The homogenate was centrifuged at 15,000g for 20 minutes and the supernatant was used for analysis of protein content and activities of antioxidative enzyme.

3.8.3.2 Protein estimation

Protein estimation was done by following the method of Lowry et al. (1951).

3.8.3.3 Principle

The blue color developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-ciocalteau (FC) reagent by the amino acids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry’s method.

3.8.3.4 Reagents

Reagent A – 2.0% sodium carbonate in 0.1N sodium hydroxide
Reagent B – 0.5% copper sulphate in 1.0% potassium sodium tartarate
Reagent C – 50 ml of reagent A and 1.0 ml of reagent B (prepared prior to use)
Reagent D – FC reagent

Protein solution (stock standard) – 50 mg of BSA dissolved in distilled water and the final volume was made to 50 ml.

Protein working standard solution was prepared by diluting the standard stock solution.

3.8.3.5 Procedure

0.1 ml of the sample and standard were pipetted into a series of test tubes. The volume of 1.0 ml was made up in all test tubes with distilled water. A tube with 1.0 ml of distilled water served as the blank. 5.0 ml of reagent C was added to each tube. After mixing it properly, it was allowed to stand for 10 minutes. Then, 0.5 ml of reagent D was
added, mixed well and incubated at room temperature in the dark for 30 minutes. Blue color was developed. The readings were noted at 660 nm.

3.8.3.6 Calculations

A graph of absorbance vs. concentration for standard solutions of proteins was plotted and the amount of protein in the samples was calculated from the graph. The amount of proteins was expressed as mg/g FW.

3.8.4 Superoxide Dismutase (SOD) (EC. 1.15.1.1)

Superoxide dismutase was estimated according to the methodology proposed by Kono (1978).

3.8.4.1 Principle

The method is based on the principle of the inhibitory effect of SOD on the reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are generated by the autooxidation of hydroxylamine hydrochloride. The reduction of NBT is followed by an absorbance increase at 540 nm.

3.8.4.2 Reagents

- Sodium carbonate buffer – 50 mM, pH 10.0
- Nitroblue tetrazolium (NBT) – 96 µM
- Triton X-100 – 0.6%
- Hydroxylamine hydrochloride (NH₂OH.HCl) – 20 mM, pH 6.0

3.8.4.3 Procedure

In the test cuvette, the reaction mixture containing 1.3 ml sodium carbonate buffer, 500 µl NBT and 100 µl Triton X-100 was taken. The reaction was initiated by the addition of 100 µl hydroxylamine hydrochloride. After 2 minutes, 70 µl of the enzyme extract was added. The percentage inhibition in the rate of NBT reduction was recorded as an increase in absorbance at 540 nm.

3.8.4.4 Calculations

Hydroxylamine hydrochloride is autoxidized by superoxide radicals to nitrite. The addition of NBT induces an increase in absorbance at 540 nm due to the accumulation of blue formazon. With the addition of enzyme SOD, superoxide radicals get trapped and hence there is an inhibition of reduction of NBT to blue formazon. The percent inhibition of NBT reduction was calculated as below:
Material & Methods

\[
\frac{\text{Change in abs./min (blank)} - \text{Change in abs./min (test)}}{\text{Change in abs./min (blank)}} \times 100 = y
\]

\( y \) (\( \% \) of inhibition) is produced by 70 \( \mu l \) of sample.

Hence, 50\% inhibition is produced by \( \frac{50 \times 70}{y} \) = \( z \mu l \) of sample

One unit of the enzyme activity is defined as the enzyme concentration required for inhibiting the absorbance at 540 nm of chromogen production by 50\% in one minute under the assay conditions. SOD activity was expressed as \( \text{SA} = \text{mol UA/mg protein} \).

3.8.5 Catalase (CAT) (EC. 1.11.1.6)

The activity of catalase was determined according to the method of Aebi (1984).

3.8.5.1 Principle

Catalase catalyzes the decomposition of \( \text{H}_2\text{O}_2 \) to give \( \text{H}_2\text{O} \) and \( \text{O}_2 \).

\[ \text{Catalase} \rightarrow \text{2H}_2\text{O} + \text{O}_2 \]

Catalase activity can be measured by following either the decomposition of \( \text{H}_2\text{O}_2 \) or the liberation of \( \text{O}_2 \). The method of choice for biological material is the UV-spectrophotometric method. In the ultraviolet range, \( \text{H}_2\text{O}_2 \) shows a continual increase in absorption with decreasing wavelength. The decomposition of \( \text{H}_2\text{O}_2 \) can be followed directly by the decrease in extinction per unit time at 240 nm. The difference in extinction per unit time is a measure of catalase activity.

3.8.5.2 Reagents

- Phosphate buffer – 100 mM, pH 7.0
- Hydrogen peroxide – 150 mM

3.8.5.3 Procedure

The rate of decomposition of \( \text{H}_2\text{O}_2 \) was followed by decrease in absorbance at 240 nm in a reaction mixture containing 1.5 ml phosphate buffer, 1.2 ml of hydrogen peroxide and 300 \( \mu l \) of enzyme extract.
3.8.5.4 Calculations

One unit of the enzyme activity is calculated as the amount of enzyme required to liberate half the peroxide oxygen from H₂O₂ and calculated from the following equation:

\[
\text{Unit Activity (Units/mg FW)} = \frac{\text{Change in abs./minute} \times \text{Total volume (ml)}}{\text{Ext. coefficient} \times \text{Vol of sample taken (ml)}}
\]

Where, Extinction coefficient = \(6.93 \times 10^{-3} \text{ mM}^{-1} \text{ cm}^{-1}\)

\[
\text{Specific Activity (mol UA/mg protein)} = \frac{\text{Unit Activity (Units/mg FW)}}{\text{Protein Content (mg/g FW)}}
\]

3.8.6 Guaiacol Peroxidase (POD) (EC. 1.11.1.7)

Guaiacol peroxidase was estimated according to the method given by Putter (1974).

3.8.6.1 Principle

Peroxidases catalyze the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc. and in particular o-cresol, pyrogallol, guaiacol etc. Activity of POD can be determined by the decrease of H₂O₂ or the hydrogen donor or the formation of oxidized compound. Usually the third method is employed using different substrates. In this case, guaiacol is used as substrate for the estimation of POD activity. The reaction is given as:

\[
\text{POD} \quad \text{H}_2\text{O}_2 + \text{DH}_2 \rightarrow 2\text{H}_2\text{O} + \text{D}
\]

One mole of H₂O₂ oxidizes one mole of guaiacol and probably more than one compound result from this reaction. Hence, the resulting end product is called guaiacol dehydrogenation product (GDHP) of which rate of formation is a measure of the POD activity and can be determined spectrophotometrically at 436 nm.

3.8.6.2 Reagents

Phosphate buffer – 0.1 M, pH 7.0
Guaiacol solution – 20 mM
H₂O₂ solution – 12.3 mM
3.8.6.3 **Procedure**

In the test cuvette, the reaction mixture comprising of 3.0 ml phosphate buffer, 50 µl guaiacol solution, 100 µl enzyme sample and 30 µl H₂O₂ solution was taken. The rate of formation of GDHP was followed spectrophotometrically at 436 nm.

3.8.6.4 **Calculations**

One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1.0 µM of GDHP/min/g FW. Enzyme activity was calculated as follows:

\[
\text{Unit Activity (Units/mg FW)} = \frac{\text{Change in absorbance/minute} \times \text{Total volume (ml)}}{\text{Extinction coefficient} \times \text{Vol. of sample taken (ml)}}
\]

Where, Extinction coefficient = 25 mM⁻¹ cm⁻¹

\[
\text{Specific Activity (mmol UA/mg protein)} = \frac{\text{Unit Activity (Units/mg FW)}}{\text{Protein Content (mg/g FW)}}
\]

3.8.7 **Ascorbate Peroxidase (APOX) (EC. 1.11.1.11)**

Ascorbate peroxidase activity was estimated according to the method of Nakano and Asada (1981).

3.8.7.1 **Principle**

Ascorbate peroxidase is specific for plants. It catalyzes the reduction of H₂O₂ using the substrate ascorbate. It is present in chloroplasts, cytosol, vacuole and apoplastic space of leaf cell in high concentrations.

\[
\text{APOX}
\]

\[
\text{Ascorbate + H}_2\text{O}_2 \rightarrow \text{dehydroascorbate} + 2\text{H}_2\text{O}
\]

One mole of H₂O₂ oxidizes one mole of ascorbate to produce one mole of dehydroascorbate. The rate of oxidation of ascorbate was followed by decrease in absorbance at 290 nm.

3.8.7.2 **Reagents**

- Phosphate buffer – 100 mM, pH 7.0
- Ascorbate – 5.0 mM
- Hydrogen peroxide – 0.5 mM
3.8.7.3 Procedure

Three (3.0) ml of the reaction mixture consisting of 1.5 ml phosphate buffer, 300 µl ascorbate, 600 µl H₂O₂ and 600 µl enzyme extract was taken and the decrease in absorbance was recorded at 290 nm.

3.8.7.4 Calculations

One unit of the enzyme activity was calculated as the amount of enzyme required to oxidize 1.0 µM of ascorbate/min/g FW. The enzyme activity was calculated from the equation given below:

\[
\text{Unit Activity (Units/mg FW) } = \frac{\text{Change in abs./minute} \times \text{Total volume (ml)}}{\text{Ext.coefficient} \times \text{Vol. of sample taken (ml)}}
\]

Where, Extinction coefficient = 2.8 mM⁻¹ cm⁻¹

\[
\text{Specific Activity (mmol UA/mg protein) } = \frac{\text{Unit Activity (Units/mg FW)}}{\text{Protein Content (mg/g FW)}}
\]

3.8.8 Glutathione Reductase (GR) (EC. 1.6.4.2)

Glutathione reductase activity was determined by following the method of Carlberg and Mannervik (1975).

3.8.8.1 Principle

Glutathione reductase catalyzes the reduction of glutathione disulphide (GSSG) involving the oxidation of NADPH

\[
\text{GR} \\
\text{NADPH + H}^+ + \text{GSSG} \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The above reaction is shown as reversible, but the reaction forming reduced glutathione (GSH) is strongly favored. Catalytic activity is measured by following the decrease in absorbance due to the oxidation of NADPH.

3.8.8.2 Reagents

- Phosphate buffer – 50 mM, pH 7.6
- EDTA disodium salt– 3.0 mM
- NADPH – 0.1 mM
- GSSG – 1.0 mM
3.8.8.3 Procedure

GR activity was determined by measuring the oxidation of NADPH at 340 nm in a reaction mixture containing 1.8 ml phosphate buffer, 300 µl each of EDTA, NADPH, oxidized glutathione (GSSG) and enzyme extract. The decrease in absorbance per minute was followed at 340 nm.

3.8.8.4 Calculation

One unit of the enzyme activity is defined as the amount of enzyme required to oxidize 1.0 µM of NADPH/min/g FW. The enzyme activity was calculated by using the equation given below:

\[
\text{Unit Activity (Units/min g FW)} = \frac{\text{Change in abs./minute} \times \text{Total volume (ml)}}{\text{Extinction coefficient} \times \text{Vol of sample taken (ml)}}
\]

Where, Extinction coefficient = 6.22 mM⁻¹cm⁻¹

\[
\text{Specific Activity (mmol UA/mg protein)} = \frac{\text{Unit Activity (Units/min g FW)}}{\text{Protein Content (mg/g FW)}}
\]

3.8.9 Monodehydroascorbate Reductase (MDHAR) (EC. 1.1.5.4)

Monodehydroascorbate reductase activity was determined according to the method of Hossain et al. (1984).

3.8.9.1 Principle

Monodehydroascorbate reductase catalyzes the reduction of monodehydroascorbate involving the oxidation of NADH to form ascorbate.

\[
\text{MDHAR} \quad \text{Monodehydroascorbate} + 2\text{NADH} \rightarrow \text{Ascorbate} + 2\text{NAD}^+
\]

3.8.9.2 Reagents

- Phosphate buffer – 150 mM, pH 7.5
- EDTA disodium salt – 0.1mM
- Triton X-100 – 0.25%
- Ascorbate – 30 mM
- NADH – 3.0 mM
- Ascorbate oxidase – 0.25 units
3.8.9.3 Procedure

MDHAR activity was determined by measuring the oxidation of NADH at 340 nm in a reaction mixture containing 1.8 ml of phosphate buffer, 300 µl EDTA, 200 µl NADH, 250 µl ascorbate, 0.25 units ascorbate oxidase and 300 µl enzyme extract. The reaction was followed by measuring the decrease in absorbance at 340nm.

3.8.9.4 Calculations

One unit of the enzyme activity is defined as the amount of enzyme required to oxidize 1.0 µM of NADH/min/g FW and was calculated using the equation given below:

\[
\text{Unit Activity (UA/min/gFW)} = \frac{\text{Change in abs./minute} \times \text{Total volume (ml)}}{\text{Extinction coefficient} \times \text{Vol. of sample taken (ml)}}
\]

Where, Extinction coefficient = 6.22 mM\(^{-1}\)cm\(^{-1}\)

\[
\text{Specific Activity (mmol UA/mg protein)} = \frac{\text{Unit Activity (Units/min/gFW)}}{\text{Protein Content (mg/g FW)}}
\]

3.8.10 Dehydroascorbate Reductase (DHAR) (EC. 1.8.5.1)

Dehydroascorbate reductase activity was measured by following the method given by Dalton et al. (1986).

3.8.10.1 Principle

Dehydroascorbate reductase catalyzes the reduction of dehydroascorbate involving the oxidation of reduced glutathione (GSH) to form ascorbate and glutathione disulphide.

\[
\text{DHAR} \quad \text{Dehydroascorbate} + 2\text{GSH} \rightarrow \text{Ascorbate} + \text{GSSG}
\]

3.8.10.2 Reagents

- Phosphate buffer – 100 mM, pH 7.0
- EDTA – 1.0 mM
- Reduced glutathione – 15 mM
- Dehydroascorbate – 2.0 mM

3.8.10.3 Procedure

The assay mixture consisted of 1.5 ml phosphate buffer, 300 µl EDTA, 500 µl reduced glutathione, 300 µl dehydroascorbate and 400 µl enzyme extract. The increase in absorbance was recorded at 265 nm.
3.8.10.4 Calculations

One unit of the enzyme activity is defined as the amount of enzyme catalyzing the formation of 1.0 μM of ascorbate/min/g FW. The enzyme activity was calculated by using the equation given below:

\[
\text{Unit Activity (Units/min/g FW)} = \frac{\text{Change in abs./minute} \times \text{Total volume (ml)}}{\text{Extinction coefficient} \times \text{Vol. of sample taken (ml)}}
\]

Where, Extinction coefficient = 14mM⁻¹cm⁻¹

\[
\text{Specific Activity (mmol UA/mg protein)} = \frac{\text{Unit Activity (Units/min/g FW)}}{\text{Protein content (mg/g FW)}}
\]

3.9 LIPID PEROXIDATION

Peroxidation of lipid was determined in the terms of malondialdehyde content by following the method proposed by Heath and Packer (1968).

3.9.1 Principle

It is based on the principle that abstraction of hydrogen atom from an unsaturated fatty acid starts lipid peroxidation, resulting in the formation of lipid radical. Lipid radical when attacked by molecular oxygen get converted to lipid peroxy radical, which starts chain reaction forming lipid peroxides. Malondialdehyde (MDA) is produced as a result of this reaction. Thiobarbituric acid (TBA) forms an adduct with MDA, which absorbs at 532nm. This adduct is used as an index to determine the extent of peroxide reaction.

3.9.2 Reagents

- Trichloroacetic acid (TCA) - 0.1%
- Thiobarbituric acid (TBA) – 0.5% in 20% TCA

3.9.3 Procedure

One gram of seedling tissue of 7 days old seedlings undergone different treatments of heavy metals and 24-EBL were homogenized in 3 ml of 0.1% TCA. Homogenized samples were centrifuged at 10000 rpm for 5 minutes. Supernatant was treated with 3 ml of TBA and kept in water bath at 95 C for 30 minutes. Then it was cooled quickly to stop the reaction. MDA content was determined after subtracting the optical density for non-specific absorbance (600 nm) from the absorbance values at 532 nm.
3.9.4 Calculation

The extent of lipid peroxidation in the seedlings was determined from the amount of MDA formed and expressed as µ mol g\(^{-1}\) FW. The concentration of MDA was calculated as follows:

\[
\text{MDA} = \frac{\text{Absorbance} \times \text{Total volume (ml)} \times 1000}{\text{Extinction coefficient} \times \text{Vol. of sample (ml)} \times \text{Wt. of plant tissue}}
\]

Where, Extinction Coefficient = 155 mM\(^{-1}\)cm\(^{-1}\)

3.10 VAPOR PRESSURE OSMOMETRY

Osmolytes are a class of total soluble compounds, ubiquitously used by living organisms to respond to cellular stress or to fine-tune molecular properties in the cell (Harries and Rösgen, 2008). The function of all these osmolytes is to give protection under osmotic stress. The dependence of water activity on the concentration of additives can be measured through the solution colligative properties mainly freezing point, boiling point, osmotic pressure and vapor pressure.

3.10.1 Principle

The vapor pressure can be measured through vapor pressure osmometer (VPO) or dew point depression. The vapor pressure over a solution is a colligative property, i.e., the concentration of solutes is determined and is termed as osmolality. The output of VPO is the osmolality (Osm) of the solution on the molal concentration scale (moles per kilogram of water), which is defined as:

\[
\text{Osm} = -\ln a_w \frac{M_w}{1000}
\]

Where \(a_w\) is the water activity and \(M_w\) is the molecular weight of water (18 g/mol), and 1000 is the conversion factor between moles per kilogram and moles per gram. The corresponding osmotic coefficient \((\theta)\) is:

\[
\theta = \frac{\text{Osm}}{M}
\]

Where \(M\) is the total molality. 10 μl of plant-sap was measured by using Wescor Vapor Pressure Osmometer 5600 and expressed as milliOsmoles/kg (mOsm kg\(^{-1}\)). These
concentration units are similar to molality (mmol kg\(^{-1}\)) except the identity of the solutes are not known, and dissociations of ions count as multiple solutes. Thus, mOsm kg\(^{-1}\) is a measure of the total number of solute particles in a given sample.

3.10.2 Procedure

i. On 30, 45 and 60\(^{th}\) day, plants treated with different concentrations of Ni, Cr and As metal were harvested.

ii. The harvested plant sample was immediately kept in liquid nitrogen followed by its storage at -80\(^{\circ}\)C for 2 hours.

iii. After 2 hours sample stored at -80\(^{\circ}\)C was taken out and the leaves were allowed to thaw. To extract the sap from thawed leaves, a plastic syringe without needle was loaded with these leaves and squeezed till drops ooze from the end.

iv. The sap was collected in the eppendorf tubes and was further stored in a deep freezer at -20\(^{\circ}\)C.

v. For the VPO measurement, the extracted sap sample (10 μl) was pipetted onto a filter paper that slides into the instrument.

vi. Before sample analysis, the instrument was first calibrated using standard solutions of NaCl of osmolalities 100, 290, and 1000 mOsm and readings were taken at 25\(^{\circ}\)C.

3.10.3 Calculations

The experiment was carried out in triplicates and for each replication three observations were recorded. The average mean was calculated for each observation and data is presented in terms of mean ± standard error (SE) as mOsm\(^{-1}\)kg.

3.11 BIOACTIVITIES ANALYSIS:

The isolated BRs from B. juncea plants grown under different metals stress viz. Ni, Cr and As were evaluated for their bioactivities analysis in terms of their potential application in the field of Medicine. The bioactivities employed in the present study include:

1) Antioxidants assays:
   a) DPPH
   b) Reducing Power assay
   c) Molybedate Ion reduction assay
2) Antiproliferative studies employing
   a) SRB assay
   b) MTT assay

3.11.1 ANTIOXIDANT ASSAYS:

3.11.1.1 DPPH Free Radical Scavenging Assay

The method given by Blois (1958) was followed to determine the hydrogen donating capacity of extract and fractions using 1, 1-diphenyl-1-picrylhydrazyl radical as a substrate.

3.11.1.1.1 Principle

DPPH assay is the preliminary test to assess the hydrogen donating capacity of methanol extracts of bark and leaves and their fractions. It offers an accurate and convenient method for determining antioxidant capacity due to relatively short time required for analysis. The methanolic solution of DPPH is a stable radical which shows peak absorbance at 517nm. The absorbance disappears due to reduction of 1, 1-diphenyl-1-picrylhydrazyl radical (purple coloured solution) to 1, 1-diphenyl-1-picryl hydrazine (yellow coloured solution), a diamagnetic stable molecule either in the presence of antioxidant or due to reaction with free radical species (Espin et al., 2000; Huang et al., 2005). The DPPH solution containing the solvent in which fractions or extracts were dissolved was used as control.

![Fig. 3.8 Mechanism of action involved in DPPH assay.](image)
3.11.1.1.2 Reagent

DPPH free radical solution- 0.1 mM in Methanol

3.11.1.1.3 Procedure

In 2ml 0.1mM DPPH solution 300 µl of various concentrations of compounds (12.5, 25, 50, 100 µg/ml) or the reference compound were added. After 30 min of incubation at room temperature, absorbance was measured at 517nm. Ascorbic acid was used as positive control. All tests were performed in triplicate.

3.11.1.1.4 Calculation

Percent inhibition of DPPH radicals was calculated by comparing the absorbance values of control and samples using the following equation:

\[
\text{Percent inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100
\]

3.11.1.2 Ferric Ion Reducing Power Assay

The reducing power of different fractions was determined by the method of Oyaizu (1986).

3.11.1.2.1 Principle

The reducing power assay was used to assess the reduction potential of fractions as this assay involves the reduction of ferricyanide ion i.e. \([\text{Fe(CN)}_6]^{3-}\) to ferrocyanide ion i.e. \([\text{Fe(CN)}_6]^{4-}\) by electron donation from polyphenols. The ferrocyanide ions combine with Fe (III) in acidic medium to give a prussian blue complex i.e. ferriferrocyanide complex, \(\text{Fe}_4[\text{Fe(CN)}_6]_3\), the intensity of which is measured spectrophotometrically at 700nm (Graham, 1992). The intensity of coloured complex increases with the electron or H donating ability of extract and its different fractions. The redox reaction may be summarized as follows:

\[
\text{AA} + 2 \text{Fe(CN)}_6^{3-} \rightarrow \text{AA oxidized} + 2 \text{Fe(CN)}_6^{4-}
\]

\[
2 \text{Fe(CN)}_6^{4-} + 4\text{Fe}^{3+} \rightarrow \text{Fe}_4[\text{Fe(CN)}_6]_3 \text{Ferric ferrocyanide (Prussian blue)}
\]

Fig: 3.9 Mechanism of action of polyphenols in reducing power assay
3.11.1.2.2 Reagents

Phosphate buffer – 200 mM, pH 6.6
Potassium ferricyanide – 1%
Trichloroacetic acid (TCA) – 10%
Ferric chloride – 0.1%

3.11.1.2.3 Procedure

1ml of extract of different concentrations was mixed with 2.5ml of phosphate buffer (200mM, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes. A volume of 2.5ml of 10% TCA was then added to the mixture and centrifuged at 3000 rpm for 10 minutes. 2.5ml of supernatant was mixed with 2.5ml of distilled water and 0.5ml of FeCl₃(0.1%) and the absorbance was measured spectrophotometrically at 700nm. Increase in absorbance of the mixture was interpreted as increase in reducing activity of extract and the results were compared with gallic acid that was used as a positive control.

3.11.1.3 Molybdate Ion Reduction Assay

The tendency of plant extract and fractions to reduce molybdate ion was determined according to the method given by Prieto et al. (1999).

3.11.1.3.1 Principle

The method is based on the reduction of Mo (VI) to Mo (V) by extract and fractions.

\[
\begin{align*}
\text{Mo}^{6+} + \text{A} & \rightarrow \text{Mo}^{5+} + \text{A}_{\text{oxidized}} \\
\text{Mo}^{5+} + \text{PO}_4^{3-} & \rightarrow \text{Mo}_3(\text{PO}_4)_5
\end{align*}
\]

Green Coloured Complex \( (\lambda_{\text{max}} = 695\text{nm}) \)

**Fig. Mechanism of action involved in molybdate reduction assay**

The phosphomolybdenum method is quantitative one to determine the antioxidant activity in terms of reduction of molybdate ions. The antioxidant activity is expressed in terms of ascorbic acid equivalents as ascorbic acid is used to plot standard curve.
3.11.3.2 Reagents

- $\text{H}_2\text{SO}_4 - 0.6 \text{ M}$
- Sodium Phosphate – 28 mM
- Ammonium molybdate – 4 mM

3.11.3.3 Procedure

It involves the mixing of 0.3ml of sample solution (12.5, 25, 50, 100µg/ml) with 3ml of reagent solution comprising of 0.6M $\text{H}_2\text{SO}_4$, 28mM sodium phosphate and 4mM ammonium molybdate. The mixture was incubated at 95°C for 90 minutes and then cooled to room temperature. The absorbance was measured at 695nm against blank.

3.11.3.4 Calculations

$$\text{Percent reduction} = \frac{\text{Absorbance of test sample} - \text{Absorbance of control}}{\text{Absorbance of ascorbic acid} - \text{Absorbance of control}} \times 100$$

3.11.2 ANTIPROLIFERATIVE STUDIES:

3.11.2.1 Sulphorhodamine Bioassay:

3.11.2.1.1 Culturing of cell lines

The human cancer cell lines were procured from National Cancer Institute, Frederick, U.S.A. Cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2mM glutamine, pH 7.4, supplemented with 10% fetal calf serum, 100 µg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator [37°C, 5% CO$_2$, 90% relative humidity (RH)]. The cells at subconfluent stage were harvested from the flask by treatment with trypsin [0.05% in PBS (pH 7.4) containing 0.02% EDTA]. Cells with viability of more than 98% as determined by trypan blue exclusion, were used for determination of cytotoxicity. The cell suspension of 1 x 10$^5$ cells/ml was prepared in complete growth medium.

Stock solutions (2 x 10$^{-2}$ M and 100µg/ml) of compounds were prepared in DMSO. The stock solutions were serially diluted with complete growth medium containing 50µg/ml of gentamycin to obtain working test solutions of required concentrations.
3.11.2.1.2 Principle

Sulphorhodamine B (SRB) is a bright pink aminoxanthene dye with two sulphonic groups. Under mildly acidic conditions, SRB binds to protein basic amino acids in TCA fixed cells to provide a sensitive index of cellular protein content, which is directly proportional to cell viability. The SRB assay provides a sensitive measure of drug induced cytotoxicity and is useful in quantitating clonogenicity and is well suited to high volume, automated drug screening.

3.11.2.1.3 Reagents

1. PBS (phosphate buffered saline)
2. 40% TCA
3. Sulphorhodamine B - 0.4% in 1% TCA
4. 1% acetic acid
5. 10mM Tris (pH 10.5)

3.11.2.1.4 Procedure

In vitro cytotoxicity against six human cancer cell lines was determined (Monks et al., 1991) using 96-well tissue culture plates. The 100µl of cell suspension was added to each well of the 96-well tissue culture plate. The cells were allowed to grow in carbon dioxide incubator (37°C, 5% CO₂, 90% RH) for 24 hours. Test materials in complete growth medium (100µl) were added after 24 hours of incubation to the wells containing cell suspension. The plates were further incubated for 48 hours in a carbon dioxide incubator. The cell growth was stopped by gently layering trichloroacetic acid (50%, 50µl) on top of the medium in all the wells. The plates were incubated at 4°C for one
hour to fix the cells attached to the bottom of the wells. The liquid of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove trichloroacetic acid, growth medium low molecular weight metabolites, serum proteins etc and air-dried. The plates were stained with Sulforhodamine B dye (0.4 % in 1% acetic acid, 100µl) for 30 minutes. The plates were washed five times with 1% acetic acid and then air-dried (Skehan et al., 1990). The adsorbed dye was dissolved in Tris-HCl Buffer (100 µl, 0.01M, pH 10.4) and plates were gently stirred for 10 minutes on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm.

3.11.2.1.5 Calculations

The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.

\[
\text{Percent cell inhibition} = \frac{(1 - \text{abs test sample} - \text{Mean abs control sample} - \text{abs blank})}{\text{Mean (abs control – abs blank)}} \times 100
\]

3.11.2.2 MTT assay

The extent of cytotoxicity of the isolated BRS in cancerous cells both in the presence and the absence of the extract was determined by the MTT dye reduction assay as described by Igarashi and Miyazawa (2001).

3.11.2.2.1 Principle

The 2-(4,4-dimethyl-2-tetrazoyl)-2,5-diphenyl-2,4-tetrazolium salt (MTT) is converted into its formazon derivative by living cells. The amount of formazon formed is a measure of the number of surviving cells. After solubilisation of the formazon in a suitable solvent, the cell viability can be measured in a microtitre plate reader.

3.11.2.2.2 Reagents

1. PBS (phosphate buffered saline)
2. MTT – 3mg/ml in PBS
3. Isopropanol in 0.04N HCl (acid-propanol)
3.11.2.2.3 Culturing of Cell lines

Rat C6 glioma and MCF-7 cell lines was obtained from NCCS, Pune, India. The cell lines were maintained on Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with streptomycin (100Uml⁻¹), gentamycin (100μgml⁻¹), 10% FCS (Himedia) at 37°C and humid environment containing 5% CO₂. Cultures at 30–40% confluency were treated with extracts for 72 hours. The medium of control culture was replaced with a fresh one.

3.11.2.2.4 Procedure

The treated cells (100 μl), were incubated with 50μl of MTT at 37°C for 3 hours. After incubation, 200μl of PBS was added to all the samples. The liquid was then carefully aspirated. Then 200μl of acid-propanol was added and left overnight in the dark. The absorbance was read at 650nm in a micro titer plate reader (Anthos 2020, Austria). The optical density of the control cells were fixed to be 100% viability and the per cent viability of the cells in the other treatment groups were calculated.

3.11.2.2.5 Calculations

The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.
Material & Methods

Percent cell inhibition = \frac{(1 - \text{abs test sample} - \text{Mean abs control sample} - \text{abs blank})}{\text{Mean (abs control – abs blank)}} \times 100

3.12 STATISTICAL ANALYSIS

All data were subjected to one-way analysis of variance (ANOVA) for scrutinizing the effect of heavy metal (Ni, Cr and As) on various morphological and biochemical parameters and expressed as the mean ± standard error of five replicates. The *Fisher LSD post hoc test* (*p* ≤ 0.05) was applied for the comparisons against control values using SigmaStat Version 3.5.

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