DISCUSSION

The world population is escalating at an alarming rate and on the contrary, there is no prospect of increasing the arable land due to urbanization, industrialization as well as water scarcity in many developing countries. Further, massive increase in the degradation of soil environment due to deposition of pollutants renders the soil unfit to support any plant or animal life. Presence of excess salts and heavy metals induce abiotic stress in plants (Jithesh et al., 2006). The term plant stress is used in a very broad sense. Selye (1936) developed the original general stress concept for living organisms. Levitt (1980) has defined stress as any environmental factor which is potentially unfavourable to living organisms. The plant ecophysiologist Larcher (1987) summarized the stress concept of plants as “State in which increasing demands made upon a plant lead to an initial destabilization of functions, followed by normalization and improved resistance” and also “If the limits of tolerance are exceeded and the adaptive capacity is overworked, the result may be permanent damage or even death.” He also stated that stress consists of both destructive and constructive elements. Stress is both a selection factor and a driving force for improved resistance and adaptive evolution.

Among various stresses being faced by plants, the stress induced by the presence of heavy metals in the ambient soil environment poses a major threat to survival of plants. In agriculture and forestry, high metal availability arising from mining and industrial activities and disposal of sewage sludge or soil acidification, is an increasing problem. On the basis of solubility under physiological conditions, 17 heavy metals are available for living cells and have importance for organisms and ecosystems (Weast, 1984). Out of these, Fe, Mo and Mn are micronutrients; Zn, Ni, V, Co, Cu and Cr are required as trace elements and are toxic at higher concentrations; As Hg, Cd, Pb, U do not have any function as nutrients and are toxic to plants (Schützendübel and Polle, 2002). If toxic heavy metals are accumulated in plentiful amounts in the plants, the absorption and transport of essential elements gets adversely affected. It further disturbs the metabolism, and has an impact on growth and reproduction (Xu and Shi, 2000). Metals affect numerous biochemical and physiological processes in plants. The phytotoxicity caused by heavy metal results from alterations of various physiological processes caused at cellular/molecular level. These inactivate enzymes, block the
functional groups of metabolically important molecules, substitute or displace the essential elements and disrupt membrane integrity (Rascio and Navari-Izzo, 2011). The consequences are changes in membrane permeability (Llamas et al., 2000), water and ion uptake (Barcelo and Poschenrieder, 1990; Cseh et al., 2000), transport and translocation (Keller et al., 2000), transpiration (Chugh and Sawhney, 1999), root exudation, enzyme activities (Gabbirelli et al., 1999), nitrogen metabolism (Hernandez et al., 1997), photosynthetic processes (electron transport, fluorescence induction, phosphorylation, CO₂ fixation) (Gadallah, 1995; Kastori et al., 1998), respiration, cell division and expansions, all kinds of synthetic processes and cell homeostasis (Eun et al., 2000; Cseh et al., 2000; Quartacci et al., 2001). The problem of heavy metal pollution is continuously getting worst because of human activities, which has resulted into an intensification of research dealing with the phytotoxicity of these contaminants as well as with the mechanisms used by plants to counteract their detrimental effects (Rascio and Navari-Izzo, 2011).

The common consequence of heavy metal toxicity is the increased production of reactive oxygen species (ROS) due to intervention with electron transport activities, especially that of chloroplast membranes (Pagliano et al., 2006; Rocca et al., 2009). The increase in ROS leads to exposure of cells to oxidative stress resulting in lipid peroxidation, membrane dismantling, ion leakage, biological macromolecule deterioration and DNA-strand cleavage (Quartacci et al., 2001; Navari-Izzo et al., 2008, 2009). Heavy metals (Zn, Mn, Co, Ni; 0, 25, 50, 100 mg l⁻¹ respectively) have been reported to reduce germination in Brassica juncea seedlings. A concentration of 100 mg l⁻¹ had maximum effect on reducing the growth of seedlings (Sharma et al., 2007). Raphanus sativus grown under copper and lead stress elicited an antioxidative response, measured in terms of lipid peroxidation, protein and proline accumulation and peroxidase and proline accumulation and altered protein content (Teklic et al., 2008). The presence of metals in excessive concentrations leads to phytotoxicity through reactions of sulphhydryl groups and nitrogen rich ligands with cations; affinity for reacting with phosphate groups of ADP or ATP; and replacement and deficiency of essential metal ions (Mendoza-Cozatl and Moreno-Sanchez, 2005).

Plants adopt a series of defence mechanisms that control the uptake, accumulation and translocation of heavy metals and detoxify them by the exclusion of the free ionic forms from the cytoplasm (Rascio and Navari-Izzo, 2011). Posing a
hinderance to entrance of heavy metals into root cells through their entrapment in the apoplastic environment by binding them to exuded organic acids is a commonly employed strategy employed by most plants (Watanabe and Osaki, 2002). Further, the heavy metals that manage to enter the plant, are then kept in root cells, where they get detoxified by complexation with organic acids, amino acids or metal-binding peptides and/or sequestered into vacuoles (Hall, 2002). Thus, the translocation of heavy metals to the above-ground organs is restricted and the leaf tissues, and particularly the metabolically active photosynthetic cells get protected from heavy metal damage. A further defence mechanism usually adopted by heavy metal-exposed plants is augmentation of cell antioxidant systems which counteracts oxidative stress (Navari-Izzo et al., 1998; Sgherri et al., 2003).

Enzymes are one of the main targets of heavy metal ions and exposure for a long duration leads to decrease in soil enzyme activity (Tyler et al., 1989). Heavy metals cause toxicity because they lead to generation of reactive oxygen species (ROS) (Pflugmacher, 2004). ROS are partially reduced forms of atmospheric oxygen (O₂). They result from the excitation of O₂ to form singlet oxygen or from the transfer of one, two or three electrons to O₂ to form a superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) or a hydroxyl radical (HO⁻) respectively. These are capable of unrestricted oxidation of various cellular components and can lead to the oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004).

To counteract oxidative stress generated under a variety of abiotic and biotic stresses, plants are equipped with several antioxidants. Antioxidant system comprising of non-enzymatic components includes glutathione, ascorbic acid, phenols and carotenoids and while the enzymatic constituents are catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPOX), glutathione reductase (GR) and mono and dehydroascorbate reductase (MDHAR and DHAR) which are directly involved in the scavenging and neutralization of ROS (Andre et al., 2010). Another strategy of defense to plants includes certain secondary metabolites and PGR’s. They include ABA, ethylene, auxin, jasmonic acid and plant steroids. Plant hormones such as auxins, abscisic acid, polyamines and brassinosteroids regulating key metabolic responses of plant growth and
Fig. 5.1 Heavy metal-mediated ROS induction and damage to the development of higher plants (After Hossain et al., 2012)
development, have also been recently found to work as vital component of stress management. A wide spectrum of physiological responses like cell expansion, vascular differentiation, reproductive development, seed germination, flowering, and fruit set in plants are affected by the exogenous application of BRs (Sharma and Bhardwaj, 2007). BRs are plant-produced compounds which are structurally similar to animal steroid hormones that can function as growth regulators (Khripach et al., 2000). Recently, BRs have been included in the category of phytohormones (Haubrick and Assmann, 2006). The application of BRs affects a broad spectrum of physiological responses like cell expansion, vascular differentiation, reproductive development, seed germination, flowering, and fruit set in plants (Yu et al., 2004; Cao et al., 2005; Montoya et al., 2005). Along with growth-promoting effects, BRs also confer resistance to plants against various abiotic and biotic stresses like heat, drought, heavy metals, infections, pesticides, salt, and even viruses. These modulate the antioxidative defence system of plants facing stress and thus help in stress amelioration (Haubrick and Assmann, 2006; Verma et al., 2011).

BRs affect cell shape and expansion via regulation of microtubule dynamics. 28-HBL and 28-HCS are known to promote cell expansion in *Arabidopsis thaliana* suspension cells by the hyperpolarization of plasma membrane (Zhang et al., 2005). Along with cell expansion, BRs are also reported to increase the rate of cell division. Clouse and Zurek (1991) reported that application of nanomolar concentrations of BRs to cultured cells of *Halianthus tuberosus* stimulated cell division by 50% in the presence of auxin and cytokinin. Nassar (2004) reported induction and elongation of shoots with the application of 28-HBL in combination with auxin to apical meristems of banana cultured *in vitro*. A positive role of BRs in vascular differentiation has also been reported (Clouse and Sasse, 1998). 28-HBL also improved the growth by increasing the net photosynthetic rate, chlorophyll content and the activities of nitrate reductase and carbonic anhydrase in nickel stressed *B. juncea* seedlings (Alam et al., 2007). Further, 28-HBL and 24-EBL enhanced the activities of antioxidant enzymes (CAT, SOD, POD) and proline content to overcome the Al-induced oxidative stress in mung beans (Ali et al., 2008a). The heavy metal stress ameliorative properties of EBL were confirmed in 15 days old *B. juncea* plants by Ali et al. (2008b).
5.1 Heavy metals effect on Morphological parameters of *B. juncea*

HM have been reported to induce oxidative damage to plant cell membranes, ultrastructural changes in organelles, impaired metabolic activities and growth retardations
(Sinha et al., 2009). In the present study, B. juncea plants were subjected to stress caused by presence of Ni, Cr and As metal ions in the soil medium. It resulted in observable changes in plant morphology parameters viz., shoot length and number of leaves as compared to control plants. A number of researches have reported morphological changes in plants which face stress during growth. Pasternak et al., (2005) have observed a decrease in number and size of leaves and the rosette diameter in Cu-treated Arabidopsis. A supplement of 10ppm dose of Cd\(^{+2}\) and Cr\(^{+6}\) to Medicago sativa plants significantly reduced the shoot growth as compared to the control plants with significance level \(P \leq 1\%\). Further, when the concentrations of Cd\(^{+2}\) and Cr\(^{+6}\) were raised to 20 ppm, the shoot size was diminished by 62.0\% and 65.0\%, respectively (Aydinalp and Marinova, 2009). In another study, Cd treatment to Albizia procera (Roxb.) Benth. seedlings showed inhibition of growth and dose dependent decrease in morphological parameters. Cd metal adversely affected root length, shoot length, leaf area and biomass of A. procera (Pandey and Tripathi, 2011).

Presence of Ni ions in soil leads to physiological alterations and varied toxicity symptoms such as chlorosis and necrosis in different plant species (Pandey and Sharma, 2002; Rahman et al., 2005). In the present study, the phytotoxicity of metal at higher doses which resulted in decrease in shoot length and number of leaves during different stages of growth i.e. 30\(^{th}\), 45\(^{th}\) and 60\(^{th}\) day old Brassica plants (Table 4.1 to 4.3; Figs. 4.1 to 4.6). It is also observed that at lower dose of Ni ions (0.2mM), there was increase in shoot length and number of leaves than control plants. This increase was occurred because Ni acted as a micronutrient at lower concentrations (Brown et al., 1988). But it decreased shoot length and number of leaves with higher dose of Ni ions because of its toxicity.

The findings of the study were fully supported by several researchers where the effect of Ni had been studied. Küpper et al. (2001) reported the decrease in shoot dry weight of Thlaspi goesingense, Alyssum bertolonii and Alyssum lesbiacum. Though Ni is an essential metal, yet presence of Ni in an excess amount in the soil might have impaired the nutrient imbalance which leads to toxicity symptoms. Vijayareengan (2004) showed that when four cultivars of blackgram (Vigna mungo (L.) Hepper) were grown in soil amended with nickel (0, 50, 100, 150 and 200 mg kg\(^{-1}\)), it reduced the length of root
and shoot, number of nodules, area of leaves and dry matter yield of root and shoot.

The toxicity caused by Cr has also been studied in many plants. The toxic effects of Cr on growth and development of plants include alterations in the germination process as well as in the growth of roots, stems and leaves. Thus, exposure to high level of Cr affects total dry matter production and yield of plants as it causes deleterious effects on plant physiological processes such as photosynthesis, water relations and mineral nutrition. There are metabolic alterations by Cr exposure in plants either by a direct effect on enzymes and metabolites or by its ability to generate ROS (Shanker et al., 2005).

The treatment of *B. juncea* L. plants with Cr has lead to occurrence toxicity symptoms. There was a notable decrease in the shoot length and number of leaves in all development stages of *Brassica* plants. The dose dependent decrease was noticed in both shoot length and number of leaves and maximum reduction in shoot length and number of leaves was seen at 0.5mM concentration of Cr ions as compared to control plants in all the stages of growth (*Table 4.14 to 4.16; Figs. 4.76 to 4.81*). In 60 days old plants, shoot length was decreased by 1.74 folds and number of leaves by 2.09 folds over the control plants at 0.5mM of Cr ion concentration (*Table 4.16; Figs. 4.80 & 4.81*). The results are in coherence with earlier studies in which the presence of Cr in growth medium lead to inhibition of chlorophyll biosynthesis, chlorosis in young leaves, imbalance of nutrients, wilting of tops, and root injury (Chatterjee and Chatterjee, 2000; Vajpayee et al., 2000; Dixit et al., 2002; Sharma et al., 2003; Scoccianti et al., 2006). A study conducted by Datta et al. (2011) on five cultivares of wheat viz., HD2956, HD2932, DBW14, KO512, WH775 grown under Cr metal stress revealed phytotoxicity of Cr in terms of decrease in rate of germination and reduced root and shoot length as compared to control plants. There are reports in which Cr mostly accumulates in roots and thus in the Cr stress, roots are affected the most (Mohanty and Patra, 2011; Nematshahi et al., 2012; Tang et al., 2012). The affect of Cr on roots might have been the cause of decreased nutrient supply to upper parts of *B. juncea* plants in the present investigation, which resulted a decrease in shoot length and leaf number.

As was the most toxic metal tested in the study and showed maximum decreased shoot length and number of leaves when compared with untreated control plants. Maximum effect has been noticed in higher dose of As ion (0.3mM) in both the cases.
when compared to control plants (Table 4.27 to 4.29; Figs. 4.150 to 4.155). Maximum decrease in overall growth was evident in terms of decrease in shoot length by 4.53 folds and number of leaves by 2.44 were assessed in 60 days old Brassica at higher dose of As ions (0.3mM). It is already reported that the excess of As is harmful to plants as it affects transpiration rate, inhibits root activity, and blocks uptake and transport of water, and essential nutrients etc. In a study conducted on ‘Lankert’ cotton (Gossypium hirsutum L.) and ‘Patterson’ soybeans (Glycine max L. merr.), treatments of As ranged from 28 to 280 kg As/ha in the Amarillo soil and from 56 to 560 kg As/ha in the Houston Black soil. It was noticed that the vegetative cotton yields were considerably decreased. Further, Soybeans were found to be more sensitive to As than cotton. Vegetative soybean yields decreased at the applied rates of 28 kg As/ha in the Amarillo soil as well as 168 kg As/ha in the Houston Black soil (Lloyd E. Deuel and Alien R. Swoboda, 1972).

It has been revealed from studies on As toxicity that plant species, which are not resistant to As, undergo considerable stress upon exposure. The symptoms range from reduced photosynthetic rate (Stoeva et al., 2004) and root growth inhibition to death (Macnair and Cumbes 1987, Paliouris and Hutchinson 1991). The decrease in photosynthetic rate under stress condition can be attributed to both stomatal and mesophyll limitations. There is inhibition of enzyme activity due to inorganic arsenic while trivalent inorganic arsenic reacts with the sulphydryl groups of proteins which affects many enzymes containing such groups. This results in abscission in plant leaves, severe inhibition of plant growth, and steep reduction of biomass (Shao et al., 2011).

5.2 Characterization of BRs in Brassica plants grown under metal stress:

In last four decade, after the finding of brassinolide (BL) (Grove et al, 1979), 70 different brassinosteroids have been isolated from 61 different plants species which includes: 53 angiosperms (12 monocotyledonous and 41 dicotyledonous), 6 gymnosperms, 1 pteridophyte and 1 bryophyte named Equisetum arvense and Marchantia polymorpha (Kutschera and Wang, 2012; Clouse, 2012). Out of these, 65 were free BRs and while 5 are BR conjugates (Bajguz, 2007).

BRs were also detected in insect and crown galls of plants of Japanese chestnut (Castanea crenata) and also been confirmed in two species of freshwater algae (Chlorophyta) (Chlorella vulgaris and Hydrodictyon reticulatum), and one of its
metabolite was present in marine brown alga *Cystoseira myrica* (Bajguz and Tretyn, 2003; Hayat and Ahmad, 2011).

![Figure 5.3. Structure of 5α-cholestane skeleton.](image)

Chemically BRs are hydroxylated derivatives of cholestane and their structural variation occurs by substitution in rings A and B and also in C<sub>17</sub> side-chain (Fig. 5.3). These compounds can be classified as C<sub>27</sub>, C<sub>28</sub>, or C<sub>29</sub> BRs, depending on the length of the side chain (Bajguz and Tretyn, 2003, Bajguz, 2007, Clouse, 2011). BRs are known to be the analogues of animal steroid hormones. These steroidal hormones were isolated from every parts of plant like pollen grains, seeds, stems, leaves, roots, anthers, flowers etc. and highest concentrations were detected in pollen and immature seeds (1–100µgkg<sup>-1</sup> fresh mass) (Konstantinova *et al.*, 2001; Bajguz and Tretyn, 2003).

In present study, plants of *Brassica juncea* L. were analyzed for the synthesis of BRs under metal stress (Ni, Cr & As) during different stages of growth viz. 30, 45 and 60 days. An attempt has been made to characterize different BRs which are expressing themselves under metal stress. Under the metals stress 7 different BRs were isolated and characterize successfully.

Nickel metal stress lead to the expression of 24-EBL in 30 days old plants (Figs. 4.10 to 4.13; Table 4.4) while CS and 28-HCS were synthesised in 45days old plants (Figs. 4.26 to 4.29; Table 4.5) and 24-EBL, CS, TY and DL were present in 60 days old plants (Figs. 4.42 to 4.45; Table 4.6).
The analyses of BRs under Cr metal stress lead to the characterization of 24-Epibrassinolide and Castasterone in 30 days old plant (Figs. 4.88 to 4.89; Table 4.17) and 24-EBL, DL and TE were isolated from 45 days old plants (Figs. 4.102 to 4.104; Table 4.18). In case of 60 days old plants, BRs isolated were 24-EBL, CS and TY (Figs. 4.117 to 4.119; Table 4.19).

The analyses of 30, 45 and 60 days old B. juncea plants for the synthesis of BRs under Arsenic metal stress lead to the isolation and characterization of 24-EBL, CS TY and TE from 30 days old plants (Figs. 4.168 to 4.171; Table 4.30). In 45 days old plants, BRs were CS and TE. Similarly, in 60 days old plants, 24-EBL and CS were present (Figs. 4.184 to 4.185; Table 4.31).

Surfing of literature revealed the scanty information regarding the status of BRs in Crucifereae family. Previously, BRs were reported from Brassica napus L. (Grove et al., 1979), Brassica campestris var. pekinensis L. (Ikekawa et al., 1984), Raphanus sativus L. (Schmidt et al., 1991) and Arabidopsis thaliana (L.) (Nomura et al., 2001) but no report is available regarding their presence in Brassica juncea L. plants (Table 5.1). Therefore finding of the study is first hand report regarding the isolation of BRs from B. juncea.

In our earlier studies, different brassinosteroids were isolated and characterized from leaves and seeds of Camellia sinensis (L.) O. Kuntze. (Bhardwaj et al., 2007) and from the leaves of Aegle marmelos Corr. (Rutaceae) and Centella asiatica (Sondhi et al., 2008, 2010).

Previously, 24-EBL was isolated from Vicia faba L. (Ikekawa et al., 1988), Gypsophila perfoliata L. seed (Schmidt et al., 1996a) and later in Ageale marmelos (Sondhi et al., 2008). Similarly, CS was also isolated from Catharanthus roses G. Don (Fujioka et al., 1995) and from the seeds of Pisum sativum L. (Nomura et al., 2001). Recently, the isolation of CS was done from the seeds of pumkin (Pachtong et al., 2006), Chlorella vulgaris Beijerinck (Trebouxiophyceae) (Bajguz, 2009) and from the leaves of Centella asiatica (Sondhi et al., 2010).

28-HCS from seeds and leaves of Brassica campestris (130 µgKg^{-1} FW) and Thea sinensis L. (<0.001 µg Kg^{-1} FW) was reported by Abe et al. (1983). The presence of 28-HCS in gymnosperm, Cupressus arizonica Greene was also reported. The isolation was done from the pollens of the plant (Griffiths et al., 1995).

The isolation and identification of six brassinosteroids, i.e. 6-deoxocastasterone, 24-epibrassinolide, 3-dehydroteasterone, typhasterol, 3-deoxotyphasterol and 28-
homodolicholide in topmost dormant leaves of tea plants was done by Gupta et al. (2004). The brassinazole treated and untreated shoots of Malus prunifolia revealed the identification and quantification of 8 endogenous BRs viz. TE, TY, CS, 6-deoxocathasterone (6-deoxoCT), 6-deoxoteasterone (6-deoxoTE), 3-dehydro-6-deoxoteasterone (6-deoxo3DT), 6-deoxotyphasterol (6-deoxoTY) and 6-deoxocastasterone (6-deoxoCS) (Netto et al., 2009).

Recent reports indicate that the occurrence of BRs in unicellular organism which are the ancestor of chlorophytes and embryophytes (Kutschera and Wang, 2012). Seven BRs, including teasterone, typhasterol, 6-deoxoteasterone, 6-deoxotyphasterol, 6-deoxocastasterone, castasterone and brassinolide, were identified in the culture of wild-type Chlorella vulgaris (Bajguz, 2009). Recently four BRs (BL, CS, DS, NorCS) have been identified in the pollen grains of adult H. annuus plants (Kurepin et al., 2012).

Naturally occurring BRs (C_{27}, C_{28} and C_{29}) are synthesized from plant sterols including campestrol, sitosterol and cholesterol. C_{28} BRs, which include BL and its precursors CS, TY and TE, are derived from campestrol, whereas C_{27} and C_{29} BRs are derived from sitosterol (Takasuto and Yokota, 1999).

It was also observed from the study that isolated BRs belonged to C_{28} group and were of 6-oxo and 7-oxolactone types (Bjaguz, 2007). It is postulated that 24-Epibrassinolide, Castasterone, Typhasterol, Dolicholide and Teasterone are synthesized during early C_{6} oxidation pathway, suggesting that C_{6} oxidation pathway is operating in B. juncea plants under metal stress.

The standard scheme for the biosynthesis of BRs (C_{28} type) occurred by 2 main route which involves 1) an early C_{6} oxidation pathway and 2) late C_{6} oxidation pathway. These pathways are interlinked at various stages with each other (Fujioka and Yokota, 2003). Biosynthesis of BL and its intermediate products occur from campesterol which is a precursor molecule via early C_{6} oxidation pathway includes following steps:
campesterol → campestanol→ 6-oxocampestanol → cathasterone → teasterone → typhasterol → castasterone → brassinolide.
Table 5.1 Status of Brassinosteroids in *Brassicace* family (modified after Bajguz and Tryten, 2003)

<table>
<thead>
<tr>
<th>Species</th>
<th>Plant part</th>
<th>Brassinosteroids</th>
<th>Isolated Quantity (µg/kg fresh wt.)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis thaliana</em> (L.) Heynh.</td>
<td>Shoot</td>
<td>6-deoxoCT 6-deoxoTY CS 6-deoxoCS 3-dehydro-6-deoxoTE TY 6-deoxoTE BL TE 28-norCS 28-norTY</td>
<td>1.96 0.95 0.75 0.71 0.31 0.11 0.10 0.04 0.025</td>
<td>Fujioka et al. 1996, 1997, 2000a Nomura et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Seeds</td>
<td>6-deoxoCS TY BL 6-deoxoTY 6-deoxoTE CS 24-epiBL</td>
<td>1.5-3 1.3 0.5-1 0.5-5.4 0.5-1 0.4-5 0.22</td>
<td>Fujioka et al. 1998</td>
</tr>
<tr>
<td></td>
<td>Seeds</td>
<td>CS 24-epiBL</td>
<td>0.36 0.22</td>
<td>Schmidt et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>BL 3-epiBL</td>
<td></td>
<td>Konstantinova et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Callus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seedlings</td>
<td>6-deoxoCT 3-epi-6-deoxoCT</td>
<td></td>
<td>Choe et al. 2001 Fujioka et al. 2002</td>
</tr>
<tr>
<td><em>Brassica napus</em> L.</td>
<td>Pollen</td>
<td>BL</td>
<td>100</td>
<td>Grove et al. 1979</td>
</tr>
</tbody>
</table>
In the present study it was also noticed that 24-EBL and CS was frequent BRs isolated from the plants of *B. juncea* grown under different metal stress during different stages of growth followed by TY, TE, DL, 24-EPICS and 28-HCS. The finding of the study was fully supported by the previous findings where the relative abundance of the 70 BRs among different groups of plants was seen (Bajguz, 2007). Among the BRs, it was found that CS is the most widely distributed BR and is reported from 53 different plant species followed by BL which was present in 37 plant species. Similarly, TY were isolated from 24 plant species, TE from 18 plant species (Bajguz and Tryten, 2003; Kutschera and Wang, 2012).

The analysis and characterization of BRs was done with ESI-QTOF-MS/MS and GC-MS (QP-2010, Shimadzu) after making their derivative and silylates and comparing their Rt’s and fragmentation pattern of different BRs with their respective standards. Electrospray mass spectrometry of 24-Epibrassinolide in positive ionization mode showed its molecular ion peak at m/z 481 [M+H]^+. Further MS/MS of m/z 481 of the Bio-active fraction showed the product ions at m/z 445 (M-2H2O+ H), 427 (M-3H2O+ H), 409 (M-4H2O+ H), 363, 349, 315, 225, 125, 113 and 95 similar to that of standard spectra. Similarly analysis 24-EBL by GC-MS showed its molecular peak at 528 (M^+), and other fragmented m/z were 457, 415, 374, 345, 332, 177, 155 & 95.

The isolated CS showed its molecular peak at 512 (M^+). The other fragmented m/z was 415, 356, 287 & 155. On the other hand, 28-HCS gave its molecular peak at 526 (M^+) and other m/z were 441, 399, 358, 287 and 169 corresponding to its standard.

Dolicholide showed its molecular peak at 526 (M^+) and other m/z were 385, 373, 343, 153, 124 & 82 and 24-EPICS showed its molecular ion peak at 512 (M^+) and m/z were 441, 399, 358, 329, 287 & 155. Typhasterol showed its molecular peak at 544 (M^+) and other m/z were 529, 515, 454, 229, 155 & 85. Teasterone showed its molecular ion peak at 544 (M^+) and other m/z were 529, 515, 454, 229, 155 & 107 respectively.
<table>
<thead>
<tr>
<th>A. 24-Epibrassinolide</th>
<th>B. Castasterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. Dolicolide</td>
<td>D. Typasterole</td>
</tr>
<tr>
<td>E. 24-Epicastasterone</td>
<td>F. 28-Homocastasterone</td>
</tr>
</tbody>
</table>

*Fig. 5.4 Structures of Isolated Brassinosteroids from plants of Brassica juncea L. grown under metal stress (Ni, Cr & As).*

5.3 Metal uptake by *B. juncea*

Heavy metals, being nondegradable in nature, keep on cycling from abiotic to biotic components and find their entry into animal systems through food chain. A number of studies keep concern with the problem of the relationship between the increased heavy-metal amounts in nature due to industrial environment and their mutagenic and carcinogenic effects (Fergusson, 1990; Memon *et al.*, 2001). Crops such as spinach, lettuce, carrot, radish, zucchini are reported to accumulate heavy metals, e.g. Cu, Cd, Mn, Pb and Zn in their tissues (Sauerbeck, 1991; Muller and Anke, 1994; Hooda, 1997; Bahemuka and Mubofu, 1999; Cobb *et al.*, 2000; Mattina *et al.*, 2003; Hough *et al.*, 2004; Zhou *et al.*, 2005). The uptake of metals is more in plants that are grown in areas with increased soil contamination. The levels of Ni, Cr and As in background soil, metalliferous (or contaminated) soil, critical load in soil above which biodiversity and ecosystem function are adversely affected are described in Table 5.2.

Plants which are capable of accumulating these metal ions in their shoots or leaves in astonishingly in higher limits than in comparison to other plants are known as metal hyperaccumulators (*Fig. 5.4*). About 450 hyperaccumulator plants are known as metallophytes, consisting of annual herbs to perennial and are spread in all continents including both in temperate and tropical environments (Shah and Nongkynrih, 2007).
Table 5.2 Summary of elemental levels in background soil, metalliferous (or contaminated) soil and critical load in soil above which biodiversity and ecosystem function are adversely affected, and Commission des Communautés Européennes (EU) and Environmental Protection Agency (USA) permissible limits (modified after Peer et al., 2005).

<table>
<thead>
<tr>
<th>Metals</th>
<th>Background Soil Levels (ppm)</th>
<th>Metalliferous Soil Levels (ppm)</th>
<th>Critical Load in loam/silt soil (ppm)</th>
<th>CCE limits (ppm)</th>
<th>EPA limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>4-55</td>
<td>11260</td>
<td>54.64</td>
<td>1</td>
<td>0.7 ppm</td>
</tr>
<tr>
<td>Cr</td>
<td>7-221</td>
<td>3450</td>
<td>64.41</td>
<td>1.5</td>
<td>100 ppb</td>
</tr>
<tr>
<td>As</td>
<td>2.2-25</td>
<td>1510</td>
<td>-</td>
<td>-</td>
<td>0.01 ppm</td>
</tr>
</tbody>
</table>

Therefore, hyperaccumulator plants could possibly be used to remove and stabilize these heavy metals so as to clean up the metal-contaminated soils and this process is known as phytoremediation (Shekhawat et al., 2010). Members of Cruciferae family like B. juncea and R. sativus are well-reported for their high capacity of accumulation of metals or metalloids (Garg and Kataria, 2010; Vamerali et al., 2010). Therefore the study was designed to observe the metal uptake capacity of B. juncea in soil amended with Ni, Cr and As doses.

The observations of the present study revealed the better uptake efficiency of B. juncea plants and metal uptake got increased with increasing dose of metal ions in 30 days old plants in all the three metals and maximum uptake of metals was noticed in higher concentration of respective metals in case of shoots (Tables 4.7, 4.20 & 4.33). Similar trend of uptake was noticed in leaves of 30 days Brassica plants in all the three metal (Tables 4.7, 4.20 & 4.33). In case of 45 days plant again increase in metal uptake was noticed with increasing concentration of Nickel and Chromium in both shoots and leaves of metal treated plants (Tables 4.7 & 4.20). But in case of Arsenic metal trend of uptake remained same but maximum uptake was shown in lower concentration of Arsenic metal in both the cases (Tables 4.33).
Fig 5.4 The mechanism of uptake of heavy metals by the hyperaccumulator plants (After Tangahu et al., 2011)

The metal uptake by plants growing in contaminated soils has been documented by various research groups. A high metal content in wheat, mustard and weed grown in the field irrigated with industrial effluents was reported by Barman et al. (2000).

The two Brassica species viz. Brassica juncea and Brassica carinata of the Indian mustard were grown in an artificially Ni-contaminated soil in order to study the
tolerance ability and Ni accumulation by Panwar et al. (2001). EDTA (chelating agent) was also supplied for promoting metal uptake. Their study showed that Ni concentration was almost double than control plants in both the species. It is also evidenced from their study that *B. juncea* found to be a little tolerant and higher accumulator of Ni ions.

In orchid soil contaminated with lead arsenate pesticides at the Hansford site in Washington State (USA), As and Pb concentrations found exceeded background levels (Yokel and Delistraty, 2003). In Hunan, China, metal contamination of soils and crops by the Chenzhou lead/zinc mine spill was studied by Liu et al. (2005). Certain soils were heavily polluted with As, Cd, Zn, Pb and Cu and the maximum permissible levels for Chinese agricultural soils were highly exceeded, particularly for As and Cd followed by Zn, Pb and Cu. This resulted in hazardous effect on the consumer's health consuming the crops grown in Chenzhou Pb/Zn mine waste affected area. Chaturvedi (2006) carried out a study using two genotypes of *B. juncea* (Varuna and DHR-9504) to check the As uptake. He observed that arsenic extraction by plants increased considerably with escalating concentrations of As in soils. It was cleared from the study that the uptake of arsenite by Indian mustard genotypes was higher than that of arsenate.

Dolenec et al. (2007) revealed that due to irrigation with mine effluents, the paddy soil of the Western part of Kocani Field, Macedonia is severely contaminated with Pb, Zn, As and Cd. The concentration of these metals was detected to be far above the threshold values that were determined in some soils of three towns of coastal Tuscany (Central Italy) by Bretzel and Calderisi (2006). The impact of wastewater irrigation on metal contaminated of *Beta vulgaris* in suburban areas of Varanasi, India was studied by Sharma et al. (2007). They reported that the use of treated and untreated wastewater for irrigation increased the contamination of Ni, Pb and Cd in edible parts of vegetables. Similarly, Srivastava et al. (2005) reported the accumulation of As in three ferns, Chinese brake (*Pteris vittata* L.), slender brake (*Pteris ensiformis* Burm. f.) and Boston fern (*Nephrolepis exaltata* L.) which were exposed to different concentrations of arsenic *i.e.* 0, 150, or 300µM . As uptake increased with an increase in its concentration in the growth medium. Diwan et al. (2010) reported that *B. juncea* accumulated two folds and three folds higher Cr in root and shoot than *Vigna radiate* respectively. A study with seedlings of *B. juncea* suggest that the Na, Mn and K mineral transporter can mediate the
uptake of Cu, and possibly of Mn, Zn and Mg, since mineral treated *Brassica juncea* accumulated more Cu and to a lesser extend Mg, Zn, Mn compared to the control seedlings (Kamaraj *et al.*, 2012).

5.4 **Response of antioxidative enzymes to metal stress in *B. juncea* plants**

Metal toxicity leads to alterations of physiological processes at cellular and molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting for essential elements and disrupting membrane integrity. The most frequent consequence of heavy metal toxicity is enhanced production of reactive oxygen species (ROS) (Pagliano *et al.*, 2006; Rocca *et al.*, 2009). This increase in ROS level exposes cells to oxidative damage leading to lipid peroxidation, biological macromolecule deterioration, membrane dismantling and DNA damage etc (Navari *et al.*, 1999; Quartacci *et al.*, 2001; Kanwar *et al.*, 2012).

The production of ROS in cells, under normal growth conditions is of the order of $240 \mu M s^{-1}$ $O_2^-$ and a steady state level of $0.5 \mu M H_2O_2$ in chloroplasts. However, during stress conditions, cellular homeostasis is disrupted and ROS production gets enhanced to $240-720 \mu M s^{-1}$ $O_2^-$ and a steady state level of 5-15 $\mu M H_2O_2$ (Polle, 2001). The increased production of ROS during stress although pose a threat to cells but it also act as signals for the activation of stress-response and defense pathways (Desikin, *et al.*, 2001; Knight and Knight, 2001). Thus, ROS can be taken as cellular indicators of stress conditions and as secondary messengers involved in the signal transduction pathway of stress-response.

As ROS are toxic, but because of their participation in signalling events, plant cells require at least two different mechanisms to regulate their intracellular ROS concentrations. One includes the fine modulation of low levels of ROS for signalling purposes, and the other enable the detoxification of excess ROS, especially during stress. In addition, the balance between the types of ROS produced and the steady-state levels of different ROS is also important. This is attained by the interplay between different ROS-producing and ROS-scavenging mechanisms. The major ROS-scavenging mechanisms of plants include SOD, found in almost all cellular compartments, the water–water cycle in chloroplasts {Fig. 5.5 (a); the ascorbate–glutathione cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes (b); glutathione peroxidise (c) and CAT in peroxisomes (d)} (Mittler, 2002).
Fig. 5.5 Pathways for reactive oxygen species (ROS) scavenging in plants. (a) The water–water cycle. (b) The ascorbate–glutathione cycle. (c) The glutathione peroxidise (GPX) cycle. (d) Catalase (CAT). Abbreviations: DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferredoxin; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; tAPX, thylakoid-bound APX. (Adapted from Mittler, 2002)
The treatment of *B. juncea* plants with heavy metals in the present finding revealed that metal stress escalated the pool of protein content and antioxidant enzymes and it was found that activities of protein content got increased with increasing concentration of metal in 30 and 45 days old plants (Table 4.8, 4.10, 4.21, 4.23, 4.34 & 4.36). This is in agreement with other reports in literature in which there has been reported an increase in protein content with increased metal stress. In a study conducted on *Lemna gibba* subjected to Mn and Ni, a significant increase in total protein content in plants stressed with excess Mn was observed as compared to the control plants (Doganlar *et al.*, 2012). However, in 60 days old plants, protein content along with the enzyme activity got decreased in all the concentration of all the metals (Table 4.12, 4.25 & 4.38). It is well documented that heavy metal induced increase in the reactive oxygen species can result in deleterious oxidation and degradation of proteins (Spychalla and Desborough, 1990). Both decreases and increases in total protein content have been reported in plants under heavy metal stress (Vajpayee *et al.*, 2000; Lei *et al.*, 2007).

In this study, the decrease in amount of total soluble protein in the 60 days old plants may be attributed to protein degradation due to oxidative damage. However, the increase in total protein content in 30 and 45 days old plants might have been due to the increase of specific stress-related proteins such as enzymes that were involved in antioxidant metabolism and phytochelatin biosynthesis (Lei *et al.*, 2007). Changes in the total soluble protein contents are taken as indicators of the physiological status as well as of the reversible or irreversible metabolic changes of the plant (Piotrowska *et al.*, 2010; Doganlar and Atmaca, 2011).

In the present work, the activities of antioxidative enzymes were modulated in the presence of heavy metals in the growth medium. There was a significant increase in the activity of SOD in metal stressed *B. juncea* plants. SOD is found in all aerobic organisms, where it plays a major role in the defence against toxic reduced oxygen species, which are generated as by products of biological oxidations. The generation of oxygen radical can be further aggravated during adverse environmental conditions and accordingly SOD has been proposed to be important for stress tolerance in plants (Apel and Hirt 2004). The first line of defence of plants is SOD which converts O$_2^-$ into
H$_2$O$_2$ at a very fast rate (Radic et al., 2010). Thus, the alterations in the activity of this enzyme are vital for overcoming the oxidative stress in plants. In the present study, increased SOD activity in Ni, Cr and As stressed plants was probably due to increase in superoxide anions. Maximum activity of SOD has been observed in higher dose of Ni (0.4mM & 0.6mM), Cr (0.5mM) and As (0.3mM) metal during different stages of development in comparison to control plants (Table 4.8, 4.10, 4.12, 4.21, 4.23, 4.25, 4.34, 4.36 & 4.38). An increase in the foliar SOD activity has been observed in many stress situations (Pastori et al., 2000). In salt stressed Catharanthus plants, alleviation of oxidative damage was reported through the action of SOD in all parts (Jaleel et al., 2007, 2008). Similar observations have been reported by Gao et al. (2009), where heavy metals induced increased expression of SOD genes in cucumber seedlings. Similarly, the stimulating effects of heavy metals on SOD activity have been reported in Elodea canadensis, Vitis vinifera, Oryza sativa and Cucumis sativus (Maleva et al., 2009, Mour et al., 2011, Srivastava and Dubey, 2011).

Further, it has been observed in the present piece of work that presence of metal ions in the substrate lead to an increase in the activities of CAT in 30 and 45 days old B. juncea plants treated Ni (Table 4.8 & 4.10), Cr (Table 4.21 & 4.23) and As (Table 4.34 & 4.36) compared to untreated plants, while there was a decrease in the activity among 60 days old plants raised in three different metals (Table 4.12, 4.25 & 4.38). This may be attributed to the fact that scavenging function of enzymes might have impaired with prolonged metal stress. The observations are in concordance with earlier reports where an early increase in CAT and SOD activities in tomato leaves was observed after 6h of Cd and Cu exposure which decreased significantly afterwards (Chamseddine et al. 2009). Similar reports with respect to unaltered CAT activity in sunflower plants grown under metal stress have been published by Laspina et al. (2005), García et al. (2006) and Wójcik et al. (2006).

Similarly, other antioxidative enzymes viz., POD (4.8, 4.10, 4.12, 4.21, 4.23, 4.25, 4.34, 4.36 & 4.38), APOX (4.8, 4.10, 4.12, 4.21, 4.23, 4.25, 4.34, 4.36 & 4.38), DHAR (Table 4.9, 4.11, 4.13, 4.22, 4.24, 4.26, 4.35, 4.37 & 4.39) and MDHAR (Table 4.9, 4.11, 4.13, 4.22, 4.24, 4.26, 4.35, 4.37 & 4.39), where alteration in activity has been observed in metal stressed plants as compared to control plants. APOX is among
important antioxidant enzymes of plants that detoxify $\text{H}_2\text{O}_2$ using ascorbate for reduction. In chloroplasts, cytosol and microsomes different isoforms of APOX are active. Its activity increases in response to a variety of biotic and abiotic stresses in different plant species (Asada, 1999). In combination with the effective ascorbate–glutathione cycle, it functions to prevent the accumulation of toxic levels of $\text{H}_2\text{O}_2$ in photosynthetic organisms (Asada, 1999; Shigeoka et al., 2002). CAT is tetrameric heme containing enzymes which catalyzes the dismutation of $\text{H}_2\text{O}_2$ into water and oxygen. Ascorbate peroxidase utilizes $\text{H}_2\text{O}_2$ to oxidize AA to MDHA radical, which disproportionates to DHA nonenzymatically. At the expense of nicotinamide adenine dinucleotide phosphate (NAD(P)H), MDHAR regenerates AA and DHAR regenerates AA utilizing GSH to form GSSG. Further, GSH is regenerated at the expense of NADPH by the action of GR, until getting the rate of limiting step of the cycle (Hoekstra et al., 2001; Gopi et al., 2007). APOX and CAT represent the major enzymes of $\text{H}_2\text{O}_2$ degradation (Vanacker et al., 1998).

In contrast to CAT, APX and GPX require an ascorbate (AsA) and/or a glutathione (GSH) regenerating cycle (Fig. 5.5 a–c). This cycle uses electrons directly from the photosynthetic apparatus (Fig. 5.5. a) or NAD(P)H (Fig. 5.5. b-c) as reducing power. Along with enzymes of the antioxidative defence system in plants, the non-enzymatic radical-scavengers such as ascorbate and glutathione have also been interpreted as the key antioxidants for the removal of $\text{H}_2\text{O}_2$ in plant cells, thus reducing the accumulation of the free radicals (Van and Clijster, 1990; Foyer, 1993; Gupta et al., 1999).

The increased activities of $\text{H}_2\text{O}_2$ scavenging enzymes POX and APOX in excess metal treated plants indicate induction of defense mechanism to protect the cell from ROS as a result of supply of the excess metal. These enzymes, responsible for quenching $\text{H}_2\text{O}_2$, are subsequently produced by SOD activity (Li et al., 2005). The high level of endogenous APOX is essential and effectively maintains the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses (Shigeoka et al., 2002). The increase in the activity of POX might result due to peroxidative damages of the thylakoid membrane or lower auxin and protein content or high phenol content in tissues inhibiting the growth of plants.
Modulation in the activities of antioxidative enzymes in seedlings of *Kandelia candel* and *Bruguiera gymnorrhiza*, grown under multiple heavy metal stresses have been reported by Zhang *et al.* (2007). An increase in activity of enzymes using ascorbate and glutathione as substrates by the Halliwell–Asada pathway (MDHAR, DHAR, APOX and GR) was observed in sunflower plants cultivated on the metal-contaminated soil in comparison to the control soil (Nehnevajova *et al.*, 2012). The activities of GPOX, SOD and CAT have been reported to be enhances in *Raphanus sativus* seedlings subjected to Cr stress (Cahoudhary *et al.*, 2012).

Plant cell membranes are usually considered to be chief sites of metal injury. Membrane destabilization is often attributed to lipid peroxidation due to an enhanced production of oxygen free radicals after being exposed to metal (Srivastava *et al.*, 2005). Malondialdehyde (MDA) is a cytotoxic byproduct of lipid peroxidation and serves as a marker of free radical generation and tissue damage in plants and animals (Wood *et al.*, 2006; Zhang *et al.*, 2008). In present study lipid peroxidation was assessed in terms of MDA content. It was found that MDA content goes on increasing with the increasing dose of Ni (*Table 4.9, 4.11 & 4.13*), Cr (*Table 4.22, 4.24 & 4.26*) and As (*Table 4.35, 4.37 & 4.39*) during different stages of growth and development.

Tomato leaves exposed to Cu or Cd stress at 25µM for 96 hours, showed increased activities of SOD, POD, CAT, APOX and MDA content (Chamseddine *et al.*, 2009). Seedlings of *Arabidopsis thaliana* treated with 50 and 100 µM of CdCl₂ showed enhanced activities of CAT, POD, SOD and MDA levels (Saffar *et al.*, 2009).

There is accumulation of osmolytes under osmotic stress in addition to antioxidant enzymes. Osmolytes are small, electrically neutral molecules and are non toxic even at high concentrations. As per Somero *et al.* (1992) osmolytes include amino acids (e.g. proline); quaternary ammonium compounds (e.g. glycine–betaine) and diverse sugar alcohols and sugars (e.g. sorbitol, sucrose and trehalose). Osmolytes (proline, glycine-betaine and sucrose) contribute to the defense mechanism probably by providing metabolic protection to the cellular machinery. This might be the reason for an increase in osmolalities of *B. juncea* plants raised in Ni (*Table 4.9, 4.11 & 4.13*), Cr (*Table 4.22, 4.24 & 4.26*) and As (*Table 4.35, 4.37 & 4.39*) in the current investigation. Similar
reports are there where pesticide stress has lead to an increase in osmolytes in Cyanobacterium (Habib et al., 2011).

5.5 Bioactivities of BRs

Cancer is the second-leading disease that causes death worldwide. Since cancer is of many types so no proper curative therapy is still available for it cures (Llanos et al., 2012).

Natural plant products represent a class of drug that have played a noteworthy role in the discovery and development of new anticancer agents (Bhuwan et al., 2011; Newmann et al., 2011; Llanos et al., 2012). Natural products obtained from medicinal plants have proved themselves significant in lowering the risk of cancer. According to the WHO norms, 80% of the world’s populations mainly residing in developing countries are dependent upon on plant-derived medicines for their health. Almost 60% of available drugs for cancer treatment are natural in nature (Gurib-Fakim et al., 2006; Boopathy and Kathiresan, 2010).

Brassica juncea L., an amphidiploid species, is commonly known as Indian mustard or mustard green and is grown as oilseed crop in India with striking medicinal value. It is also used as a folk remedy for arthritis, foot ache, lumbago, and rheumatism (Duke and Wain, 1981). Mustard greens are rich source of vitamin A, vitamin C, iron, calcium and contains novel phytochemicals which are protective against carcinogenesis (Steinmetz and Potter, 1996). Besides other phytohormones, the presence of BRs, a special group of steroidal compounds have been reported from few members of Cruciferae family (Abe et al., 1983, Nomura et al., 2001, Kanwar et al., 2012).

BRs stimulate numerous physiological processes, like cell expansion, cell division, xylem differentiation, proton-pump activity, ethylene biosynthesis and photosynthesis (Sasse 1997; Clouse and Sasse 1998; Yu et al. 2004). In addition, BRs also reported to modulate the plant response to different environmental stress and pathogen infection (Dhaubhadel et al., 2002; Krishna 2003; Bajgu and Hayat, 2009; Bajguz, 2011; Kanwar et al., 2012b).

Although brassinosteroids are extensively studied in plant system and reported to be vital for many growth and development processes in plants (Tanaka et al., 2003; Clouse, 2011). On the same side, information about the effects of BRs on animal cells is
very insufficient. Recent studies indicate their antiviral (Romanutti et al., 2007),
antiproliferative (Malikova et al., 2008), antibacterial (Nakashita et al., 2003),
antigenotoxic potential (Sondhi et al., 2008, 2010) and neuroprotectors (Ismaili et al.,
2012).

BRs are natural compounds which are safer to use and the confirmation of their
safety had been obtained from toxicological studies made in the Sanitary-Hygienic
Institute of Belarus for 24-EBL. It was observed that LD$_{50}$ (orally) in mice (female) was
more than 1000 mg kg$^{-1}$ and LD$_{50}$ (orally and dermally) in rats (male/female) was more
than 2000 mg kg$^{-1}$ (Khripach et al., 2000).

Therefore an attempt has been made to study the bioactivities of different isolated
BRs named 24-epibrassinolide (Fig. 5.4 A), Castasterone (Fig. 5.4 B), 24-Epicastasterone
(Fig. 5.4 E), Typhasterol (Fig. 5.4 D), Dolicholide (Fig. 5.4 C) and Teasterone (Fig. 5.4
G). The isolated natural BRs were found to possess considerable antiproliferative
activity. All the compounds effectively suppressed the growth of cancerous cells. 24-
Epibrassinolide was most effective in Lung (A-549) cancer cell line employing SRB
assay (Table 4.40). In case of C-6 glioma cell line, maximum reduction was shown by
TE with its IC$_{50}$ value was 17.09µgml$^{-1}$ followed by CS, TY, 24-EBL and DL (Table
4.41). Similarly pattern of reduction in growth of cancer cell was noticed in MCF-7
cancer cell, where the most active BRs was again TE with IC$_{50}$ value was 7.87µgml$^{-1}$
followed by DL, CS, TY and 24-EBL (Table 4.42).

The findings of the study clearly highlighted the possible potential of these
compounds for the development of cytotoxic drugs and are in concordance with the
earlier reports on anticancer potential of these compounds. Swaczynova et al. (2006)
studied the cytotoxic activities of BRs against CEM, MCF7, T98, HeLa and RPMI 8226
cancer cell lines. They found that two cell lines viz. CEM and RPMI 8226 were more
sensitive to the BR tested and could inhibit proliferation of cancer cells via apoptosis.
Similarly, Malikova et al. (2008) reported the interesting anticancer properties of 28-
homocastasterone and 24-EBL, on the viability, proliferation, and cycling of hormone-
sensitive/insensitive (MCF-7/MDA-MB-468) breast and (LNCaP/DU-145) prostate
cancer cell lines. Their study highlighted that growth inhibition in cancer cell lines by
both BRs was dose dependent. Flow cytometric studies further confirmed that BRs
treatment have the capacity of arresting MCF-7, MDA-MB-468 and LNCaP cells in G1 phase of the cell cycle and induced apoptosis in MDA-MB-468, LNCaP, and to some extent in the DU-145 cells. Another metabolite of BRs (3-keto-22-epi-28-norcathasterone), isolated from brown algae (Hamdy et al., 2009) was evaluated for its anticancer potential against HEPG-2 (liver) and HCT116 (colon) human cancer lines. This compound was found to be active against both the cell lines with its IC_{50} value which was 2.96 µM for liver and 12.38 µM for colon cell line. Similarly, Oklešková et al. (2010) observed series of brassinosteroids which were effective for the growth inhibition of many cancer cell lines at micro molar range despite of its minimal effects in normal cells. They have patented certain techniques that could arrest the cell cycle by BRs which ultimately leads to apoptotic changes in cancer cells. Brassinosteroids treatment therefore can adversely affect the hyperproliferation on mammalian cells \textit{in vitro} and \textit{in vivo}, particularly treating the hyperproliferative diseases in mammals. Activity of 24-epibrassinolide to protect neuronal PC12 cells from 1-methyl-4-phenylpyridinium- (MPP+) induced oxidative stress and resulting apoptosis in dopaminergic neurons was studied by Carange et al. (2011). Their study highlighted the antioxidative potential of 24-EBL that caused the inhibition of MPP+-induced apoptosis by controlling DNA fragmentation, Bax/Bcl-2 protein ratio and cleaved caspase-3.

Recently, Ismaili et al. (2012) also reported the neuroprotective activity of 2 natural and 5 synthetic analogues of synthesized BRs in neuronal PC12 cells, against 1-methyl-4-phenylpyridinium (MPP+), a neurotoxin known to induce oxidative stress and degenerescence of dopaminergic neurons characteristic of Parkinsonian brains. Their study showed that 6 of the 9 BRs and their analogs protected the neuronal PC-12 cells against MPP+ toxicity.

Free radicals attacking cellular constituents and bringing DNA damage are considered as cancer causing agents. Thus, the search for compounds capable of scavenging free radicals and act as antioxidants has gained much interest in recent years. The evaluation of BRs isolated from \textit{B. juncea} plants for their potential to act as antioxidant compounds was undertaken using DPPH radical scavenging assay, Ferric ion reducing antioxidant power assay and Molybdate ion reduction assay. There are reports regarding the antioxidant potential of steroidal compounds leading to protection from
oxidative damage. 2-hydroxyestradiol has been reported to inhibit lipid peroxidation of erythrocyte membranes induced by the systems of xanthine oxidase-hypoxanthine and ascorbate. Further, lipid peroxidation induced by tbutyl hydroperoxide-Fe$^{3+}$ was strongly inhibited only by 2-hydroxyestradiol (Miura et al., 1996). Studies have shown that steroids play important pharmacological roles against oxidative stress (Maximo et al., 2009). A steroid [17-(4-hydroxy-1,5-dimethylhexyl)-2,3,7-(acetyloxy) gona-1,3,5(10)- trien-15-ol], isolated from *Cleome Arabica* have been reported to exhibit greater capacity to scavenge free radicals as compared to standard antioxidant compounds *viz.*, ascorbic acid, a-tocopherol, Trolox, (+) catechin, p-coumaric acid and gallic acid (Djeridane et al., 2010). In a study conducted on *Raphanus sativus* L. seedlings treated with 24-EBL and grown under Cu metal stress, a major improvement in DPPH radical scavenging activities, and elevated deoxyribose and reducing powers have been reported (Choudhary et al., 2010).

5.6 CONCLUSION

BRs are important plant steroidal hormones which regulate a number of growth responses in plants and also enable the plants to face biotic and abiotic stresses. In the present study, a number of BRs have been successfully isolated and characterized from *B. juncea* plants given metal stress of Ni, Cr and As. These BRs are active constituents of early C$_6$ oxidation pathway. The HM stress ameliorating properties of the isolated BRs were also explored in plants. The activation of antioxidant defense system in *B. juncea* by the treatments of BRs further revealed their potential in providing resistance to metal stressed plants. The free radical scavenging activities & antiproliferative activities of isolated BRs, further points to a future area of research on potential uses of BRs in drug development.