CHAPTER 5
Discussion

5.1 Analysing the Presence of Bixin in Leaves of *B. orellana*
We have studied a comparative quantification study for the presence of bixin in both leaves and seeds of *B. orellana*. Characterization and quantification of the apocarotenoid bixin in leaves extract were done through leaves section, HPLC, FTIR, NMR and GC-MS comparatively with *B. orellana* seed extract for the confirmation of bixin.

Availability of seeds is seasonal whereas leaves occur throughout the year; our research compared and quantified the presence of Bixin in seeds and leaves. We also confirmed the presence of Bixin in leaves by characterization studies through HPLC, FT-IR, GC-MS and NMR analysis, these studies revealed the presence of Bixin which was significant when compared with seeds. Hence leaves of *B. orellana* can be an alternative source for the highly demanded apocarotenoid bixin. The presence of pigments in leaves was reported by (Rodríguez-Avila et al., 2011)

5.2 Interaction of Bixin with Bovine and Human Serum Albumin
Bovine (BSA) and human (HSA) serum albumins are frequently used in biophysical and biochemical studies since they have a similar folding, a well-known primary structure, and they have been associated with the binding of many different categories of small molecules (Gelamo and Tabak, 2000; Gelamo et al., 2002). We attempted a comparative between the HSA and BSA using computational analysis. We employed pairwise alignment between protein sequence of BSA and HSA, and observed a similarity of 88%. In addition, by conservation analysis, we predicted that all of the regions in the corresponding proteins are either completely conserved or semi conserved (Fig. 4.28). To have a better comparative functional analysis, we performed molecular docking and molecular dynamics analysis to understand the change in binding pattern between the protein (HSA and BSA) and the ligands (bixin). Noticeably, we found equal interaction of the ligands (Bixin) with both the proteins (BSA and HSA).
BSA and HSA exhibit 88% similarity in the amino acid sequence. In addition, by conservation analysis, we can predict that all of the regions in the corresponding proteins are either completely conserved or semi-conserved. Serum albumin is the most abundant protein, within this Bovine serum albumin (BSA) is important protein model due to its 76% high structural homology with human serum albumin and as the soluble protein ingredient of the circulatory system with many physiological functions (Papadopoulou et al., 2005; Kumari et al., 2014). The most significant property of BSA is that they serve as a depot protein and as a transport protein for a variety of compounds (Li et al., 2014). They enhance the apparent solubility of hydrophobic compounds in plasma and modulate their delivery to cells. So the absorption,
distribution, metabolism and excretion properties, stability and toxicity of chemical substances can be affected because of their binding to serum albumins (Shahabadi et al., 2014; Mir et al., 2014). Furthermore, there exists evidence that the conformation of serum albumin changes upon interacting with low molecular weight molecules, which may affect the secondary and tertiary structures of albumins (Hushcha et al., 2005). BSA has been more widely used in the development of serum-free media than human serum albumin (HSA) until recently, oftenly due to issues of cost and supply, rather than for any advantageous functionality (Francis, 2010; Koly et al., 2015).

BSA is a globular protein which contains single polypeptide chain with a molecular weight of 66 kDa (583 amino acid residues). BSA contains three homologous domains (I, II and III) and each domain consists of two sub domains (A and B) (Chatterjee and Mukherjeee, 2014). BSA has two tryptophan residues, at the position of 134 is located on the surface of the sub domain IB and Trp-212 in the hydrophobic cavity of sub domain IIA respectively (Tong et al., 2012). Up to now many studies have been carried out to analyze the binding of BSA with many compounds such as drugs (Chaturvedi et al., 2015; Korkmaz et al., 2015), organic small molecules (Zsila et al., 2005; Rodriguez Galdon et al., 2013; Li et al., 2015). However, the binding of food colorants to serum albumins is a developing field to study the role of pigments in the biological systems.

Recently, our group has reported the interaction of various small molecules with biological macromolecules (Hridya et al., 2015; 2016; Amrita et al., 2016; Hemachandran et al., 2016). Studies involving binding of apocarotenoids with serum albumin will help us to understand the metabolism and transport of these bioactive compounds, and it is useful to infer the relationship between the structure and function of serum albumin. To the best of our knowledge, there is no information on the interaction of bixin with BSA. Here, for the first time, we report the interaction of important apocarotenoid bixin with BSA using the observed structural changes, fluorescence quenching behavior, binding and thermodynamic parameters. In addition, molecular docking and stimulation were carried out to reveal the binding mechanism of the bixin to BSA. The present study contributes to the basic knowledge for the binding mechanism, the interaction of bixin with BSA and is helpful for understanding its effect on protein function during the biological activity. As both BSA and HSA can be used interchangeably, in the current study, we used BSA for all
of the experimental aspects and a comparative study between BSA and HSA interaction using molecular docking and molecular simulation has been studied for clarity. This kind of combinational experimental and theoretical analysis will not only help in understanding the interaction but provide better insights for future experimental designs to include them and enhance the findings. In our present study, we analysed the interaction of the apocarotenoids with bovine serum albumin (BSA) at physiological pH using spectroscopic techniques and in-silico tools. The binding constant and the mode of binding sites have been studied. The enthalpic and entropic contribution to the intermolecular binding event was analysed and it was found that the contribution of hydrogen bonding and hydrophobic interactions were dominant. The adverse temperature dependence in the unusual static quenching is found to be a reasonable consequence of the large activation energy requirement in the binding process, which is required to overcome the fundamental block and is a direct result of the unique microstructure of the binding sites. To confirm the experimental analysis, we investigated the binding patterns using different in silico tools. A combination of molecular docking, molecular dynamics, and toxicity analysis was performed, and the obtained results revealed that both the apocarotenoids had high binding affinity with a binding energy of -5.44 and -5.93 kcal/mol for bixin respectively with no toxic effects and are in accordance with our experimental analysis. The results directly revealed the flexibility of the protein towards bixin which has a great impact on the interaction. Thus bixin can guardedly be used as food colorants in food industries.

5.3 Identification of Carotenoid Cleavage Dioxygenase4a Gene in B. orellana

In biosynthetic pathway, the breakdown of carotenoids into apocarotenoids was catalyzed by the enzyme carotenoid cleavage oxygenases (CCOs). The CCOs are categorized into 9-cis epoxy carotenoid dioxygenase (NCEDs) and carotenoid cleavage dioxygenase (CCDs). CCDs are further divided into four groups viz, CCD1, CCD4, CCD7 and CCD8. The CCD1 enzyme cleaves numerous cyclic and linear all-trans-carotenoids (C5-C6, C7-C8, C9-C10 double bonds) to produce multiple apocarotenoid products. The CCD4 are involved in production of plant volatile compounds like β-ionone and damascene and also reported to help during drought stress. The other two CCD enzymes, CCD7 and CCD8 are involved in shoot
branching and strigolactones production. The CCD7 enzyme cleaves β-carotene to produce β-ionone and C27 10'-apo-β-carotenal. The CCD8 cleaves the C27 aldehyde at its 13, 14 double bond, which result in the formation of apocarotenoids (Vogel et al., 2008; Vallabhaneni et al., 2010; Rodríguez-Avila et al., 2011). In B. orellana, CCD1 gene was isolated by (Rodríguez-Avila et al., 2011) and reported that BoCCD1 enzymes cleaves the lycopene the double bonds 5-6/5'-6' leading to the formation of bixin. It is also interesting to note that production of bixin in different developmental stages of seeds of B. orellana and BoCCD1 plays an important role in bixin production. However, in several other plant species the expression of CCD1 was controlled by the production of important apocarotenoids such as beta-ionone, a fragrance volatile in petunia flowers, or C13-norisoprenoids, considered flavor compounds in grapeberries (Simkin et al., 2004; Mathieu et al., 2005).

In the present study, a new CCD gene, BoCCD4a, was isolated and its sequence showed high homology with CCD4a of C. sativus. The function of CCD4 gene has been reported in several plant species like C. sativus, Chrysanthemum morifolium. In C. sativus, the function of CCD4 expression pattern revealed that CsCCD4a and b emissions of high level of β carotene and emission of β ionone formation during stigma development (Rubio et al., 2008). In CCD4 enzymes belongs to the N-terminal domain for plastid targeting, CsCCD4a and CsCCD4b proteins are targeted to plastid plastoglobules in plant and the activity was determined in this enzyme have a 9,10 (9',10') cleavage activity on β carotene (Rubio et al., 2008). The silencing of CCD4a gene in Chrysanthemum morifolium by RNA interference (RNAi) resulted in the change of petal color from white to yellow. It occurs by the accumulation of carotenoid in Chrysanthemum petals by cleaving β-carotene, which finally confirms the CCD4a gene expression resulting in white color petal formation (Ohmiya et al., 2006). Similar studies have been carried out in potato tubers, by using RNA interference (RNAi) approach by down regulation of CCD4 gene in tubers resulted in high level of carotenoid formation (Campbell et al., 2010).

The evolutionary relationship of CCD sub-class genes were well documented by (Priya and Siva, 2014). The tree result suggested that, the isolated B. orellana CCD4 gene was closely related to C. sativus CCD4 gene family. According to previous report, In Glycine max CCD4a gene sequence and Chrysanthemum morifolium CCD4a gene sequence showed the presence of two exons and one introns
(Ahrazem et al., 2010; Priya and Siva, 2014). However, in *Arabidopsis thaliana* CCD4a and *Rosa domestica* CCD4a gene sequence showed the absence of introns (Huang et al., 2009).

5.3.1 Callus Culture and *Agrobacterium* Mediated Genetic Transformation

Our result shows that most of the nodal region explant exhibits callus initiation after two weeks and developed compact, creamish white calli after two weeks of culture initiation. The highest growth of callus was observed in the lower concentration of hormones, while in higher concentration of hormones showed the browning of callus and also affected further proliferation in sub-cultured medium. In order to study the *Agrobacterium* mediated transformation, callus were further sub-cultured for every two weeks, the high amount of gelling agent would reduces the water potential in medium and resulting in the formation of hard and fragile embryogenic calli that are supposed to be more responsive for transformation (Kumar et al., 2005). In the present study, we observed the nodal region were most suitable for the induction of callus as compared to any other explants. Only the fresh nodal region segments were totipotent and produced with the ability to regenerate the callus. In our observation, any callus that turned brown was found to be unsuitable for regeneration and transformation.

At higher concentration the antibiotics will decreased the transformation efficiency. Apart from this, the selection could be based on the antibiotics like kanamycin or hygromycin. The plant cells regenerate only in the selective agent, the non- transformed cell dies in selectable marker genes. In this study, we used hygromycin as selectable marker gene for the efficient transformation, it is widely used to select the *Agrobacterium* transformed explants or callus for further study (Ziemienowicz, 2014). Many researchers have worked in transformation of *B.orellana* using different strains of *Agrobacterium*. For instance Parimalan et al., 2011 transformed somatic embryos with *A. tumefaceins* strain GV 3101 harbouring pCAMBIA 1305.2 vector in RBANGT medium containing 10 µm of acetosyringone, 10 mg l⁻¹ of hygromycin and 250 mg l⁻¹ of cefotaxime (Parimalan et al., 2011). Zaldivar–Cruz et al., 2003 used hypocotyl as explant for agroinfection using LBA4404 strain containing pBI.121 and pCAMBIA 2301 in PC-L2 medium (Phillips and Collins, 1979) containing 200 µm of acetosyringone and 250 mg l⁻¹ of cefotaxime.
The chemical acetosyringone produces natural phenolic compound that induce the virulence and enhances the genetic transformation in plant (Andreussi et al., 2015). In loblolly pine, the effect of different antibiotics like carbellicillin, claforan and timentin was involved in the removal of *Agrobacterium* overgrowth through *Agrobacterium*-mediated transformation (Tang et al., 2004). In *Agrobacterium* transformation, the co-cultivation time and density of *Agrobacterium* are important to affect T-DNA delivery and integration.

The transformation efficiency was affected by several important factors such as MS medium with nutrient supplement, hormones, co-cultivation time, *Agrobacterium* vectors and selection (Katiyar et al., 1999). The main problem in *Agrobacterium* transformation was necrotic response, because it decreases the transformation efficiency after co-cultivation and that leads to callus browning and tissue shrinkage. It was caused due to the cell death at the site of *Agrobacterium* was applied. In our observation, any callus that turned brown within two weeks was untransformed. Such kind of activity was recognized as hypersensitivity defence mechanisms of plants to *Agrobacterium* infection (Ombori et al., 2013). The callus that grow rapidly were found to be transformed and further investigated for the β-glucuronidase (GUS) assay. Other factors like culture medium, incubation condition, mode of injury for *Agrobacterium* infection to improve the T-DNA delivery in plant species and acetosyringone concentrations plays a vital role in transformation efficiency (Aggarwal et al., 2011). In this study, we used *Agrobacterium tumefaciens* strain EHA 105 harbouring pCAMBIA 1301 for stable transformant study in *B. orellana*. The *Agrobacterium* transformation work was carried out in *Vitis vinefera* CCD1 (Lashbrooke et al., 2013). The vector pCAMBIA 1301 was widely used in *Agrobacterium* mediated transformation in rice (Hiei et al., 1994), *Citrus paradise* (Burkhardt et al., 1997) *Arabidopsis thaliana* (Yan et al., 2015). For GUS assays pCAMBIA 1301 vector used for stable transformation and it could be used for suitable reporter gene system. Similar study was carried out in rice, where, the gene was constructed into the pCAMBIA 1301 vector to study the expression of the *Agrobacterium* mediated transformation in rice seedlings (Pooja et al., 2015).

GUS gene expression was extensively applied in many plant species like rice (Karthikeyan et al., 2011; Andrieu et al., 2012), tomato (Sharma et al., 2009), corn (Cao et al., 2014), yam (Nyaboga et al., 2014) and maize (Frame et al., 2002). In GUS
assay, acetosyringone plays major role and it enhance the transformation efficiency (Humara et al., 1999), the removal of acetosyringone from the co-cultivation medium and inoculation medium has decrease the GUS gene expression. It was observed that the low level GUS expression in 100 mM concentration of acetosyringone and showed significant result in GUS activity. The GUS gene expression was reported in two varieties of *B. orellana* and in hypocotyls of annatto seedings (Zaldivar–Cruz et al., 2003). A similar GUS transformation efficiency was reported in *B. orellana* by (Parimalan et al., 2011). The amplified products were observed in all transformants tested, confirming the presence of both transformed GUS and hptII. The molecular confirmation of the GUS and hptII was reported in many plants such as in yam (Nyaboga et al., 2014), GUS gene in maize (Ombori et al., 2013) GFP fluorescence visualization is useful tool for selecting transformed plants. It was widely used in gene transformation of many plant species such as yam (Nyaboga et al., 2014), barley (Murray et al., 2004), alfalfa (Duque et al., 2007), apple (Maximova et al., 1998). In this study, we have examined explants from hypocotyls, leaf, roots for GFP fluorescence. Visualizing GFP expression is an advantage to select transformation in early stage, thus avoiding regeneration of non transformants.

RT-PCR was evaluated to study the expression pattern in vegetative organs, reproductive tissues and also the expression was carried out to study the *BoCCD1* expression level with Bixin accumulation in mature seeds (Rodríguez-A vila et al., 2011). After *Agrobacterium* transformation to study the presence and expression level of the gene with control has been carried out in previous report such as in mint with gluthathione synthetase gene (Kumar et al., 2009), Potato (Bhaskar et al., 2009).

5.4 The Effect of UV-B and UV-C Stress Towards *B. orellana* L

In nature, plants are affected by various environmental stresses like salinity, drought, metal toxicity, high temperature and Ultraviolet radiations. Sunlight is the major source of energy for plants. The electromagnetic/solar radiations from sun is subdivided into three classes such as UV-A (315-400 nm) is less harmful radiation and not attenuated by atmospheric ozone, UV-B (280-315 nm) is the most effective radiation that is absorbed by the ozone layer and UV-C (200-280 nm) radiations are high energetic and absorbed by ozone in the stratosphere but not present in terrestrial sunlight (Casati and Walbot, 2003; Kataria et al., 2014; Chen et al., 2015; Verdaguer
et al., 2017). UV-B results in stratospheric ozone depletion which has a role in damaging agriculture and plant ecosystems. In general, UV radiations induce both direct (changes in morphological, physiological and molecular aspects) and indirect effect such as conformational changes in macromolecules (DNA, RNA, and proteins) (Rai et al., 2001; Hectors et al., 2007; Nenadis et al., 2015).

Plant species may differ in their response towards UV stress; some plant species tolerate UV radiation through certain modifications such as increased leaf thickness, production of secondary compounds and having antioxidant activity (Mackerness et al., 2001; Caldwell et al., 2003; Agarwal et al., 2008; Kumari et al., 2009). Several works have been carried out to study the impact of UV radiation in crops and herbaceous plants which result in a various alterations in morphological characters of the plant such as plant stunting, leaf discoloration reduction in leaf area and biomass and plant productivity, reduced number of stomata, smaller internodes, increase axillary branching, changes in antioxidant enzymes activities, release of free radicals, reduced synthesis of chlorophyll and inhibited photosynthesis (Rai et al., 2001; Santos et al., 2004; Hectors et al., 2007; Zu et al., 2010; Hui et al., 2013). The antioxidant enzyme such as superoxide dismutase, peroxidase, and catalase plays a major role in protecting the plants from oxidative damage (Santos et al., 2004; Martinez-Luscher et al., 2013). Several studies have been reported to determine the effect of UV radiation in cellular reactive oxygen species that generate oxidative stresses (Santos et al., 2004).

In this present study, we focused the effects of UV-B and UV-C on the morphological, antioxidant activity, photosynthetic pigment level alterations and their gene expression variation in *B. orellana*. Several studies have been reported that inducing UV radiations to plants might inhibit or promote the growth and development of the plants and the effect of this radiation may be different between species to species (Day et al., 1999; Giuntini et al., 2005; Hectors et al., 2007; Ibáñez et al., 2008). Researchers have analysed the changes in gene expression level in UV exposed plants. In *Arabidopsis*, the gene expression analysis has been studied using short term UV-B radiation just 15 min (Ulm et al., 2004). In maize, long term exposure to UV radiation has been studied to analyse the expression of the genes using DNA arrays under field condition (Casati et al., 2003). The enhanced effect of UV-B radiation and their combination of photosynthesis and antioxidant defense
system reported in seedlings of *Picea asperata* (Yao and Liu, 2007). Likewise, the short and long term exposure of UV-B radiation on leaves of grapevine *Vitis vinifera* reveals that the exposure of long-term acclimation capacity of grapevine to high UV-B levels shows the high accumulation of UV-B absorbing compounds in leaves and plants are tolerant to moderate doses of UV-B (Martinez-Luscher et al., 2013). Chen et al. (2015) was studied the effect of UV-B stress causes the changes in gene expression and transcriptome profiling in *Lycium ruthenicum*. In *Chrysanthemum* flower, the effect of enhanced UV-B radiation on the nutritional importance and active ingredients compounds during floral development has been analysed (Ma et al., 2016). The effect of preharvest UV-C radiation on strawberry was analysed to study the yield of fruits, fruit quality and the endogenous phytohormones (Xu et al., 2017). The UV-B radiations reaching the earth surface are predicted to be increased in the near future due to high absorption level, However, UV-C does not penetrate to the earth comparatively. Henceforth researchers are focussing on UV-B radiations when compare to the UV-C (Urban et al., 2016). In our study, we aimed to analyse the role of UV-B and UV-C stress towards the bixin biosynthesis genes in *B. orellana*. Though seeds are the major source of bixin production, it is also reported present in young leaves of in *B. Orellana* (Rivera-Madrid et al., 2006). Hence the effect of UV treatment in the young seedlings of *B. orellana* was assessed. *B. orellana* is a tropical tree/shrub where the germination of seedlings is comparatively slow as the seed dormancy period is longer henceforth for this study 45 days grown young seedlings were used for further study.

5.4.1 Morphological Effects after Treatment with UV Radiations

After treatment with UV radiations such as UV-B and UV-C the leaves of *B. orellana* did not result in any changes in the loss of the basic structural level and also no significant changes had been observed in the leaf structure. This result may differ from other reports because the UV induced radiations ultrastructural changes vary from species to species. The UV-B exposure to potato plants exhibits various changes to protect the plant from UV-B radiation. The potato plant activates several defense mechanisms to protect the cell structural integrity to enable the plant to survive with UV-B radiation (Santos et al., 2004). In cotton leaves the UV-B treatments resulted in decreased leaf thickness of palisade and spongy mesophyll layer (Kakani et al.,
2003a,b). In 2014, Inostroza-Blancheteau et al. studied the anatomical changes observed in leaves of two highbush blueberry genotypes such as Brigitta and Bluegold treated to UV-B radiations and observed the increase in secondary metabolites production and stable induction of the VcMYBPA1 transcription factor against UV-B radiation had been observed. In 2010, Zu et al. analyzed the *Taxus chinensis* var.mairei under increased UV-B radiation indicated that the abaxial stomatal density was higher than comparing to untreated conditions. In our study with *B. orellana*, though there were no structural variation in control and treated leaves, the difference in pigmentation pattern can be a notable variation which can be related to the photo protective adaptability of the plants to mask the effect of UV-B and UV-C. As this can be evident by the previous reports of (Andreussi et al., 2015; Solovchenko and Neverov, 2017).

5.4.2 Effect of UV Radiations towards Photosynthetic Pigments

The photosynthetic pigment has been studied in various plants such as in *Chrysanthemum* (Ma et al., 2016), olive trees (Koubouris et al., 2015), *Artemisia annua* L. (Rai et al., 2001), high bush leaves (Inostroza-Blancheteau et al., 2014), sunflower cotyledons (Costa et al., 2002). In *B. orellana*, the β carotene content was high in both the UV-B and UV-C treated young leaves when compared with control. The secondary metabolite pigment bixin and ABA content were decreased compared with the control. Both the chlorophyll a and b level was high in UV-B, and UV-C treated seedlings when compared to the control. When comparing with the chlorophylla/b with total carotenoid level, if the chlorophyll a/b is high the total carotenoid level was low. Likewise if the chlorophyll a/b is low the total carotenoid level was high. In 2006, Fedina et al. reported on the effect of salt stress on barley seedling where a decrease in chlorophyll a and b content with increased carotenoid content and reduced chlorophyll/carotenoid ratio. The carotenoids play a major role on effective scavengers and protect the plant from photooxidative damage by dissipating the excessive excitation energy (Rai et al., 2001).

5.4.3 Effects on Antioxidant Enzyme Activity

The oxidative stress towards UV-B and UV-C radiation has been reported in several studies, and the information was limited (Mazza et al., 1999; Rai et al., 2001; Costa et
The activity of POD, CAT, and SOD has been analysed under UV B, and UV C radiations and the plant response towards the stress has been observed. The POD activity was increased in UV-B treated young seedlings when compared to the control, and the UV-C treated leaves. The POD activity was involved in the detoxification of excess hydrogen peroxide. The increased POD activity under UV-B stress was observed in sunflower cotyledons (Yannarelli et al., 2006) and *Artemisia annua* L (Rai et al., 2001). The present study showed that UV-B exposure also increased the activity of CAT and SOD. The increase in the antioxidant enzymes reveals that *B. orellana* might respond to UV-B radiation by activation of the antioxidant defense system required for control of endogenous superoxide and hydrogen peroxide level providing cells with protection against deleterious effects of the activated oxygen species triggered by the UV exposure. The response of the antioxidant activity was varying among different species (Zlatev et al., 2012). In *Arabidopsis thaliana*, UV-B radiations showed the increased activity in SOD, but no significant effect has been observed in CAT activity (Rao et al., 1996). In 2002, Costa et al. observed UV-B radiations showed increased CAT activity with decreased SOD activity in sunflower cotyledons. In potato, the UV-B radiations showed increased antioxidant activity such as SOD and CAT (Santos et al., 2004). The enhanced UV-B radiation increased the antioxidant activity in *Picea asperata* seedlings (Yao et al., 2007). Likewise, in *Vitis vinifera*, the short term and long term UV radiation significantly increased the antioxidant activity (Martinez-Luscher et al., 2013). In cucumber cotyledons also the effect of UV-B radiations increased the antioxidant activity (Rybus-Zajac and Kubis, 2010). The current study shows that the *B. orellana* exposed to UV-B radiation activated various defence mechanisms to protect the plant from antioxidant defence system.

### 5.4.4 Gene Expression Analysis of *B.orellana* towards UV Radiation

UV-B radiation can either upregulate or downregulate the transcripts of carotenoid biosynthesis enzymes (Swindell, 2006). Few reports are available on the effects of UV radiations on biosynthetic pathways such as *V. vinifera* (Matus et al., 2009) *Artemisia annua* L (Rai et al., 2001), high bush blueberry leaves (Inostroza-Blancheteau et al., 2014), *Populus* spp (Mellway et al., 2009), strawberry cv. Camarosa (de Oliveira et al., 2016). In this study, the genes of the carotenoid
biosynthesis pathway essential for bixin synthesis are differentially affected, and distinctive expression pattern was observed by UV-B and UV-C radiation. The UV radiations affected the carotenoid biosynthesis in plants in the early stages of the pathway and it is correlated with the production of the pigment bixin. The gene encoding DXS gene remained at the low level of response when compare to the control, but UV-C is showing upregulation when compare to control and UV-B. The expression of PSY gene was downregulated when treated with the UV-B and C radiations when compare to the control. The effect of UV radiations in PDS expression of B. orellana exhibited a lower level when compared with the control. These results were similar to those previously reported for Arabidopsis Hy-1 mediated UV-C hypersensitivity showed downregulation in the PSY gene (Xie et al., 2012). The transcriptional regulation of β-LCY gene resulted in downregulation of the gene expression when compared with the control. In certain cases, the accumulation of β-carotene might be associated with the β-LCY gene. But downregulation of mRNA expression was detected, but the increased amount of β-carotene was detected in both UV-B and UV-C treated seedlings. In 2011, Ma et al. reported in transgenic tomato plant while silencing the β-LCY gene the β-carotene was decreased. Likewise, bixin was treated with the herbicide norfluoran, in 10 µM concentration and the mRNA expression of β-LCY was increased, and high amount of β-carotene was detected. However 1 µM and 100 µM concentration of norfluoran exposed B. orellana showed downregulation of β-LCY and reduced concentration of β-carotene (Rivera-Madrid et al., 2013). At the same time, bixin concentration was decreased however β-carotene content increased suggesting the cyclization regulating the flow of lycopene to the cyclic carotenoid formation and also controlling the pool of precursor molecules available for the synthesis of linear carotenoids, such as bixin.

In 2012, Ma et al studied the effect of red and blue light in the citrus plant. The expression of β-LCY in the citrus plants treated with the red light on third and sixth days of exposure upregulated CitLCYb1 and CitLCYb2, but when treated with the blue light had a temporary effect on the expression LCYb1, and LCYb2 gene only upregulated on the third day; however, there is no upregulation on the sixth day. But the upregulation in the expression of genes did not lead to accumulation of carotenoids (Ma et al., 2012). In the present study, ε-LCY mRNA transcription level was upregulated in the UV-C treated seedlings of B. orellana when compared to UV-
B radiation and control. In 2015, Ma et al. studied the expression of the flavedo of citrus fruit with the combination of ethylene and red light irradiation and reported the $\text{CitLCY}_\varepsilon$ was upregulated when treated with the red LED light, but it showed downregulation when treated with ethylene alone, but combination of both ethylene and red LED light showed upregulation of $\text{CitLCY}_\varepsilon$ gene. The $\varepsilon$-LCY was consistent with the photosynthetic role which was closely related with the chlorophylls in photosynthesis reactions as $\varepsilon$-LCY involve in the formation of $\varepsilon$-carotene. The increase in $\varepsilon$-LCY mRNA expression indicates the positive correlation of this gene through chlorophyll depletion. In 2013, Rivera Madrid et al. reported that the Bixin leaves treated with norfluran, upregulated $\varepsilon$-LCY mRNA expression.

The expression of the $\text{CCD}$ gene reveals no correlation between the $\text{CCD}$ expression and the pigment production. The mRNA transcription level of $\text{CCD}$ genes has been involved with the pigment production. In this study, significant changes have been observed when compared to the control with the UV-C and UV-B $\textit{B. orellana}$ young seedlings. Rivera-Madrid et al. (2013) demonstrated the expression of $\text{CCD1}$ genes in $\textit{B. orellana}$ using the herbicide norflurazon. The $\text{CCD1}$ genes showed high transcriptional level after treated with norflrazon. But no correlation was observed between the $\text{boCCD1}$ gene expression and bixin accumulation. The enzymatic cleavage of carotenoids leads to the production of a carotenoid enzyme such as $\text{CCD}$ gene (Vogel et al., 2008; Walter and Strack, 2011). Among these genes, especially $\text{LCD}$, $\text{ADH}$, $\text{CMT}$ genes are involved in the bixin biosynthesis pathway (Bouvier et al., 2003). The effect of UV radiation on the young seedlings the $\text{CMT}$ gene doesn’t show any mRNA transcriptional regulation between the control and treated leaves. In $\text{ADH}$ gene only the UV-B treated has been upregulated when compared to the control and UV-C treated leaves. But in the $\text{LCD}$ gene both the UV-C radiations showed higher expression of the genes when compared to the control. The $\text{LCD}$ gene plays a key regulator of the bixin biosynthesis. Not much data are available for the expression of the bixin biosynthesis genes. The mRNA expression has been studied in carotenoid biosynthetic genes and bixin biosynthetic genes in $\textit{B. orellana}$ by (Cardenas-Conejo et al., 2015). The carotenoid biosynthesis genes such as $\text{PSY}$, $\text{LCY}$ showed better expression in leafs whereas the bixin biosynthetic gene does not show any differences and also the expression level was varied.
When a gene interaction analysis was carried out using in silico analysis, we predicted similar kind of interactions between the proteins. *CCD, LCY, PDS, ZDS* and *PSY* proteins showed interaction and hence suppression of one gene can interfere with the function of other genes. Similar kind of interactions was observed through other experimental analysis. Zinati et al. (2016) studied the co-expression analysis pattern for the carotenoid/ apocaotenoid biosynthetic pathway in miR414 and miR837-5p transcription factors in *Crocus sativus*. The transcription factors are involved for identification of candidate genes involved in the carotenoid/apocarotenoid pathway.

In rice, induced by UV-C stress, the functional gene network analysis was carried out to determine the phytoalexin biosynthetic and UV-induced genes involved in the signalling components and transcription factors to study the regulatory networks involved in receptor kinases, G-proteins and transcription factors which control flavanoid and phytoalexin biosynthesis in rice (Park et al., 2013). In rice (*Oryza sativa*) coexpression analysis was carried out in starch biosynthesis genes. A starch synthesis gene has been grouped into type 1 and 11 and nearly 307 and 621 coexpressed genes are putatively involved in the regulation of starch synthesis in rice seeds and vegetative tissues. Among these genes rice starch regulator1 (RSR1), APETALA2/ethylene-responsive element binding protein family transcription factor was found to negatively regulate the expression of type 1 starch synthesis gens, and RSR2 deficiency results in the enhanced expression of starch synthesis genes in seeds. Likely the gene expression study was carried out to analysis the expression pattern. The rsr1 mutant, the RSR1 deficiency stimulated the expression of type 1 starch synthesis genes throughout seed development; however these genes were significantly up-regulated and the RSR1 gene mainly function at early stages of seed development. (Fu and Xue, 2010). From these reports we determined that the pigment production and gene expression were varying between different plant species. The present study revealed the expression of bixin biosynthesis genes were expressed differentially upon the exposure of UV-B and UV-C treatments. This co-expression network analysis study help to find the unravelling regulatory networks involved in pathways and also used to designing the metabolic engineering pathway to enhance the secondary metabolites.
5.5 Effect of Salt Stress on *B. orellana*

5.5.1 Effects of Salt Stress towards Photosynthetic Pigments

In general, abiotic stress known to affect growth and yield of the plant. Salt stress is one such abiotic stress that adversely affect growth and development of plants and also affects their physiological process such as respiration rate, changes in mineral distribution, ion imbalance and hyperosmotic stress (Sairam and Tyagi, 2004; Parida and Das, 2005). Researchers have studied the effect of salt stress towards various plant species like Potato (Rahnama and Ebrahimzadeh, 2005), Tomato (Hui-Kin et al., 2012; Horvath et al., 2015) and Wheat (Sairam, 2002). Salt stress may affect the biosynthetic pathway, it may enhance or diminish the secondary pigment level, photosynthetic pigments and also effect the light harvesting reactions.

The objective of present study is to understand the role of pigment production, antioxidant enzyme activities and analyse the transcriptional regulation of genes involved in carotenoid biosynthesis pathway in response to salt stress in *B. orellana*. These findings will contribute further insight into the molecular mechanism when plant cells are subjected to various stresses.

5.5.2 Effect of Salinity Stress on Carotenoid and Bixin Content in the Leaves.

The effect of different concentration of salinity stress towards *B. orellana* was analysed to determine the photosynthetic pigments and their secondary metabolite pigment production. The photosynthetic pigment such as chlorophyll a and b performance was altering in different concentration of salinity stress. The highest amount of chlorophyll a and b was detected in 50 mM concentration of salinity stress. While increasing the concentration of salinity stress at 75 mM and 100 mM the chlorophyll a and b content was decreasing. When compare to the control a significant changes has been observed among the chlorophyll a and b. Rivera et al. 2013 observed a significant change between chlorophyll a and b content in *B. orellana* leaves treated with herbicide norflurazon. The total carotenoid content was decreased in all treated salinity stress compete with control. Kim et al. (2012) reported that in high salt conditions, the total carotenoid content was decreased in RNAi-*lbCHY* transgenic lines of sweet potato. Loss of carotenoid content by salinity stress was revealed to be due photoinhibition. Compared with control highest amount of β-carotene was detected in 100 mM concentration of salinity stress and the lowest level
was observed in 75 mM concentration. The increase in the β-carotene level indicates the adaptability of the seedlings to decrease the effects of salinity stress by eradicating ROS (Kim et al., 2012). The highest amount of bixin has been detected in high salinity stress such as 100 mM on par with control. But in remaining stress induction conditions, the bixin content was found to be lower than control. The ABA content was high with increased salinity stress concentration. Highest level was recognized in 100 mM concentration. In 2012, Kim et al reported in RNA-\(l_b\)CHY-b calli increased ABA content enhanced tolerance of salinity stress (Kim et al., 2012). Jia et al. (2002) reported the effect of salt stress induced ABA accumulation in 6-day old leaves but high amount has been occurred in root tissues. The accumulation salt stress in roots which triggers the osmosensing mechanism for the ABA accumulation in leaves. Ruiz-Sola et al. (2014) analysed in Arabidopsis thaliana root and shoot when treated with 200 mM of NaCl an accumulation of ABA content was observed in roots and aerial tissues and the results revealed that the salt stress triggers enhanced production xanthophylls. In Maize, the effect of salt stress has been reported in two cultivars such as salt resistant SR03 and senstitive hybrid. Among these two cultivars the increased concentration of ABA has been observed in salt resistant SR03. ABA is a phytohormone which plays a major in plant abiotic stress conditions such as signaling adaptation and environmental stress such as drought, water and salinity (Zorb et al., 2013). Thus our results were in concordant with previous reports.

5.5.3 Antioxidant Defense Enzymes Activity towards Salinity Stress

The antioxidant enzyme such as POD, CAT, SOD activity plays an important role in oxidative defense mechanism in plants (Xue et al., 2008) and the antioxidant enzymes increase under salt stress and a association of these enzymes levels and salt tolerance exists (Parida and Das, 2005). The oxidative stress is the imbalance between the reactive oxygen species and scavenging/detoxification (Pastor et al., 2013). Excess ROS cause oxidative damage to various cellular components such as structural protein, chlorophylls, lipids, enzymes and nucleic acids and also inhibition of plant growth and development. So it is important to reduce the negative impact of ROS to an appropriate level through the coordination of ROS production and scavenging (Ibrahim, 2016). To scavenge excess ROS, plants have evolved extensive enzymatic systems such as SOD, POD and CAT. SOD is the important ROS scavenging
enzyme, it catalyses the conversion of superoxide radicals to H₂O₂ and O₂ and CAT catalysed H₂O₂ into water and O₂ (Wang et al., 2009; Jaspers and Kangasjarvi, 2010; Gill and Tuteja, 2010; Ma et al., 2017). The antioxidant activity towards salinity stress has been reported in various plants such as cotton (Meloni et al., 2003), potato seedlings (Rahnama and Ebrahimzadeh, 2005), Cakile maritima and Arabidopsis thaliana (Ellouzi et al., 2011), Broussonetia papyrifera (Zhang et al., 2013), Rice (Khare et al., 2015; Kordrostami et al., 2017), maize (Zorb et al., 2013). In the present study we analysed the changes in POD, CAT and SOD enzyme activities under different concentration of salinity stress and their oxidative response towards salinity stress was studied. The results exhibit the antioxidant enzymes such as POD, CAT and SOD activities was increased with increasing concentration of salinity stress. This result was match with previous reports such as the antioxidant activities POD, CAT and SOD had been studied in four cultivars of potato seedlings and the results suggest the salt tolerant cultivars of potato showed better protection against ROS by increasing the antioxidant enzymes activity (Rahnama and Ebrahimzadeh, 2005). Hu et al., 2012 reported that salinity stressed plant leaves revealed greater activity of SOD, POD and CAT in the salt sensitive genotype of Perennial ryegrass. Likewise, in rice a positive involvement of antioxidant enzymes SOD, POD and CAT with salt tolerance of two rice cultivators such as Oryza sativa L. Cv. Lunishree and cv. Begunbichi was differing in salt tolerance has been reported (Khan and Panda, 2008). Ellouzi et al. (2011) reported the antioxidant activities of leaves in C. maritima and A. thaliana was showed the increased activity on salt stress treatment. The antioxidant activity such as SOD, POD and CAT reached the higher activity when treated with the salinity stress on par with control leaves. Many reports have been proposed that the salt tolerant species showed higher antioxidant activity and the salt sensitive showed the lower antioxidant activity (Shalata and Neumann, 2001). In the present study reveals that the B. orellana seedlings under salinity stress activated defense system to protect the plant from oxidative stress mechanism.

5.5.4 Expression Pattern of Bixin Biosynthesis Genes towards Salt Stress
Earlier the expression pattern of carotenoid biosynthesis gene in B. orellana under stress has been studied using herbicide such as norflurozan (Rivera-Madrid et al., 2013). In this study, we focussed on expression of carotenoid biosynthetic genes
under salinity stress. Various reports are available on the effects of salinity stress on carotenoid biosynthetic genes such as tomato (Babu et al., 2011; Hui-Kun et al., 2012), capsicum (Maurya et al., 2015), Solanum nigrum (Ben Abdallah et al., 2016). From our findings, observed the differential expression pattern of bixin biosynthesis genes under different concentration of salinity stress. The DXS gene involved in the early stage of carotenoid biosynthesis pathway, expressed high in 25 mM concentration of salinity stress while increasing the stress the expression level was decreased continuously compete with control. The regulation of PSY and PDS gene was constitutively decreased with high concentration of salinity stress. Similar results were obtained when the leaves treated with high concentration of herbicide norflurazon (NF), the expression of PSY gene was decreased in B. orellana and the high concentration of stress might be due to the damage caused to the photosynthetic apparatus (Rivera-madrid et al., 2013). The response of the PDS mRNA expression showed a downregulation in all salinity treated leaves on par with control. The obtained results revealed the lower level regulation of the PSY and PDS genes involved in initial biosynthetic pathway and it might affect the carotenoid pathway but there is no mandatory reduction in the pigment level (Rivera-madrid et al., 2013). Similar results has been obtained in Solanum nigrum when exposed to high level of salt stress mRNA regulation of SnPSY1 was reduced, but the SnPSY2 gene was enhanced with increasing concentration of salt stress. In some plants while induced to the salt stress the expression of PSY gene has been increased. In Solanum lycopersicon cv. moneymaker the expression of the PSY1, 2 and PDS had been high when enhancing the salt concentration (Hui-kin et al., 2012). The results suggest the different homologous of PSY genes were expressed differentially in plant tissues. The cyclization of β-LCY and ε-LCY is the key branch in carotenoid biosynthesis pathway and it decide the pathway to further produce with α. Branch or β branch to synthesis α. Carotene and β carotene (Li et al., 2017). In our study, the results reveal the downregulation of β-LCY and ε-LCY mRNA transcript which was observed in increasing concentration of salinity stress. The expression of LCY gene was associated with the accumulation of β-carotene pigment in some cases. We observed the increased amount of β-carotene was detected in all concentration of salinity stress than control. Rivera Madrid et al. (2013) reported in B. orellana treated with the herbicide norflurazan, in 1 µM and 100 µM concentration showed the downregulated
expression of the β-LCY and β-carotene content was also decreased. Kim et al., 2014 reported that the downregulation of lbLCY-β in transgenic sweet potato calli enhanced the β-carotene content and also α-carotene, β-crythoxanthina and zeaxanthin under salt stress condition. Similarly downregulation of lbLCY-ε –RNAi leads to the high content of β-carotene, β-crythoxanthina, violaxanthin and ABA derived from the β branch pathway of carotenoid conferring high tolerance to salt-mediated oxidative stress (Kim et al., 2013). The result suggests that the downregulation of lbLCY-ε induce an increase in carotenoid synthesis by the β branch pathway which protects against salt-mediated oxidative stress.

The upregulation of the CCD gene has been observed in the lowest concentration (25 mM) of the salinity stress while increasing the salt stress the mRNA transcript level was reduced. Although the pigment bixin level was observed high in 100 mM concentration compared with control at the same time the remaining concentration of salinity such as 25 mM, 50 mM and 75 mM has been observed to be low when compete with control. Rivera et al. (2013) reported that when the B.orellana was treated with the herbicide Norflurazon at the particular concentration such as 0.1 and 1.0 µM the BoCCD1 has showed an increased mRNA transcript level but no correlation has been observed among BoCCD1 gene and the pigment accumulation. However, Higher ABA content was accumulated with increasing concentration of salt stress. It affirms ABA act as plant stress hormone which serve as endogenous messanger in the regulation of the plant’s water condition (Swamy and Smith, 1995; Mahajan et al., 2005). In stressed condition, ABA response to desiccation of the cell and osmotic imbalance and it splits the common elements which involve in cross talk with each other to maintain the cellular homeostatis in the signaling pathway (Tuteja, 2007). The oxidative breakdown of carotenoid produces CCD gene (Rodriguez Avila et al., 2011). In 2003, Bouvier, reported that LCD, ADH, CMT gene has been involved in bixin biosynthetic pathway. The LCD gene play important role in bixin biosynthesis. The response of LCD and CMT under salinity stress shows decreased transcript level upon rising stress. The ADH mRNA level was increased in low salinity stress but was reduced with further high concentration on par with control. Though, no much data was available on bixin biosynthesis genes towards salinity stress. From our in silico analysis, we predicted LCY, PDS, PSY to be showing close relation and in the gene expression analysis, we found that these
proteins showed similar reaction at varying salt concentration. Further ADH showed varying expression which was not similar to any of the protein addressed above. In addition, we found ADH to be showing no correlation with the salt stress genes in our insilico analysis. With this result we can predict that, a change in expression of one gene may affect the expression of other genes. Our result suggests that under high salt stress condition the mRNA transcript was decreasing however, no correlation can be attributed towards the expression of the mRNA transcripts and the pigment accumulation. This co-expression network investigation help us to study the deciphering regulatory networks implicated in pathways and also used to designing the metabolic engineering pathway to improve the secondary metabolites. Based on co-expression network analysis, the protein interaction study helps us to understand the corresponding interacting proteins of the genes involved in the UV and salt stress.