CHAPTER 4

Results

To compare the presence of bixin from *B. orellana* leaves extract was done by using various spectroscopic analysis. Both the seed and leaves extract was used to compare the presence of bixin in leaves and to study the beneficial biological activity and binding of bixin with bovine serum albumin has been studied.

4.1 Presence of Bixin in Both Seeds and Leaves

4.1.1 Microscopic Analysis

Presence of Bixin was visually observed as reddish orange spots under the microscope on the leaves tissues of petiole (Fig. 4.1). The presence of pigment in different tissues like root apex, cortex, cytosol, and also surface of seeds was observed.

![Fig. 4.1 Presence of Pigment in Different Part of Leaves. a-d. Adaxial and Abaxial Surface, e. Midrib Region, f. Petiole, g. Dry Leaves](image-url)
4.1.2 HPLC Analysis

HPLC analysis was carried and the chromatogram was obtained. The presence of bixin present in *B. orellana* seed and leaf extract was determined based on their retention time. The retention time for bixin observed for the seed extract was 24 min and the leaves extract was 22 min. Comparatively, the presence of Bixin is confirmed by similar retention time through HPLC analysis (Fig. 4.2).

![HPLC Chromatogram](image)

**Fig. 4.2** HPLC Chromatogram of Bixin Pigment From (a) Seeds and (b) Leaves, Which Showed Retention Time at 22.6 min in Seeds and 24 min in Leaves
4.1.3 FTIR Analysis

FTIR is an important method for characterization of biomolecules. Fig. 4.3 (a, b) depicts the FTIR spectrum of *B. orellana* seed and leaf respectively. The crude extracts of leafs and seeds shows the presence of vibrational bands at 1718 and 1616 may be due to the stretching frequency of carbonyl and conjugated olefin functions present in the molecule. The bands appeared in both seed and leaf at 1261 corresponds to the C-O stretching frequency. The vibration at 2924 in seed and 2918 in leaf shows the presence of C-H stretching of CH3 and a band at 3111 in leafs indicates OH stretching of COOH. The obtained results in seed were correlated with earlier reported results (Yusá-Marco et al., 2008, Siva et al., 2010).

![FT-IR Spectrum of Bixin Pigment from both (a) Leaves and (b) Seeds](image)

**Fig. 4.3 FT-IR Spectrum of Bixin Pigment from both (a) Leaves and (b) Seeds**
4.1.4 NMR

NMR analysis was done using deuterated methanol for bixin. $^1$H NMR affords the direct evidence for the structures of bixin based on the position off the molecules and the magnitude of shift off the hydrogen atoms and their relation for stability (Fig 4.4). NMR spectrum was obtained with respect to absorption (Hz) and chemical shift (PPM). TMS (trimethylsilane) was used as a reference point for NMR spectra due to the fact that, it has the maximum number of identical protons that gives maximum shielding effect and thereby providing a single sharp peak at a high applied field that is well separated from most of the peak of interest in any spectrum. Peak at 2.1 ppm shows the presence of methyl group attached with a carboxyl group. The spectrum shows that it is found that Bixin is present at around 3.7 ppm. In both the seeds and leaf extract showing the same pattern.

![Fig. 4.4 $^1$H NMR Spectrum of Bixin Pigment from Both Seeds and Leaves](image-url)
4.1.5 GC MS
MS analysis for confirmation of the structure of molecule by the mass fragmentation was done using MS. The molecular weight of bixin is 394.51. It shows 396 in both leaves and seeds. (Fig. 4.5)

Fig. 4.5 GC-MS Spectrum of Bixin Pigment from both Seeds and Leaves
4.2 Biological Interaction of BSA with Bixin

4.2.1 Fluorescence Quenching Spectra and Mechanism

Fluorescence quenching is a widely used technique to study the interaction between ligands and bio-macromolecules. The binding affinity of Bixin with BSA was determined by fluorescence quenching. The data was corrected for the inner filter effect to reduce the effect produced due to the absorption of light at the excitation wavelength ($\lambda_{ex}$) and emission wavelength ($\lambda_{em}$). The inner filter effect was calculated using the following equation in Eqn. 4.1 (Kumari et al., 2016; Patel et al., 2016 a,b Maurya et al., 2017).

$$F_{corr} = F_{obs} 10^{\frac{(A_{ex}+A_{em})}{2}}$$

(4.1)

Where $F_{corr}$ and $F_{obs}$ are the corrected and background subtracted observed fluorescence intensities respectively. The $A_{ex}$ and $A_{em}$ are the sum of measured absorbances at the excitation and emission wavelengths.

The fluorescence emission spectra of BSA in the absence and presence of bixin was recorded at 340 nm. It was observed that the fluorescence intensity of BSA constantly decreased upon increasing concentration of the apocarotenoids at 340 nm comprising tryptophan as major residues. There was a slight blue shift implying the interaction of bixin enhanced hydrophobicity to the protein (Malde, 2011). Further the interaction of bixin with tryptophan residues induced to quench the intrinsic fluorescence intensity of the BSA (Fig. 4.6)

![Fluorescence Emission Spectra of BSA with Various Concentrations of Bixin](image)

Fig. 4.6 Fluorescence Emission Spectra of BSA with Various Concentrations of Bixin. The Concentrations of the Compounds are 0-10 µm. Dotted Lines Represents Free Bixin.
We have analyzed fluorescence quenching at varying temperatures to study the effect of temperature on the quenching mechanism. There are two types of quenching that could be either static quenching where a ground state complex in formed between the fluorophore and quencher or dynamic quenching in which occurs by a collision between the fluorophore and quencher (Zhang, et al., 2008). The quenching mechanism and the quenching constant can be determined by we used the Stem-Volmer equation in Eqn. 4.2 (Tan., 2004; Kim et al., 2006; Lakowicz, 2006; Han et al., 2009; Hemachandran et al., 2017).

\[ \frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{sv} [Q] \]  

(4.2)

Where \( F_0 \) and \( F \) are the fluorescence intensities of BSA before and after addition of the quencher, respectively, \( k_q \) is the biomolecular quenching constant, \( \tau_0 \) is the lifetime of the fluorophore in the absence of the quencher, \( [Q] \) is the concentration of the quencher, and \( K_{sv} \) is the stem-Volmer quenching constant. Hence, the above equation is used to determine the \( K_{sv} \) using linear regression of a plot of \( \frac{F_0}{F} \) against \( [Q] \). A linear Stern-Volmer plot is generally indicative of a single class of fluorophores all equally accessible to the quencher.

The interaction between bixin with BSA at different temperatures was studied by Stern-Volmer plots (Fig. 4.7 (a,c)) (double logarithmic plot). Stern-volmer plots for the fluorescence quenching of various concentration of bixin with BSA was anlayes (Fig.4.8 (a,b,c,d,e,f). The \( K_{sv} \) and \( K_q \) value for the bixin with BSA was shown in Table 4.1. The \( K_{sv} \) and \( K_q \) value decreased with increasing temperature. This decrease in quenching constant can be attributed to decrease in stability of the apocarotenoid- BSA complex upon increasing temperature. The decrease in quenching constant \( (K_{sv} \) and \( K_q \) upon increasing temperatures denotes static quenching. It is well-known that the maximum scatters collision quenching constant for dynamic collision is \( 2.0 \times 10^{10} \) mol\(^{-1}\)s\(^{-1}\) (Ishtikhar et al., 2014; Chaturvedi et al., 2015). However in this present study, the quenching constant \( (K_q) \) has a larger magnitude of \( 10^{13} \), which is higher than \( 2.0 \times 10^{10} \) mol\(^{-1}\)s\(^{-1}\) thus indicating the probable quenching mechanism is of static type occurred for bixin with BSA.
4.2.2 Analysis of Binding Constant and Binding Sites

The binding constant ($K_b$) and the number of binding sites ($n$) was determined from the Eqn. 4.3 (Zhou et al., 2007; Chakraborty and Basu, 2009; Zhang et al., 2011; Kumari et al., 2014; Shen et al., 2015; Shi et al., 2016)

$$\log \left( \frac{F_0 - F}{F} \right) = \log K_b + n \log [Q]$$  \hspace{1cm} (4.3)

Where $K_b$ is the binding constant and $n$ is the number of binding sites is represented in Table 4.1. The binding constant of BSA with bixin increased temperature-dependently. The binding number was near to one upon increasing temperature (Hu et al., 2004; Li et al., 2014; Ishtikhar et al., 2014).

4.2.3 Thermodynamics parameters of BSA with Bixin

The interaction between the ligand and protein by a number of binding modes such as Van der Waals, hydrogen bonds, hydrophobic interaction, electrostatic interaction. The importance of thermodynamic parameters such as free energy changes ($\Delta G$), enthalpy changes ($\Delta H$) and entropy changes ($\Delta S$) of the reaction is very essential to study the binding modes.

The enthalpy change ($\Delta H$) and entropy ($\Delta S$) can be evaluated from the van’t Hoff equation in Eqn. 4.4.

$$\ln K_b = \frac{\Delta H}{RT} + \frac{\Delta S}{R}$$  \hspace{1cm} (4.4)

In this, $K_b$ is the binding constant of the related temperature, $R$ is the gas constant. The enthalpy change ($\Delta H$) was calculated from the slope of the van’t Hoff equation. The free energy change ($\Delta G$) was calculated from the below Eqn. 4.5.

---

**Fig. 4.7** Stern-Volmer Plots for the Fluorescence Quenching of Various Concentration of (a) Bixin with BSA. Double Logarithmic Plot for the Fluorescence Quenching of (c) Bixin with BSA.
\[ \Delta G = \Delta H - T \Delta S \]  

(4.5)

**Fig. 4.8** Stern-Volmer Plots for the Fluorescence Quenching of Various Concentration of Bixin with BSA at (a) 298 (b) 310 and (c) 313 K. Double Logarithmic Plot for the Fluorescence Quenching of Bixin with BSA at (d) 298 (e) 310 and (f) 313 K.

The thermodynamic parameter was determined from the linear second law of thermodynamics. To investigate the enthalpy-entropy relation in the BSA-Bixin interaction is very important to study the binding mode. In three different temperature,
the temperature-dependent binding constant was studied without any structural degradation of BSA (Han et al., 2009; Ishtikhar et al., 2014; Li et al., 2015, Anantharaman et al., 2015).

Table 4.1 shows $\Delta G$, $\Delta H$, $\Delta S$ value. From the results we can find that the binding process was driven by entropy and the negative value of entropy support the binding process non-spontaneously. Negative entropy implies the increase in molecular order upon complex formation. Ross and Subramanian (1981) discussed the sign and magnitude of the thermodynamics parameter related to the many individual kinds of interaction taking place in protein binding process. From the result, the thermodynamics showed hydrogen bond, and hydrophobic force played a major role in the bixin with BSA interaction.

Table 4.1 Stern-Volmer Quenching Constant ($K_{sv}$), Binding Constant ($K_b$) and Thermodynamics Parameters of the Bixin with BSA Interaction at Different Temperatures.

<table>
<thead>
<tr>
<th>T (K)</th>
<th>$10^{-5} K_{sv}$ (L mol$^{-1}$)</th>
<th>$10^{-13} K_b$ (L mol$^{-1}$)</th>
<th>$N$</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (JK mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>0.181±0.0054</td>
<td>1.064±0.04</td>
<td>1.266±0.045</td>
<td>74.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bixin</td>
<td>310 0.1751±0.015</td>
<td>1.729±0.071</td>
<td>1.817±0.08</td>
<td>78.01</td>
<td>1.507±1.46</td>
<td>-25.16±4.7</td>
</tr>
<tr>
<td>313</td>
<td>0.1306±0.009</td>
<td>2.028±0.10</td>
<td>1.95±0.108</td>
<td>78.76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.2.4 Three-Dimensional Fluorescence Spectroscopic Analysis

Recently three-dimensional fluorescence spectra are an eminent technique to understand the conformational changes of proteins. We employed three-dimensional spectra and contour map of BSA and BSA with bixin to study the conformational change of the protein. Fig. 4.9 (a, b) represents the fluorescence spectra of BSA in which the inclined contour surface denoted the typical Rayleigh scattering peak, where the excitation wavelength equals to the emission wavelength (peak 1). The emission peak of tryptophan residues of BSA is represented as peak 2, and the spectra of the polypeptide backbone residues are denoted as peak 3. Upon the addition of Bixin, there is no change observed in the Rayleigh scattering peak. However there was a remarkable change in the microenvironment of the fluorophore (Chen et al.,
The intensity of fluorescence peak decreased after interaction of bixin demonstrating the conformation changes induced by the apocarotenoids (Fig. 4.9(c,d)). The bixin interacted with BSA by exposing the hydrophobic regions which is buried inside the protein revealing the conformational change.

**Fig. 4.9** Three Dimensional Fluorescence Spectra and Contour Map of (a,b) BSA Alone, (c,d) Bixin with the Presence and Absence of BSA.

4.2.5 Secondary Structural Analysis of Bixin with BSA

CD spectrum is widely used to monitor the change in secondary structures of proteins with ligands which results in conformational alterations. Spectra in the far-ultraviolet
wavelength range from 200 to 250 nm provide the information about \( \alpha \)-helix, \( \beta \)-sheets, \( \beta \)-turns, and random coils structures. To study the structural changes of BSA interaction with apocarotenoids such as Bixin, CD spectrum has been recorded.

Fig. 4.10 shows that BSA exhibited two negative minima in the UV region at 208 and 222 nm, which is the characteristic of \( \alpha \)-helix in the structure of protein attributing \( n \to \pi^* \) and \( \pi \to \pi^* \) transitions (Han et al., 2009). Upon interaction with bixin, the \( \alpha \)-helix and \( \beta \)-sheets decreased, and random coils increased which denotes that the binding of bixin with BSA unfolds the native helical structure of BSA to more random coiled structure by unfolding the polypeptide depicting the conformational changes induced by bixin. The relative intensity of the negative ellipticity decreased significantly with the addition of bixin which suggested a conformational change in the protein secondary structure (Zhang et al., 2008; Dubeau et al., 2010; Zhang et al., 2011; Anantharaman et al., 2015). The fraction of secondary structures of BSA in the presence or absence of bixin with different ratio was determined. Due to the interaction of these apocarotenoids, there was a significant conformational change in the secondary structural contents of BSA as it is evident from table 4.2. The obtained result indicates the bixin caused a major conformational change in the secondary structure of BSA suggesting the interaction of this apocarotenoid with BSA (Hu et al., 2004)

![Circular Dichroism Spectra of the Bixin with BSA](image)

**Fig. 4.10** Circular Dichroism Spectra of the Bixin with BSA. Bixin Concentration 1, 2 and 10 \( \mu \)m Respectively. Dotted Line Represents the Bixin Value Alone.
Table 4.2 The CD Spectra Values Shows the Secondary Structure Analysis for Bixin with BSA Interaction

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Helix</th>
<th>Beta</th>
<th>Turns</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>1:0</td>
<td>26.4%</td>
<td>15.9%</td>
<td>22.9%</td>
<td>34.7%</td>
</tr>
<tr>
<td>Bixin</td>
<td>1:1</td>
<td>27.6%</td>
<td>13.3%</td>
<td>21.0%</td>
<td>38.1%</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>26.6%</td>
<td>10.1%</td>
<td>20.4%</td>
<td>42.9%</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>16.5%</td>
<td>14.4%</td>
<td>20.9%</td>
<td>48.2%</td>
</tr>
</tbody>
</table>

4.3 Computational Analysis

4.3.1 Toxicity Prediction
The prediction of toxicity for each ligand was made using two servers; ProTox and pkCSM server. According to ProTox tool, both the ligands were classified as Class 6 drugs, and the LD50 values were 5600 mg/kg for bixin. From pkCSM tool, we identified that; these compounds do not have any mutagenic and hepatotoxicity, making the ligands more applicable in the food industry (Table 4.3). The toxicity analysis predicts no toxic effect of these ligands which further add as a credit to use these compounds as food colorants.

Table 4.3 Toxicity Analysis of the Ligands using Protox and pkCSM Servers

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Toxicity</th>
<th>Predicted (mg/kg)</th>
<th>LD50</th>
<th>AMES Toxicity</th>
<th>Water Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bixin</td>
<td>Class 6</td>
<td>5600</td>
<td>No</td>
<td>-6.113</td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Molecular Docking Analysis
The molecular docking analysis could help us to understand the molecular interactions and prior studies have used this technique to identify the interactions between BSA and HSA with small compounds (Zhang et al., 2011; Fatima et al., 2016). Consequently, in the current study, we have used GOLD docking software to analyze the various binding interactions observed between bixin with BSA and HSA.
The docking analysis was performed with the fixed binding site in BSA to accurately compare the binding patterns of the two ligands. The Gold Score is the docking score, and Gold X score is the binding affinity (Table 4.4). The docking result suggests that both ligands showed better binding to BSA. In addition, we observed that there was similar binding energy exhibited by the HSA molecule during a docking analysis (Table 4.4). We found bixin having more affinity. The surrounding amino acids present around bixin along with their interactions with the proteins were visualized using PyMOL software to understand the binding patterns (Fig. 4.11 (a, b, c, d)). When analysing the interaction pattern, we observed that BSA showed more number of surrounding amino acids with 4 Å around Bixin. Among the various amino acids, the most favored residue by the ligands was Arg144 and Arg185 in the case of BSA. In the case of HSA, we did not observe existence of similar residues significantly. Overall, the surrounding amino acids show that bixin showed similar kind of hydrophobic interaction with BSA as well as HSA. Finally, from the docking score, we predicted both ligands showed better binding affinity with a very narrow increased affinity shown by bixin. Since bixin possess high binding affinity it is also used as a safe alternative tracking dye (Siva, 2007).

**Table 4.4** Docking score of the Ligands using Gold Software. Gold Score is the Docking Score and Gold X Score is the Binding Affinity of the Ligands with BSA and HSA

<table>
<thead>
<tr>
<th>Ligand</th>
<th>BSA</th>
<th>HAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gold Score</td>
<td>Gold X-Score</td>
</tr>
<tr>
<td>Bixin</td>
<td>75.34</td>
<td>-6.28</td>
</tr>
</tbody>
</table>

4.3.3 Molecular Dynamics Simulation Study

To understand the interaction specificity of BSA and HSA with ligands, molecular dynamics simulation studies were performed. The trajectories obtained as a result of simulation were analyzed using various utilities of the Gromacs Software. The initially convergence analysis were made by plotting the root mean square deviations
for the two complex molecules. When comparing the RMSD plots, we observed negligible deviation patterns from the beginning of the simulation until 7 ns, which then was found to be converging by the end of 10 ns. Along with convergence, the RMSD plot also renders helps to analyze the level of stability attained by the complex molecules, where a higher RMSD value indicates a lesser stable structure (Yun and Guy, 2011; Sneha and George Priya Doss, 2017). The RMSD conveys that bixin show similar stabilizing character with BSA (Fig. 4.12 a) converging with an RMSD value between ~0.35 Å and 0.45 Å at 10 ns simulation. Similarly, with HSA, we observed bixin showing comparable deviation pattern that showed convergence with an RMSD value 0.35 Å for Bixin (Fig. 4.13 a). This predicts that bixin have similar kind of effect.
with only -0.44 kcal/mol in the case of BSA and -.10 in the case of HSA. Concluding, both the ligands should have a similar effect on the serum albumins. Another, interesting factor of analyzing the level of compactness of the protein and the ligand was analyzed using Radius of Gyration (Rg). The lower Rg values predict a better compactness attained by the protein-ligand complex (Lobanov et al., 2008; Sneha and George Priya Doss, 2016a). From the radius of gyration plot, we predict bixin showed a similar range of compactness in BSA (Fig. 4.12 b) and HSA (Fig. 4.13 b). The increase or decrease in the level of compactness is due to the change in the binding nature of the protein throughout a simulation. Consequently, elucidates that bixin can bind to the serum albumin with greater affinity which correlates with the experimental analysis. Though docking tools are enhanced for speed rather than precision, more accurate techniques for predicting binding affinity do exist as advanced technologies (Sneha and Doss, 2016b). To investigate this compact nature of bixin, MM-PBSA analysis was carried out. Of the various available technologies, MM-PBSA approach where the entropies are calculated in a much similar manner as performed in quantum mechanics has proved better analysis to understand the binding energies (Genheden and Ryde, 2015). The various interactions observed between the ligands and proteins through the simulation are tabulated in Table 4.5. This analysis predicts that bixin have higher affinity with BSA as well as HSA. We also found the binding energies obtained from molecular docking analysis correlated with the results from MM-PBSA analysis (Table 4.4 and Table 4.5). This combinational study helps us to elucidate the binding of ligands to the proteins more accurately (Alonso et al., 2006).

Table 4.5 Binding Energy Values as obtained from MM-PBSA Analysis of the Trajectories of BSA and HSA

<table>
<thead>
<tr>
<th>Components</th>
<th>BSA</th>
<th>HAS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VanderWaal Energy</strong></td>
<td>-103.411± 73.66</td>
<td>-101.333 ± 67.32</td>
</tr>
<tr>
<td><strong>Electrostatic Energy</strong></td>
<td>-12.553± 22.413</td>
<td>-10. 430 ± 19.34</td>
</tr>
<tr>
<td><strong>PolarSolvation Energy</strong></td>
<td>45.229 ± 41.648</td>
<td>51.167 ± 20.564</td>
</tr>
<tr>
<td><strong>SASA Energy</strong></td>
<td>-5.132 ± .241</td>
<td>-3.76 ± .443</td>
</tr>
<tr>
<td><strong>Binding Energy</strong></td>
<td>-72.104± 63.787</td>
<td>-68.11 ± 59.455</td>
</tr>
</tbody>
</table>
Fig. 4.12 a) Root Mean Square Deviation of the Complexes for 10 ns.  b) Radius of Gyration of the Complexes. Color scheme. Black : BSA, Red: Bixin Docked with BSA
Fig. 4.13 a) Root Mean Square Deviation of the Complexes for 10 ns. b) Radius of Gyration of the Complexes. Color scheme. Black: BSA, Red: Bixin Docked with HSA
4.4 Isolation of CCD Genes from B. orellana

4.4.1 Cloning of Partial Genomic DNA Clone of CCD4a Gene

Bixin of B. orellana and crocin of Crocus sativus are apocarotenoids having similar chemical structure. Taking this advantage, the primers were designed from sequence of C. sativus CCD4a gene. The primers has been designed from Crocus sativus based on 100% sequence similarity. The primers were designed from the region of 112-132bp for forward primer and 1719-1738bp for reverse primer. A partial CCD4a gene was obtained by using two gene specific oligonucleotides. The obtained 1083 bp (Accession number: KT378217) product was cloned into pGEMT vector and selection of recombinant was done by blue-white screening method and recombinants were confirmed by the same fragment size obtained from colony PCR. The obtained nucleotide sequence showed similarity with C. sativus. The relationship between B. orellana CCD4a sequence with other plant CCD4 sequences were analysed by sequence comparison using CLUSTALW and by construction of a phylogenetic tree.

4.4.2 Phylogenetic Tree Construction

Phylogenetic tree was constructed to explore the evolutionary relationship of isolated CCD4a gene (BoCCD4a) using MEGA v6.0. The best-fit model suggested by ModelTest v2.4 was (GTR + I + G) for nucleotide substitution. The sequence alignment of BoCCD4 gene shows the conserved region with other plant CCD4 genes. The phylogenetic tree shows relatively high sequence homology of BoCCD4a gene with various plants CCD4 (Fig. 4. 14). The different plant families included for comparing the phylogenetic tree construction are Rosaceae (Malus domestica, Prunus persica, Rosa domascena), Rutaceae (Citrus clementine, Citrus unshiu), Asteraceae (Chrysanthemum morifolium), Oleaceae (Osmanthus fragans), Iridaceae (Crocus sativus), Vitaceae (Vitis vinifera), Fabaceae (Medicago truneatula), Brassicaceae (Arabidopsis thaliana), Lamiaceae (Scutellaria baicalensis), Solanaceae (Nicotiana tabacum, Lycium barbarum, Lycium ruthenium), Liliaceae (Lilium ronnie), Cucurbitaceae (Momordica charantia) and Bixaceae (B. orellana).

To study the structural variation of isolated CCD4 genes, the exon regions were predicted through FGENESH software and identified that two exons were present in our isolated BoCCD4 genes from B. orellana. The isolated sequence showed the presence of exon region from 373-455 and 645-830bp. It has been
evidenced by comparing the *B. orellana* CCD4 exons position with already reported CCD4 genes in the species of *AtCCD4*, *RdCCD4*, *GmCCD4a*, and *CmCCD4a* (Fig. 4.15, Table. 4.6).

**Fig. 4.14** Phylogenetic Tree Analysis was Constructed based on Sequence Similarity of CCD4 Genes. Bootstrapping Values 1000 Replicates are Shown. Accession Numbers Indicated Aside
Fig 4.15 Structural Analysis of CCD Genes to Detect the Exon and Intron Region. Red: First Exon Region in the Sequence, Blue: Last Exon Region in the Sequence, Orange: Exon Region

Table 4.6 CCD4a Gene Structural Information in Selected Plant Species

<table>
<thead>
<tr>
<th>Organism Name</th>
<th>Chromosome Location</th>
<th>Genomic Sequence</th>
<th>Number of Exons</th>
<th>Length of Exons</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Glycine max CCD4a</em></td>
<td>I</td>
<td>NC_016088.1</td>
<td>2</td>
<td>599-1250 1940-2562</td>
</tr>
<tr>
<td><em>Chrysanthemum morifolium CCD4a</em></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>190-840 1000-1800</td>
</tr>
<tr>
<td><em>Rosa domestica CCD4a</em></td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>15-1790</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana CCD4a</em></td>
<td>IV</td>
<td>NC_003075.7</td>
<td>1</td>
<td>78-1900</td>
</tr>
<tr>
<td><em>Bixa orellana CCD4a</em></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>373-455 645-830</td>
</tr>
</tbody>
</table>

The modelled protein was visualized to study the various secondary structures present in the protein molecule. Vp14 (viviparous14) structure of the *Zea mays* could possibly serve as a template for all the plant carotenoid cleavage dioxygenases (CCDs). The Vp14 Protein folds as a seven blade beta-propeller with four alpha-helical inserts that constitutively form an alpha-helical domain on top of the beta-propeller. Similar to
the Vp14, our CCD proteins are showed complete formation of beta-propeller in the case of CsCCD4a and CsCCD4b (Fig. 16). Thus, the modelled structure shows similar conformation to that of the template Vp14 protein.

**BoCCD4**

**CsCCD4a**

**CsCCD4b**

*Fig. 16* Modelled 3D Structure of *B. orellana* CCD4 Gene Comparison with *Crocus sativus* (*CsCCD4a* and b)
4.4.3 Expression of *BoCCD4a* Gene in pCAMBIA 1301

The expression vector was generated and named as pCAMBIA 1301: *BoCCD4a* in which the *BoCCD4a* gene was under the control of caMV35s promoter. The regenerated expression vector pCAMBIA 1301: *BoCCD4a* was then introduced into *Agrobacterium* strain EHA 105, and the recombinant strain EHA105/pCAMBIA1301: *BoCCD4a* was used for transformation experiments (Fig. 4.17). The transformation of pCAMBIA1301: *BoCCD4a* gene into *Agrobacterium* strain EHA105/pCAMBIA1301: *BoCCD4a* was verified by colony PCR (Fig. 4.18a).

![Structure map of the plant expression vector pCAMBIA 1301:CCD4a and the reporter genes used for transformation experiments](image)

**Fig. 4.17** Structure map of the plant expression vector pCAMBIA 1301:CCD4a and the reporter genes used for transformation experiments

76
4.4.4 Optimization Conditions for Callus Induction

For callus initiation in *B. orellana*, different explants like cotyledon, hypocotyls, leaves and nodal region were used. Here, we observed maximum growth of callus was obtained from the nodal explants. For callus induction study, we have followed protocol and accordingly, different concentration and combination of NAA, BA and 2,4-D were used. In brief, NAA (1.07-2.14 µM) with BA (10.2 µM) and 2,4-D (0.45 µM and 0.90 µM) with BA (10.2 µM) were supplemented with MS medium.

Among the different combination, MS medium supplemented with NAA (1.07 µM) with BA (10.2 µM) showed good response (57 %). The combination with 2,4-D (0.45 µM ) with BA (10.2 µM) not show the proper growth (Table. 4.7).

4.4.5 *Agrobacterium* Mediated Transformation and Co-Cultivation, Selection of Callus

Three weeks old callus and explants were infected with *Agrobacterium* EHA105 strain for transformation study. The callus and explants were co-cultured on MS medium supplemented with 100 µM acetosyringone to enhance the transformation efficiency. Co-cultivation for two days in the presence of 100 µM acetosyringone was found to be most suitable for optimum transformation. After co-cultivation, callus and explants were transferred to selection medium containing MS medium supplemented with 50 mg/L hygromycin and 250 mg/L cefatoxime. The antibiotic cefatoxime was widely used to avoid the overgrowth of *Agrobacterium* and the selection marker will be useful in selection of transformed tissue without effecting the transformation. In this study, cefotaxime with a concentration of 250 mg/L was found to be more efficient for growth and differentiation of transformed callus.

**Table 4.7** MS Medium with Hormones for Callus Induction using Nodal Region

<table>
<thead>
<tr>
<th>Medium</th>
<th>Plant Growth Hormone(µM)</th>
<th>No. of Explants Cultures</th>
<th>No. of Callus Formed</th>
<th>Percentage of Explants Inducing Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA</td>
<td>2-4-D</td>
<td>BAP</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>1.07</td>
<td>10.2</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>MS</td>
<td>0.45</td>
<td>10.2</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>
4.4.6 Histochemical GUS Expression in Transformed Plants

To further confirm the transformed plants, GUS assay was used as one of the markers. The result has shown successful agro-infection and transient expression of GUS gene expression, which was indicated as blue color staining in transformed explants and callus (Fig. 4.18 b (2,4,6)). The frequency of transient GUS expression was 84.4% in explants and 80% in tested callus (Table. 4.8).

Table 4.8 Transformation Efficiency of *B.orellana* Explants and Callus. Values Represent Mean ± Standard Error (SE) of Explant and Callus (Values are the Mean of Three Replicates)

<table>
<thead>
<tr>
<th>No. of Explants Infected</th>
<th>No. of Callus Infected</th>
<th>GUS Positive Plants</th>
<th>GUS Positive Callus</th>
<th>Percentage of Transformation Efficiency in Explants</th>
<th>Percentage of Transformation Efficiency in Explants</th>
<th>Mean ±S.E. of GUS Positive Explants</th>
<th>Mean ±S.E. of GUS Positive Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5</td>
<td>38</td>
<td>12</td>
<td>84.4</td>
<td>80</td>
<td>12.67±0.47</td>
<td>4±0.82</td>
</tr>
</tbody>
</table>

4.4.7 Visualization of GFP Fluorescence in Transformants

GFP fluorescence visualization is useful tool for selecting transformed plants. As (Fig.4.19. (b,d,f,h)) shows GFP expression in transformed hypocotyls, leaf and root. In control GFP was not observed (Fig. 4.19. (a,c,e,g). Visualizing GFP expression is an advantage to select transformation in early stage, thus avoiding regeneration of non transformants.
Fig. 4.18 (a) Confirmation by Five Randomly Selected Colonies after Transformation. Lane 1: 1kb ladder, Lane 2-6: Transformed Colonies, (b) Transient GUS Gene Expression Analysis. (1) Non-Transformed Explant and (3,5) Non-Transformed 3 Weeks Old Calli. (2) Stable Expression of Transformed Explants, (4,6) Stable Expression of 3 Weeks Old Calli, showing GUS Expression in following Co-Cultivated in Presence of 100 µM Acetosyringone. (c) PCR Amplification of Partial hptII gene. 1. 100 bp Ladder, 2. 407 Amplicon of Partial hptII Gene. (d) PCR Amplification of GUS Gene. Lane 1: 100 bp Ladder, Lane 2: 589 bp Amplicon of Partial GUS Gene. (e) BoCCD4 Expression Profile Obtained for B.orellana Callus. RT-PCR Analysis was carried out with CCD4 and 18S rRNA Gene. Lane 1: 100 bp ladder, Lane 2: Amplification of Specific CCD4 Gene and Amplification of 18S rRNA
4.4.8 Molecular Analysis of Transformants

To confirm the occurrence of foreign genes into the genome of transformed callus, antibiotic resistant plants were examined for PCR. PCR analysis was performed with transformed genomic DNA to confirm the presence of transformants. We obtained the expected result in the partial amplified product of about 589 bp (Fig. 4.18.c) corresponding to the GUS gene was observed from genomic DNA using GUS gene-specific primers. An amplified fragment of 407 bp (Fig. 4.18.d) was also observed from transformed plants using \textit{hptII} specific primers that confirm the presence of \textit{hptII}. 

\textbf{Fig. 4.19} Transient Expression of GFP Gene a,c) Without Agro-Infection Hypocotyls ; b,d) 2 Days after Agro-Infection. e) Leaf without Agro-Infection ; f) 2 Days after Agro-Infection in Leaf; g) Root without Agro-Infection; h) Root with 2 Days after Agro-Infection
gene. The amplified products were observed in all transformants tested, confirming the presence of both transformed GUS and hptII.

4.4.9 RT PCR Analysis
To investigate the CCD4a gene expression of transformed callus, RT-PCR was carried out. The cDNA from the transformed callus were subjected to RT-PCR to analyse the expression level. The expected size of the band 390 bp (Fig. 4.18.e) was observed in transformed callus. It is noticeable that the CCD4a gene was expressed in transformed callus. In parallel the 18s rRNA was used as an mRNA expression as a control.

4.5 Effect of UV and Salt Stress towards Bixin Synthesis in B. orellana
4.5.1 UV Stress induced Changes in B. orellana Leaves
B. orellana seedlings were treated with UV-B, and UV-C radiations and their morphological alterations were observed in the abaxial region are depicted in (Fig.4.20). In control leaves, the pigment accumulation pattern was observed as rod shaped spots. There was a significant difference observed in UV-B treated leaves when compared to control leaves. In UV-B treated leaves the pigmentation pattern was more elongated than the control pigmentation pattern. The pigmentation pattern in UV-C was observed to be of a similar pattern of control leaves; however, the pigmentation pattern was found to be numerous in an observed surface area than the control leaves. These morphological alterations were keenly observed in UV treated leaves depicting the effect of UV treatment. Further morphological alterations in the UV- B and UV- C treated leaves with control leaves has been analysed using SEM. In three different samples, the morphological changes near the stomatal region have been observed. There are no significant morphological changes in the leaf surface observed when compared to control leaves (Fig. 4.21).
4.5.2 Photosynthetic Pigment Analysis in *B. orellana* Leaves

The primary carotenoids such as chlorophyll a, b, carotenoid, β-carotene, bixin and abscisic acid content were analysed to study the effect of UV stress on *B. orellana*. UV-B treated plants shows higher chlorophyll a and b content than control as well as UV-C. The total carotenoid level was more in UV-C treated leaves comparing with the carotenoid content in control and UV-B treated leaves. The β-carotene level increased in UV-B and UV-C treated leaves, however β-carotene level in control was
found to be less. The apocarotenoids such as bixin and ABA level of UV treated leaves were comparatively low than the control. The effect of UV B and UV C, on the photosynthetic and apocarotenoid pigment levels like chlorophyll a and b, carotenoid, β-carotene, bixin and abscisic acid is detailed in Table 4.9.

**Table 4.9** Effects of UV-B and UV-C Treatments on Photosynthetic Pigments of *B. orellana*. The Data is Expressed as Mean ± SD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll a (mg/g)</th>
<th>Chlorophyll b (mg/g)</th>
<th>Bixin (mg/g)</th>
<th>Abscisic acid (mg/g)</th>
<th>β carotene (mg/g)</th>
<th>Total Carotenoid (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.177±0.0005</td>
<td>0.718±0.002</td>
<td>26.8±0.0258</td>
<td>13.82±0.0164</td>
<td>1.583±0.022</td>
<td>2.87±0.004</td>
</tr>
<tr>
<td>UV-B</td>
<td>2.57±0.003</td>
<td>3.482±0.002</td>
<td>25±0.0078</td>
<td>13.36±0.117</td>
<td>3.21±0.077</td>
<td>1.563±0.005</td>
</tr>
<tr>
<td>UV-C</td>
<td>1.94±0.0015</td>
<td>0.842±0.001</td>
<td>23.5±0.0072</td>
<td>11.86±0.0046</td>
<td>3.0±0.0087</td>
<td>2.924±0.002</td>
</tr>
</tbody>
</table>

4.5.3 Antioxidant Enzyme Activities

The antioxidant enzymes activities such as CAT, POD, and SOD were measured. The POD activity was high in the UV-B treated leaves, whereas POD level in control and UV-C treated leaves does not vary. The high level of CAT activity was found in the UV-B treated leaves when compete with the control, but there is significant decreased in UV-C treated leaves when analyzed with the control. The SOD activity was more in UV-B treated leaves than control and UV-C treated leaves. Overall, the antioxidant result reveals the UV-B radiations showed an increased antioxidant activity in *B. orellana* than the UV-C and control (Fig.4.22).
4.5.4 Expression of Genes Involved in Bixin Biosynthesis Pathway

Gene expression analysis of bixin biosynthesis pathway under UV-B and UV-C radiations was observed. We analyzed the transcriptional regulation of nine different genes under UV-B, and UV-C radiation in *B. orellana* leaves. The expression of *DXS*, *PSY*, *PDS*, β-*LCY*, ε-LCY, *CCD*, *LCD*, *ADH* and *CMT* were assessed using qRT-PCR. The *DXS* transcript level was high in UV-C treated seedlings compared with UV-B stress and control. In both the UV-B and UV-C treatments the mRNA expression of *PSY* genes was reduced differentially to the control. The mRNA transcript level of the *PDS* was high in control compared with UV-B and UV-C treated seedlings. The highest level of *LCD* was observed in both, UV-B and UV-C
treated leaves on par with the control. The expression level of CMT and β-LCY was downregulated in seedlings treated with UV stress on contrast to the control. The CCD gene transcript level was low in both the UV treated seedlings distinctly with the control. The ε-LCY mRNA expression level was downregulated in UV-B treated seedling at the same the expression level was upregulated in the UV-C treated seedlings showing dissimility expressions with the control. The mRNA transcript of ADH was high in UV-B treated young leaves however UV-C treated seedlings and the control showed low ADH transcript level. The remarkable high level of expression of LCD and ADH has been observed in UV-B treated seedlings. Similarly the mRNA transcript level of DXS, LCD, ε-LCY was upregulated in UV-C treated young leaves. From the results, we observed that UV-B and UV-C treated young leaves showed significant induction of the transcriptional expression in carotenoid biosynthesis gene (Fig. 4.23 (a-i)).

4.5.5 Correlation Analysis
A gene expression and interaction analysis was carried out using in silico analysis to understand the interaction patterns of the protein. Cytoscape 3.0.4 was used to understand the interaction patterns and functional factors involved in particular biological processes. To explore the interaction patterns of genes Cytoscape 3.0.4 was used to understand co-expressed genes. 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 stress. When comparing the expression of the proteins, CCD, LCY, PDS, ZDS and PSY showed closer interaction in comparison to other proteins. Hence expression or suppression of these proteins may also affect the expression of the other important proteins (Fig. 4.24).
Fig 4.23 The Effects of UV-B and UV-C Treatment on the Expression of a) DXS, b) PSY, c) PDS, d) \( \varepsilon \)-LCY, e) \( \beta \)-LCY, f) CMT, g) ADH, h) LCD, i) CCD Genes in *B. orellana* Leaves. The Transcripts were Detected Through RT-PCR using Gene-Specific Primers. An 18S rRNA was included as a Control. The Treatments included a Control (no stress) and UV B and UV C Administration at 5 Days on 1 Hour. The Data are Presented as the Means ± Standard Deviation (SD). A Completely Randomized Design was Utilized. The Data were Subjected to Analysis of Variance (ANOVA), and the Mean Comparison (Tukey test at P<0.05) was Performed using OriginPro 8 Software. The Data Represent the Means ± Standard Deviation. Different Codes Denote Significant Differences among Treatments (P≤0.05).
Fig 4.24 Co-Expression Network Analysis of Related Target Genes
4.6 Effect of salt Stress towards Bixin Synthesis in *B. orellana*

4.6.1 Morphological Analysis

No significant morphological variation occurred in the pigmentation pattern or structural pattern in the control and salinity stress induced seedlings.

4.6.2 Estimation of Pigment Content

The effect of salinity stress on photosynthetic pigment contents such as chlorophyll a, b, total carotenoids, β-carotene, bixin and ABA content was analysed at different concentration of salinity stress (0, 25 mM, 50 mm, 75 mM, 100 mM). Among different concentration of salinity stress seedlings in the chlorophyll a and b content, there is an increase in pigment production in response to salinity when compared to the control. The highest level of chlorophyll a and b content was observed in 50 mM concentration. The lowest level of chlorophyll a and b content was observed in 100 mM concentration. The total carotenoid level was declined with the increase in salinity stress. The high level of β-carotene content was determined in 100 mM concentration of salinity stress when compared to the control. The lowest level of β-carotene content was observed in 75 mM concentration of salinity stress. The secondary metabolite contents such bixin and ABA was observed to be high in 100 mM and 25 mM concentration respectively (Table 4.10).

4.6.3 Response of Antioxidant Activity towards Salinity Stress

The antioxidant enzyme assay was determined for the different concentration of salinity stress in *B. orellana* seedlings. The salinity stress induced an increase in antioxidant defence activity among different stress. The antioxidant activity such as CAT, POD, and SOD was examined to study the changes among the different concentration of stress (Fig. 4.25). The obtained results showed different effects on different antioxidant enzymes. The CAT enzyme showed increased activity with increase in the salt concentration when compared to the control. The highest level of antioxidant activity was observed in 100 mM concentration of salinity stress. The POD enzyme activity showed significant changes in different concentration of salinity stress while increasing the concentration, peroxidise activity was increased. In SOD
antioxidant enzyme activity significant changes has been observed in different concentration of salinity stress. In the SOD activity salinity stress seedlings showed the increasing in antioxidant activity than the control. The highest antioxidant activity has been observed in 100 mM concentration of salinity stress.

Table 4.10 Effects of Different Concentration of Salt treatments on Photosynthetic Pigments of *B. orellana*. The Data is Expressed as Mean ± SD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll a (mg/g)</th>
<th>Chlorophyll b (mg/g)</th>
<th>Bixin (mg/g)</th>
<th>Abscisic acid (mg/g)</th>
<th>β carotene (mg/g)</th>
<th>Total carotenoid (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.177±0.0005</td>
<td>0.718±0.002</td>
<td>26.8±0.0258</td>
<td>13.82±0.0164</td>
<td>1.583±0.022</td>
<td>2.87±0.004</td>
</tr>
<tr>
<td>25 mM</td>
<td>6.913±0.000</td>
<td>4.27±0.001</td>
<td>23.87±0.004</td>
<td>18±0.0023</td>
<td>3.542±0.0011</td>
<td>0.016±0.0025</td>
</tr>
<tr>
<td>50 mM</td>
<td>7.644±0.004</td>
<td>6.302±0.002</td>
<td>24.91±0.005</td>
<td>46±0.0031</td>
<td>3.208±0.048</td>
<td>1.0632±0.006</td>
</tr>
<tr>
<td>75 mM</td>
<td>5.563±0.0035</td>
<td>4.151±0.005</td>
<td>20±0.005</td>
<td>33±0.0025</td>
<td>1.667±0.020</td>
<td>0.572±0.0015</td>
</tr>
<tr>
<td>100 mM</td>
<td>5.042±0.002</td>
<td>3.04±0.003</td>
<td>43.16±0.003</td>
<td>58.33±0.005</td>
<td>4.75±0.005</td>
<td>0.213±0.001</td>
</tr>
</tbody>
</table>

4.6.4 Gene Expression Level of Carotenoid Biosynthesis Pathway

The gene expression analysis of nine different genes in bixin biosynthesis pathway was determined in *B. orellana* seedlings under different concentration (0, 25 mM, 50 mM, 75 mM, and 100 mM) of salinity stress. The carotenoid biosynthesis genes such as *DXS, PDS, PSY, β-LCY, ε-LCY, ADH, CCD, LCD, and CMT* were used for this study. The genes showed varied expression pattern in different concentration of salinity stress using quantitative expression analysis using RT-PCR. The expressions of these genes were decreased with increasing concentration of salinity stress. The *DXS* gene mRNA expression was higher in 25 mM concentration of salinity stress, while increasing the concentration of salinity stress the expression pattern was
declined. The *PSY* gene activity was reduced in all different concentration of salinity stress significantly in comparison to the control. The *PDS* mRNA transcript level was 

![Fig. 4.25](image)

**Fig. 4.25** Effect of Salt Stress (0, 25 mM, 50 mM, 75mM, 100 mM) on Different Antioxidative Enzymes Activities  a) CAT, b) SOD and c) POD of *B. orellana*. The Data is Represented as Mean ± SD

significantly downregulated in seedlings of *B. orellana* treated with different salinity stress on compete with control. The expression pattern of the cyclization enzymes such as *β-LCY*, *ε-LCY* was downregulated with increasing concentration of salinity stress. In the case of *LCD* gene significant reduction of mRNA expression was observed in salinity stress seedlings on par with control. The expression pattern of *CMT* gene was down regulated with increasing concentration of salinity stress. The *ADH* and *CCD* gene expression showed upregulation in the 25 mM concentration of the salinity stress, but increasing concentration of stress induced declined expression
pattern has been observed when compete with control. In general, all the bixin biosynthesis genes have been downregulated with increasing concentration of salinity stress (Fig. 4.26a-i).

4.6.5 Correlation Analysis
To predict the protein protein interaction, we observed that DXS, LCY, PDS and PSY showed close interaction, Meanwhile, ADH, CCD and CMT showed very less correlation with any of the salt stress protein. When comparing the results of correlation study using in silico analysis and gene expression study made using experimental methods, suggest a similarity pattern between the various gene expression (Fig. 4.27).
Fig. 4.26 The Effects of Salt Stress Treatment on the Expression of a) DXS, b) PSY, c) PDS, d) ε-LCY, e) β-LCY, f) CMT, g) ADH, h) LCD i) CCD Genes in B. orellana Leaves. The Transcripts were Detected Through RT-PCR using Gene-Specific Primers. An 18S rRNA was included as a Control. The Treatments included a Control (no stress, Control) and Different Concentration (25 mM, 50 mM, 75 mM, 100 mM). The Data are Presented as the Means ± Standard Deviation (SD). A Completely Randomized Design was Utilized. The Data were Subjected to Analysis of Variance (ANOVA), and the Mean Comparison (Tukey test at P<0.05) was Performed using OriginPro 8 Software. The Data Represent the Means ± Standard Deviation. Different Codes Denote Significant Differences among Treatments (P≤0.05)
Fig. 4.27 Co-Expression Network Analysis of Related Target Genes