MATERIALS AND METHODS

3.1. Breaking of dormancy of the seed

*Caesalpinia bonducella*, pods were collected from a healthy plant grown in the forest range of Bhadra Wild Life Sanctuary of Shivamogga District, Karnataka, during the month of March-April 2011. Healthy seeds were divided into 8 groups of 100 seeds each and each group was subjected to different kinds of mechanical and chemical stress treatments. The first set of 100 seeds were punctured on both sides of seed and soaked in water for 48 hrs. The second sets of seeds were incubated in boiling water for 10 min and soaked in water at room temperature for 48 hrs. Third set of seeds were kept in concentrated Sulfuric acid for 10 min, thoroughly washed with water for 5 to 6 times and soaked in water for 48 hrs. Fourth, fifth and sixth sets of seeds were soaked in different concentrations of Gibberellic acid solutions viz., 2, 5, 10 mg/l respectively, for 48 hrs. The seventh sets of seeds of 100 seeds were pretreated with boiling water for 10 min, washed with distilled water and surface sterilized with 0.1% Mercuric Chloride solution for 10 min. then were aseptically inoculated on MS media supplemented with 5mg/l GA₃. The eighth sets of seeds were kept as control under normal condition without subjecting to any stress. After stress treatment, the seeds were surface sterilized with 0.1% mercuric chloride for 10 min. Then the seeds were rinsed with sterilized cooled distilled water for five times and were sown individually in polythene bags containing sterilized soil and moistened with sterilized water. The bags were maintained in green house at 25 to 30°C temperatures and 65 to 70% relative humidity. The percentage viability of the seeds was calculated after 45 days of sowing. The Seeds inoculated on cultured media were maintained at 22 ± 5°C temperature and 60 -70% relative humidity.
3.2. Standardization of *in vitro* regeneration protocol

Plant tissue culture is the classic field of Biotechnological investigations in which techniques are employed for the growth of plant organs, tissues and cells in *in vitro* conditions. The literary meaning of *in vitro* is in glass, and this is because the culturing is carried out within the glass vessel. This technique has proven its potential for the practical application in improvement of many agricultural, horticultural and medicinally important plant species. Development of the Science of tissue culture is historically linked to the discovery of the cell and subsequent propounding of the Cell Theory. Plant tissue culture could be attributed to the cell doctrine, which implicitly admitted that a cell is capable of autonomy and the potential totipotency. The explants have to be provided with a suitable nutrient medium and proper conditions required for its growth and development. Among them, the general techniques that are essential in all experiments have to be carried out to regenerate the plantlets from explants. To ensure the growth and development of explants under *in vitro* condition it is very essential to maintain the germ free system at all operations.

The following techniques have been employed to regenerate plantlets *in vitro*:

3.2.1. Laboratory organization

To attain optimal aseptic condition, the media preparation room, anteroom, inoculation chamber and growth room were cleaned, sprayed with disinfectant and fumigated with potassium permanganate and formaldehyde. The laminar airflow hood table and its accessories were swabbed with 70% ethanol and UV radiated for 30 minutes
before use. The glasswares required for the preparation of the media and the maintenance of cultures were soaked over night in 40% chromic acid solution, followed by running under a jet of tap water. Later they were dipped in 5% ‘Tween–20’ solution and again washed with tap water. The glasswares were finally rinsed with distilled water and hot sterilized in an oven at 150°C for an hour. The contaminated culture vessels prior to washing were autoclaved at 20 lb/in² for 20 min and the contents were discarded.

3.2.2. Preparation of culture media

Initially, three nutrient formulations were tested to know the organogenic responses in vitro namely, MS medium (Murashige & Skoog, 1962), LS medium (Linsmaier & Skoog, 1965) and Gamborg’s B₅ medium (Gamborg et al., 1968). Since, the response was more in the MS medium, it is used as the basal medium to initiate callus from different explants and regeneration of plantlets from the direct and indirect organogenesis.

All the chemicals used for the preparation of media were of analytical grade (Hi–Media, India). The growth regulators used were procured from Sigma Research Laboratory, America. The macro, micro and organic nutrients were categorized as stocks and were prepared separately. All the nutrient stocks were prepared at 25X concentrations. The iron stocks were kept in amber color bottle and preserved in darkness. It was made a point that stocks once prepared were not used after three months. The stocks for growth regulators such as 2, 4–D (2, 4 dinitrophenoxy acetic acid), NAA (α-naphthalene acetic acid), IAA (Indole 3–acetic acid), IBA (Indole 3–butyric acid),
BAP (6–Benzy1 amino purine), Kn (6–Ferfuryl amino purine) were prepared at 0.1% concentration and coconut milk at 5ml/l.

Coconut water has been shown to stimulate shoot proliferation in many species of plants. Coconut water is prepared from selected coconuts and processed to remove most of the protein. The product is then filter sterilized and frozen prior to use. Remaining protein levels in the water may vary from one lot to the next and may result in precipitate when the product is frozen. This precipitation should not effect the growth of the plant tissue. The precipitate can be removed by filtering or by allowing it to settle to the bottom of the bottle and then decanting. Coconut water can be divided into smaller aliquots, corresponding to your standard medium batch size, and refrozen until needed. Coconut water should be used at a concentration of 5-20% (v/v).

During preparation of the media appropriate quantity of stocks of nutrients and growth regulators were added to the volumetric flask of one-liter capacity. The carbon source, sucrose was added to the solution at the concentration of 3% and made up to desire volume using distilled water. The pH of the medium constituent was adjusted between 56-58 by adding 0.1N hydrochloric acid or 0.1N sodium hydroxide. The media was gelled with 0.8% bacteriological grade Agar-agar (Hi-Media, Mumbai) and homogenized in an autoclave at 15 lb/in$^2$ and at a temperature of 120$^0$C for 5 min. After homogenization about 20 ml of molten medium was dispensed into each culture tube of 25 X 150 mm dimension and 40 ml of media was dispensed into culture bottles of 250 ml capacity and the bottles were covered with sterilizable polypropylene screw caps. The culture media were steam sterilized at 15 lb/in$^2$ and at a temperature of 120$^0$C for 15 min. Then media was allowed to solidify in laboratory condition.
3.2.3. Maintenance of aseptic condition

In order to initiate aseptic cultures, inoculations were carried out under laminar airflow hood. The transfer area with all the paraphernalia (culture vessels, forceps, scalpel, burner, alcohol, stereomicroscope, etc.) were conveniently arranged and sterilized by exposing to UV radiation for a period of one hour. Before and after inoculation, forceps and scalpel were dipped in alcohol and flamed. The mouth of the tube and conical flasks were opened over the flame and inoculation of the explants and subculture of calli was carried out over the flame. To each flask two to three explants or small calli were inoculated. The cultures were checked daily to verify their aseptic condition. If any contamination found in the culture, they were immediately discarded from the batch and killed by autoclaving.

3.2.4. Disinfestation of the explants

The juvenile explants of the seedlings and the matured plant were used as the source of the explants. The plant material which is to be cultured were thoroughly washed in running water followed by 5% liquid detergent ‘Tween-20’ for few minutes to remove all detritus. The required segmental parts were surface sterilized with 0.1% (W/V) aqueous Mercuric Chloride (HgCl₂) for 8-10 min followed by 5-6 rinses with sterilized distilled water and were taken into previously UV radiant sterilized laminar airflow chamber. With the help of a sterilized blade, different parts of the explants were cut into transverse segments of required length and were carefully inoculated onto the organogenic and callogenic media.
3.2.5. Seedling explant culture

The seeds were washed under running tap water, treated with 0.1% Tween 20 for 15 min, then with 70% ethanol for 2 min and disinfected with 2% Mercuric Chloride for 3 min followed by 3 to 4 times wash with sterilized distilled water. Seeds of *C. bonducella* were germinated *in vitro* on MS basal medium (solidified). The aseptically excised segments (0.5 – 1.0 cm long) of cotyledon, hypocotyl and root explants were used culture purposes.

i) **Cotyledon explant culture**

Cotyledon of the *in vitro* germinated immature seed was removed in aseptic condition under laminar air flow hood and aseptically excised in to transverse halves and placed on to the MS medium supplemented with 3 to 6 mg/l BAP and 0.1 to 0.4 mg/l TDZ for high frequency shoot induction.

ii) **Hypocotyl explant culture**

Hypocotyl segments (5-10 mm) of hypocotyl were excised from such aseptically raised hypocotyl and served as explants for both shoot regeneration directly and indirectly from the calli. The direct shoot organogenic media consisted with a range of 2-5 mg/l BAP and 0.1 - 0.7 mg/l TDZ. For callus mediated regeneration, callogenic media consists of 1-4 mg/l 2,4-D and 0.1 -0.9 mg/l BAP.

iii) **Root explant culture**

The root explants were selected from the *in vitro* grown seedlings. After 45 days of culture profuse root initials developed from the seedlings. Root segments of 1.0 - 1.5 cm in length were aseptically harvested from 45 days old culture and used as explants for
the exploration of callogenetic and caulogenetic potentialities. For direct organogenesis, aseptically excised root segments were placed horizontally on to MS medium fortified with 2 mg/l Adenine sulfate, 300 mg/l Polyvinyl pyrrolidone (PVP) and a range 1.0 to 5.0 mg/l BAP and 0.5 to 2 mg/l IBA. For indirect organogenesis the callogenetic media consisted of MS basal salts supplemented with 4mg/l Adenine sulfate, 500 mg/l Polyvinyl pyrrolidone (PVP) and the growth regulators 2, 4-D and BAP are at the range of 0.75 to 3.0 mg/l and 0.1 to 0.5 mg/l respectively. The shoot bud differentiation media fortified with a range of 2.0 – 6.0 mg/l BAP and 0.5 – 2.0 mg/l IAA.

3.2.6. Mature plant explant culture

The tender, stem tip, node leaves flower buds and anther explants of *C. bonducella* were collected from an elite plant maintained in University herb garden. The plant material which is to be cultured were thoroughly washed in running water followed by 5% liquid detergent ‘Tween-20’ for few minutes to remove all detritus. The required segmental parts were surface sterilized with 0.1% (W/V) aqueous mercuric chloride (HgCl₂) for 8-10 min followed by 5-6 rinses with sterilized distilled water and were taken into previously UV radiant sterilized laminar airflow chamber. With the help of a sterilized blade, different parts of the explants were cut into transverse segments of required length and were carefully inoculated onto the MS organogenic and callogenetic media. The effect auxins and cytokinines on organogenic and callogenetic potentialities of each of the juvenile and mature plant explants were tested individually.

i) Stem explant culture

Stem explants were collected between 8-9 AM and were thoroughly washed with running tap water for 5 min and surface sterilized thoroughly with diluted Tween 20
for 2-3 min then with 70% ethanol for 5 min followed by 3 to 4 times rinses with tap water and finally with double distilled water. The explants were disinfested with 0.1% Mercuric Chloride for 10–15 min followed by 3-4 time wash with double distilled water. The stem was aseptically cut in to 1–1.5 cm long segments and was carefully inoculated onto the culture media to induce organogenesis either directly or through the calli. Direct organogenesis media consisted with a range of 2 to 5 mg/l BAP and 0.1 - 0.7 mg/l IBA. For multiple shoot bud differentiation, the media supplemented with the range of 1–6 mg/l BAP and coconut milk at 5ml/l. For indirect organogenesis the callogenic media consisted with a range of 0.75 to 3 mg/l 2,4-D, and 0.1 to 0.5 mg/l BAP. The shoot bud differentiating media supplemented with a range of 2–6 mg/l BAP and 0.1-0.3 mg/l IBA.

ii) Leaf explant culture

The tender leaves of *C. bonducella* were rinsed with running tap water for 5 min and surface sterilized thoroughly with 1% Tween 20 for 2 min then with 70% ethanol for 30 sec followed by 0.1% Mercuric Chloride for 3-4 min and finally rinsed with distilled water for 3 to 4 times. The leaf explants were aseptically cut in to transverse segments and inoculated onto the culture media. The callogenic media consisted with a range 0.5 to 2.0 mg/l 2, 4-D and 0.1 to 1.0 mg/l Kinetin. The caulogenic media augmented with the range of 2.0 to 5mg/l BAP and 1to 2 mg/l IAA.

iii) Flower bud culture

Immature Flower buds (3-5 mm) of *C. bonducella* were collected from the mother elite plant grown in the forest range of Bhadra Wild Life Sanctuary in the month of December. The flower buds are aseptically cultured on callogenic media. Floral callus
was initiated on the media at the growth regulator levels of 0.5 to 3 mg/l 2,4-D and 0.1 to 1.0 mg/l BAP. The primary calli subcultured on to the shooting media which containing MS basal salts supplemented with a range of 2 to 5 mg/l BAP and 0.3 -0.9 mg/l IBA. After four weeks of shoot initiation, the shootlets grew above 3-4 cm length were transferred to rooting media augmented with a range of 0.1-1.0 mg/l of IBA. The regenerants were hardened at greenhouse condition and transferred to the pots containing sterilized soil.

iv) Anther culture

Anthers were collected from young flower buds at appropriate stage. Before anther culture similar stage of flower buds were squashed with acetocarmine and observed for meiotic development. If the meiotic stages are at tetrad or microspore development without complete organization of pollen grains, such anthers are considered for culturing purposes. In C. bonducella the flower buds are collected as soon as the enveloping foliage leaves had exposed the buds. After cleaning with sterile distilled water, flower buds were surface sterilized with freshly prepared 0.1 % aqueous Mercuric Chloride solution for 5 min with intermittent shaking followed by a quick rinse in 70 % ethanol and then washed thoroughly 3 to 4 times in sterile distilled water. Young anthers were dissected out in aseptic condition under laminar air flow hood by looking through the previously sterilized microscope. The anther lobes without the filament were carefully isolated and inoculated in 25 × 150 mm glass tubes (Borosil, Bombay, India) containing with a range of 1 mg/l to 3 mg/l 2,4-D and 0.1 mg/l to 0.4 mg/l Kn. The shoot differentiating media consists of 2- 4 mg/l BAP and 0.3 - 0.9 IBA.
3.2.7. Root induction from the regenerants

The regenerated shoots grew above 4 - 5 cm with 2 to 4 biparipinnately compound leaves, were aseptically transformed to rooting medium consists of MS half strength nutrients with a range of 0.2-1.0 mg/l IBA. After the development of profuse root initials, the rooted plantlets were subjected to hardening process.

3.2.8. Hardening of regenerants

Plantlets with well-grown roots were removed from the culture bottles and roots were washed in running tap water and the regenerants were transformed to pots filled with garden soil, cocopeat dust, and cattle dung manure in the ratio 1:1:2. Initially maintained in the culture condition (25 ± 2°C temperature, 12 h. photoperiod and 60 to 70% relative humidity) by covering with a thin perforated transparent polythene bag to maintain humidity and were watered with 1/10th strength of MS/LM basal salt solution. After two weeks of incubation, the polythene bags were removed and the plants were exposed to garden environment (25 ± 2 °C and 40 – 50% relative humidity) and subsequently they were transformed to forestland adjacent to campus.

3.2.9. Culture condition and result evaluation

All plant growth regulators were added to the medium before autoclaving. The pH of the medium was adjusted to 5.6 to 5.8, autoclaved at 121°C at 15 psi (1.06 kg/cm) pressure for 20 min. The cultures were maintained at 12 h photoperiod with a light intensity of approximately 2000 Lux at 25 ± 2°C with 65 to 70% relative humidity.
A minimum of 10 culture tubes were raised for each combination and all the experiments were performed 10 times. Analysis of variance (ANOVA) and mean separations were carried out using Duncan’s multiple range test, followed by Tukey’s multiple comparison tests to assess the statistical significance. P ≤ 0.05 was considered to be statistically significant, using statistical software SPSS ver. 11 (SPSS Inc., Chicago, USA). The nature and percentage of response was recorded at an interval of two week and sub-culturing was periodically carried out at 25 days intervals.

3.3. **Mass multiplication of calli for secondary metabolite production**

The optimal PGRs concentration for callogenesis from the leaf and stem explants has standardized from the previous experiments. The same concentrations have tried for mass multiplication of calli from each of the explants. The leaf calli was mass multiplied on MS media fortified with 1mg/l Kn + 0.5 mg/l BAP. Similarly the stem calli was mass multiplied on the media supplemented with on 3 mg/l 2,4-D + 1 mg/l BAP. The calli were harvested after four weeks of incubation and ten replicates were used for each of the concentration. The weight of the calli at the initial stage of inoculation and after harvesting period was recorded. The calli dried separately in oven at 50°C and used for the evaluation of phytochemicals.

3.4. **Phytochemical investigation**

3.4.1. **Collection of plant materials**

The leaves and stem bark of *C. bonducella* were collected from forest ranges of Bhadra Wild Life Sanctuary (1 Km from Kuvempu University) of the Western Ghats,
The plant was authenticated by Prof V. Krishna, author of Flora of Davanagere District, Karnataka (Majunath et al., 2004) and Prof. Y.L Krishnamurthy, Department of Applied Botany, Kuvempu University, Shankaraghatta, Shivamogga, District, Karnataka comparing with the herbarium voucher specimen (KUAB.301) deposited at Kuvempu University herbaria. The leaves and stem bark materials were shade dried, powdered mechanically (sieve No. 10/44), stored in airtight containers and subjected to phytochemical investigations. The oven dried leaf calli and stem calli of *C. bonducella* induced on optimal concentrations of growth regulators were also used for secondary metabolite extraction.

### 3.4.2. Preparation of extracts

The shade dried, powdered materials of both the plants were subjected to successive solvent extraction as described below.

The powdered materials of *C. bonducella* leaves, stem bark and their shade dried calli were taken separately in one liter capacity thimble of soxhlet apparatus and refluxed successively with the solvents petroleum ether (40 to 60, E-Mark Mumbai, India), Chloroform (E-Mark Mumbai, India) and ethanol (E-Mark Mumbai, India) for 48 h in 16 batches of 600 gm each. Each time, the solvent from the marc was removed completely before extracting with the next solvent.

All the extracts were filtered and concentrated in vacuum using rotary flash evaporator (Buchi, Flawil, Switzerland). Left over solvent was completely removed on water bath and finally dried in the desiccators. The crude extracts so obtained from each of the solvents were labeled, weighed and the percentage of yield was recorded.
3.4.3. Qualitative phytochemical analysis

The petroleum ether, chloroform and ethanol sequential solvent extracts of leaves and stem bark of *C. bonducella* were subjected to qualitative phytochemical screening using standard methods (Harborne, 1984; Trease & Evans, 1989, Kumara Swamy & Krishna, 2008).

i) Qualitative tests for Alkaloids

**Dragendorff’s test:** To 2 mg of the extract, 5 ml of distilled water was added. To this 2M hydrochloric acid was added until an acid reaction occurs. Then 1 ml of Dragendorff’s reagent was added. Formation of orange red precipitate indicated the presence of alkaloids.

**Hager’s test:** 2 mg of the extract taken in a test tube, a few drops of Hager’s reagent were added. Formation of yellow precipitate confirmed the presence of alkaloids.

**Wagner’s test:** 2 mg of extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner’s reagent were added. A yellow or brown precipitate indicated the presence of alkaloids.

**Mayer’s test:** A few drops of the Mayer’s reagent were treated with 2 mg of extract. Formation of white or pale yellow precipitate indicated the presence of alkaloids.

ii) Qualitative tests for Flavonoids

**Shinoda’s test:** In a test tube containing 0.5 ml of the extract, 10 drops of dilute hydrochloric acid followed by a small piece of magnesium ribbon were added. Formation of pink, reddish or brown color indicated the presence of flavonoids.
**Ferric chloride test:** Test solution with few drops of ferric chloride solution shows intense green color.

**Zinc-Hydrochloric acid reduction test:** Test solution with zinc dust and few drops of hydrochloric acid shows magenta red color.

**Alkaline reagent test:** Test solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow color, which becomes colorless on addition of few drops of dilute acid.

**Lead acetate solution test:** Test solution with few drops of lead acetate (10%) solution gives yellow precipitate.

**iii) Qualitative tests for Triterpenoids**

**Liebermann - Burchard’s test (LB test):** 2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulfuric acid was added along the sides of the test tube. Formation of a violet colored ring indicated the presence of triterpenoids.

**Salkowaski test:** When few drops of conc. sulfuric acid was added to the test solution, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.

**iv) Qualitative tests for Saponins**

**Foam test:** In a test tube containing about 5 ml of an extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 min. Formation of honeycomb like froth indicated the presence of saponins.
Haemolysis test: 2 ml each of 18% sodium chloride solution in two test tubes were taken. To one test tube, distilled water was added and to the other 2 ml of filtrate. Few drops of blood were added to both the test tubes. Mixed and observed for haemolysis under microscope.

v) Qualitative tests for Steroids

Liebermann-Burchard’s test: 2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulfuric acid was added along the sides of the test tube. Formation of green color indicated the presence of steroids.

Salkowaski reaction: 2 mg of dry extract was shaken with chloroform, to the chloroform layer sulfuric acid was added slowly by the sides of test tube. Formation of red color indicated the presence of steroids.

Sulphur test: Sulphur when added to the test solution, it sinks to the bottom.

vi) Qualitative tests for Tannins

Ferric chloride test: To 1-2 ml of the extract, few drops of 5% w/v FeCl₃ solution were added. Formation of green color indicated the presence of gallotannins, while brown color indicated the presence of pseudotannins.

Gelatin test: Test solution when treated with gelatin solution gives white precipitate.

vii) Qualitative tests for glycosides

Baljet test: The test solution when treated with sodium picrate gives yellow to orange color.
**Keller-Killiani test:** The test solution was treated with few drops of ferric chloride solution and mixed. When conc. sulfuric acid containing ferric chloride solution was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green.

**Raymond’s test:** The test solution when treated with dinitrobenzene in hot methanolic alkali gives violet color.

**Bromine water test:** Test solution when dissolved in bromine water gives yellow precipitate.

**Legals test:** Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red color.

viii) **Qualitative tests for Carbohydrates**

**Benedict’s test:** 0.5 ml of extract, 5 ml of Benedict’s solution was taken and boiled for 5 minutes. Formation of brick red colored precipitate indicated the presence of carbohydrates.

**Fehling’s test:** To 2 ml of extract, 1 ml mixture of equal parts of Fehling’s solution A and B were added and boiled for few minutes. Formation of red or brick red colored precipitate indicated the presence of reducing sugar.

**Molisch’s test:** In a test tube containing 2 ml of extract, 2 drops of freshly prepared 20% alcoholic solution of α-naphthol was added. 2 ml of conc. sulfuric acid was added so as to form a layer below the mixture. Red-violet ring appeared, indicating the presence of carbohydrates which disappeared on the addition of excess of alkali.
Barfoed’s test: To the test solution, Barfoed’s reagent was added, boiled on water bath, brick red precipitate was formed.

ix) Qualitative tests for Proteins

Biuret’s test: To 1 ml of extract, 5-8 drops of 10% w/v sodium hydroxide solution, followed by 1 or 2 drops 3% w/v copper sulphate solution were added. Formation of a violet red color indicated the presence of proteins.

Millon’s test: 1 ml of extract was dissolved in 1ml of distilled water and 5-6 drops of Millon’s reagent was added. Formation of white precipitate which turns red on heating indicated the presence of proteins.

Xanthoproteic test: Test solution after treating with concentrated nitric acid and on boiling, gave yellow precipitate.

Ninhydrin test: Test solution when treated with Ninhydrin reagent gives blue color.

3.4.4. Determination of total phenols

Total phenol content in sequential extracts of leaves and stem bark of C. bonducella was measured by the method described by Chang et al., (2002). Briefly, 1 ml of each extract (200 µg) was mixed with Folin–Ciocalteu reagent (2 ml) (diluted 1:10, v/v) followed by the addition of 2 ml of sodium carbonate (7.5%, w/v) and mixed, allowed to stand for 90 min at room temperature and absorbance was measured against the blank at 750 nm using spectrophotometer (Systronics, PC based double beam spectrophotometer 2202). Total phenol content of the extracts was expressed in terms of gallic acid equivalent (GAE, µg/mg of dry mass).
3.4.5. Determination of total flavonoids

Total flavonoid content in sequential extracts of leaves and stem bark of *C. bonducella* was determined according to the modified method of Zhishen *et al.*, (1999). Briefly, 5 ml of extract (200 µg) was mixed with 300 µl of 5% sodium nitrite and 300 µl of 10% aluminum chloride followed by the addition of 2 ml of 1 M sodium hydroxide after the incubation of reaction mixture at room temperature for 6 min. The volume in each test tube was made up to 10 ml by adding 2.4 ml of millipore water. Absorbance was measured at 510 nm against the blank. Total flavonoid content of the extract was expressed in terms of quercetin equivalent (QE, µg/mg of dry mass).

3.4.6. Quantitative estimation phenolics and flavonoids by HPLC-UV analysis

Phenolic acids and flavonoids from various extracts of leaves and stem bark of *C. bonducella* were analyzed by HPLC (Model LC-10ATVP. Shimadzu Corp, Kyoto, Japan) on a reversed phase Shimpak C18 column (5 µm, 250 mm × 4.6 mm). Phenolic content in the extracts were detected using octadecylsilyl silica gel as stationary phase. Solvent system consisting of [A] phosphoric acid:water (0.5:99.5, v/v), [B] acetonitrile was used as mobile phase at a flow rate of 1ml/min. Phenolic acid standards such as gallic acid, p-coumaric acid, ellagic acid, hydroxy benzoic acid and vanillic acids were employed for identification of phenolic acids present in various extracts of leaves and stem bark by comparing the retention time under similar experimental conditions. The detector used for analysis was UV detector at 220 nm. Flavonoid content in the extracts was detected using octadecylsilyl silica gel as stationary phase. Solvent system consisting of methanol, water and phosphoric acid (50:49.6:0.4, v/v) was used as mobile phase at a flow rate of 0.5 ml/min. Rutin, Quercetin, Myricetin, Kaempferol and Luteolin were used as reference standards.
3.4.7. Isolation and characterization of phytoconstituents

i) Leaf chloroform extract

Leaves chloroform extract was subjected for Thin Layer Chromatography (TLC) studies, using the solvent system hexane: chloroform in the ratio of 9:1, which showed separation of three distinct spots, with R$_f$ value: 0.71, 0.57 and 0.52. The constituents were eluted by column chromatography (60×4 cm, 60–120 mesh, 200g silica gel) by gradient elution method using chloroform/hexane in combination and the fractions were collected at the intervals of 5 min. These yielded three different pure compounds; the purity of the isolated compounds was monitored by TLC examination, based on single spot separation. These isolated pure compounds were coded as LC1, LC2, LC3. Melting point of the isolated compounds was determined using scientific melting point apparatus and the observation was recorded.

ii) Stem bark chloroform extract

Initially, the chloroform extract was subjected for thin layer chromatography studies using the solvent system petroleum ether : chloroform at the ratio of 8:2, which showed 5 separated spots. Among them 2 spots were UV active, as observed on UV Chamber and the R$_f$ values were 0.68 and 0.56. The chloroform extract (15g) of stem bark was subjected to silica gel column chromatography (60×4 cm, 60–120 mesh, 200 g), eluted with a stepwise gradient of chloroform-methanol combination and fractions were eluted at the intervals of 5 min. These yielded two different pure compounds, the purity of the isolated compounds was monitored by TLC examination, based on single spot separation. These isolated pure compounds were coded as SC1, SC2. Melting points of the isolated compounds were determined using scientific melting point apparatus.
iii) Calli extract

Initially the leaf callus extract was subjected for thin layer chromatography studies using the solvent system Hexane: Chloroform at the ratio of 9:1, which showed 3 separated spots with $R_f$ value: 0.71, 0.57 and 0.52. The constituents were eluted by column chromatography (60×4 cm, 60–120 mesh, 200g silica gel) by gradient elution method using hexane/ chloroform combination.

The extract of stem callus was subjected to silica gel column chromatography (60×4 cm, 60–120 mesh, 200 g), eluted with a stepwise gradient of petroleum ether - chloroform combination (8:2) and fractions were eluted at the intervals of 5 min. These yielded two different pure compounds, the purity of the isolated compounds was monitored by TLC examination ($R_f$ values: 0.68 and 0.56), based on single spot separation. Melting points of the isolated compounds were determined using scientific melting point apparatus.

The pure compounds isolated through column chromatography were once again tested qualitatively for the presence of phytochemical groups and subjected to IR, $^1$H-NMR and mass spectral studies. Based on the corresponding spectral data the compounds were characterized. The structure of the compound was drawn by using Chem Draw software.

3.5. Pharmacological evaluation

3.5.1. In vitro antioxidants assay

i) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Extracts of leaves and stem bark, and isolated constituents were screened for free radical scavenging activity by DPPH method (Braca et al., 2001). Each extract (100, 200,
300 µg/ml) and isolated constituents (25, 50 and 100 µg/ml) at different concentrations were added to 3 ml of 0.004% DPPH in 95% ethanol and the mixtures were incubated at room temperature in dark condition for 30 min. The scavenging activity against DPPH radical was determined by measuring the absorbance at 517 nm. DPPH radical scavenging activity of BHT was assayed for comparison. Radical scavenging activity was calculated using the formula: % inhibition = [(A_{control} - A_{test})/A_{control}] × 100, where A_{control} is the absorbance of the control reaction and A_{test} is the absorbance of the extract reaction. IC_{50} value was calculated using the formula: IC_{50} = [(ΣC/ΣI) × 50], where ΣC is the sum of extracts and pure compounds concentrations used to test and ΣI is the sum of percentage of inhibition at different concentrations.

ii) Superoxide radical scavenging assay

Superoxide anion radical scavenging activity of leaves and stem bark extracts, and isolated constituents was measured according to the method of Nishikimi et al., (1972) with slight modifications. All the reagents were prepared in phosphate buffer (pH 7.4). 1ml of NBT (156µM), 1ml of NADH (468µM) and 2ml of extracts and pure compounds at different concentrations were added to each test tube. The reaction was initiated by adding 100µl of PMS (60µM) and incubated at 25°C for 5 min followed by the measurement of absorbance at 560 nm against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion radical scavenging activity. Ascorbic acid was taken as reference standard. The percentage inhibition was calculated using the following formula, % inhibition = [(A_{control} - A_{test})/A_{control}] × 100, where A_{control} is the absorbance of the control reaction and A_{test} is the absorbance of the extract reaction. IC_{50} value was calculated using the formula: IC_{50} = [(ΣC/ΣI) × 50], where ΣC is the sum
of test sample concentrations used to test and $\Sigma I$ is the sum of percentage of inhibition at different concentrations.

iii) **Lipid peroxidation inhibition assay**

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected according to the method of Halliwell & Guttridge (1989). 10% of egg yolk homogenate in 0.15M potassium chloride was prepared. 0.5 ml of liver homogenate and 1.0 ml of extract/isolated constituent at different concentrations were taken in test tubes. Lipid peroxidation was induced by adding ferrous sulfate (50µl, 0.07M) and incubated at room temperature for 30min. The reaction was stopped by adding chilled acetic acid (1.5 ml, 20%, pH 3.5) containing 20% TCA followed by the addition of TBA (50µl, 0.8% TBA in 1.1% SDS). The content was mixed thoroughly and incubated in boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added and centrifuged at 3,000 rpm for 10 min. Absorbance of the organic supernatant was measured at 532 nm. Percentage of inhibition was calculated using the following formula, % inhibition = \[ \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100 \], where $A_{\text{control}}$ is the absorbance of the control reaction and $A_{\text{test}}$ is the absorbance of the extract reaction. IC$_{50}$ value was calculated using the formula: IC$_{50}$ = \[ \frac{(\Sigma C/\Sigma I) \times 50}{\Sigma I} \], where $\Sigma C$ is the sum of extract/isolated constituent concentrations used to test and $\Sigma I$ is the sum of percentage of inhibition at different concentrations.

iv) **Nitric oxide radical scavenging activity**

Nitric oxide radical scavenging activity of various extracts of leaves and stem bark and their isolated constituents were determined according to Marcocci *et al.*, (1994).
The reaction mixture containing sodium nitroprusside (2 ml, 10 mM in 0.5 M phosphate buffer, pH 7.4) and 250 µl of test samples at different concentration was incubated at 25°C for 150 min. Aliquot (0.5 ml) of reaction mixture was added to test tube having sulfanilamide (1 ml, 1% in 5% phosphoric acid) and incubated at 25°C for 5 min. 1 ml of 0.1% (α-napthyl)-ethylenediamine was added to the reaction mixture and incubated for 30 min at 25°C. Absorbance of the reaction mixture was measured at 546 nm. Ascorbic acid was used as positive control. The percentage of nitric oxide radical scavenging was calculated using the formula, \%

\[
\text{% inhibition} = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

where $A_{\text{control}}$ is the absorbance of the control reaction and $A_{\text{test}}$ is the absorbance of the extract reaction. IC$_{50}$ value was calculated using the formula: IC$_{50}$ = \left( \frac{\Sigma C}{\Sigma I} \right) \times 50, \] where $\Sigma C$ is the sum of test samples concentrations used to test and $\Sigma I$ is the sum of percentage of inhibition at different concentrations.

v) **Hydroxyl radical scavenging activity**

The hydroxyl radical scavenging activity was determined according to the method of Klein *et al.*, (1981). Extracts/isolated constituents at different concentrations were taken in different test tube and evaporated to dryness followed by the addition of iron-EDTA solution (1 ml, 0.13% ferrous ammonium sulfate and 0.26% EDTA), EDTA (0.5 ml, 0.018%), and DMSO (1 ml, 0.85%, v/v in 0.1M phosphate buffer, pH 7.4) and the reaction was initiated by adding ascorbic acid (0.5 ml, 0.22%). Test tubes were capped tightly and heated on a water bath at 80–90°C and the reaction was terminated by the addition of chilled TCA (1 ml, 17.5%, w/v) after 15 min of incubation, 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and made up to 1l by water) was added and left at room temperature.
for 15 min for color development. The intensity of the yellow color was measured at 412 nm against blank. The percentage hydroxyl radical scavenging was calculated by the formula, \( \% \) inhibition = \( 1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \) \times 100, where \( A_{\text{sample}} \) is the absorbance of the reaction mixture contains extract; \( A_{\text{blank}} \) is the absorbance of the blank. IC50 value was calculated using the formula: \( IC_{50} = \left( \frac{\Sigma C}{\Sigma I} \right) \times 50 \), where \( \Sigma C \) is the sum of test samples concentrations used to test and \( \Sigma I \) is the sum of percentage of inhibition at different concentrations.

vi) Metal chelating activity

Metal chelating activity was measured by according to the method of Dinis et al., (1994). About 3ml of test samples and EDTA at different concentrations were taken in different test tubes followed by the addition of ferrous chloride (50\( \mu l \), 2mM) and ferrozine (20\( \mu l \), 5mM). Tubes were allowed to stand for 10 min at room temperature. Absorbance of reaction mixture was measured at 562 nm against blank. EDTA was used as standard for comparison. Percentage of metal chelating activity was calculated by using the following formula, \( \% \) inhibition = \( \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \) \times 100, where \( A_{\text{control}} \) is the absorbance of the control reaction and \( A_{\text{test}} \) is the absorbance of the test sample reaction. IC50 value was calculated using the formula: \( IC_{50} = \left( \frac{\Sigma C}{\Sigma I} \right) \times 50 \), where \( \Sigma C \) is the sum of extracts/isolated constituents concentration used to test and \( \Sigma I \) is the sum of percentage of inhibition at different concentrations.

vii) Total antioxidant capacity

Total antioxidant capacity of extracts and isolated constituents was performed by phosphor molybdenum method (Prieto et al., 1999). 300 \( \mu l \) of each extract (100, 200,
300 µg) or isolated constituents (25, 50 and 100 µg) at different concentrations were combined with 3 ml of reagent mixture (4 mM ammonium molybdate, 0.6 M sulfuric acid and 28 mM of sodium phosphate). Test tubes were kept for incubation at 95°C for 90 min and allowed to cool. Absorbance of the content was measured at 695 nm against blank. Antioxidant capacity of each extract/pure constituent is expressed as ascorbic acid equivalents (µg/mg of dry mass).

viii) Total reductive capability

Total reductive capacity of extracts and isolated constituents was determined according to the method of Oyaizu (1986). 1 ml of each test sample at different concentration were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.5) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. At the end of the incubation period, trichloroacetic acid (2.5 ml, 10%) was added and centrifuged at 3000 rpm for 10 min. To the 2.5 ml of supernatant, 2.5 ml of millipore water and ferric chloride (0.5 ml, 0.1%) was added. The absorbance of the reaction mixture was measured at 700 nm against blank. Increased absorbance of the reaction mixture indicated increased reducing power. Total reducing capacity of each test sample is expressed as pure constituents equivalents.

3.5.2. Acute toxicity studies

The experimental animals were procured from the Central Animal House, National College of Pharmacy, Shivamogga. These animals were maintained at standard housing conditions (temperature 27±1°C; relative humidity 60 ± 5%) and were fed with commercial diet (Hindustan Lever Ltd., Bangalore) and water ad libitum, during the
experiment. The institutional animal ethical committee (Ref No. NCP/IAEC/CL/10/12/2010-11. Dated: 28-11-2012) approved the study.

The staircase method (Ghosh, 1984) was adopted for the determination of acute toxicity by oral administration. This method involved the determination of LD$_{50}$ value in biphasic manner. The animals were starved of feed but allowed access to water 24 h prior to the study. In the initial investigatory step (phase I), a range of doses of the extract and the constituent producing the toxic effects was established. This was done by oral administration of widely differing doses of the extract (10, 100, 1000, 2000, 3000 mg/kg b.wt.) to the rats and mice based on the pharmacological study. Based on the results obtained, a phase II investigatory step was followed by giving more specific doses (100, 200, 300, 400 and 1600 mg/kg b.wt.). DMSO (1% v/v) was used as a vehicle to suspend the extracts and isolated constituents, and were administered orally. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days for changes in their behavioural pattern (tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma) and mortality. The maximum non-lethal and the minimum lethal doses were recorded. One tenth of LD$_{50}$ dose was considered as the safer dose for oral administration for the evaluation of pharmacological activity of the extracts and the constituents (Litchfield & Wilcoxon, 1949).

3.5.3. Drug formulations

The crude sequential solvent extracts and their isolated compounds were used for drug formulation based on their water solubility/insolubility factors. The one tenth of the lethal dose of extracts and their isolated compounds were mixed with 1% DMSO (v/v) for oral administration. Based on traditional medicinal claim and reviews the crude
extracts and the isolated constituents of *C. bonducella* were used for the evaluation of following pharmacological activities.

### 3.5.4. Evaluation of hepatoprotective and *in vivo* antioxidant activity

Rats were divided into 9 groups consisting of six animals in each group. Group-I served as vehicle control and received 1% (v/v) DMSO (1ml/kg body weight, p.o); Group-II (Toxic control) received 50% CCl$_4$ in olive oil (1ml/kg b.wt., i.p); Group-III received leaves chloroform extract (300 mg/kg b.wt.); Groups-IV received leaves ethanol extract (300 mg/kg b.wt.); Group-V received stem bark chloroform extract (300 mg/kg b.wt.); Group-VI received stem bark ethanol extract (300 mg/kg b.wt.); Group-VII received LC3 (60 mg/kg b.wt.); Group-VIII received SC2 (70 mg/kg b.wt.); Group-IX received standard drug silymarin (25 mg/kg b.wt.) once in a day. Treatment duration was 15 days and all the groups received the intraperitoneal dose of 50% CCl$_4$ after every 72h (Khadeer Ahamed *et al.*, 2010).

At the end of the experimental period, animals were sacrificed by cervical decapitation. Blood was collected and serum was separated. The liver tissue was excised, part of the excised liver was homogenized in ice-cold saline and utilized for biochemical analysis.

#### i) Estimation of liver function marker enzyme activities in serum

Liver damage was assessed by estimating serum marker enzymes such as ALT, AST and ALP using commercially available test kits. The results were expressed as units/litre (U/l). In addition, the levels of cholesterol, triglycerides (TG), total bilirubin
and total protein were estimated in the serum of experimental animals using assay kits and semi auto-analyzer, which were obtained from the Robonik India Pvt. Ltd., New Mumbai.

ii) **Estimation of oxidative stress marker enzyme activities in the liver homogenate**

Hepatic tissues were homogenized (10%) in frozen normal saline and centrifuged at 4000 rpm for 5 min. The supernatant was used for the measurement of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione S-transferase (GST).

a) **Superoxide dismutase**

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp & Fridovich (1971). The 3ml reaction mixture contained 50mM phosphate buffer, pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100μl liver homogenate. Riboflavin was added at the end and the tubes were shaken and placed 30cm below the light source consisting of two 15W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 15 min. The reaction was stopped by switching off the light and the tubes were covered with a black cloth. The absorbance of the reaction mixture was read at 560 nm. Percentage inhibition of NBT reduction was plotted as a function of the enzyme extract used in the reaction mixture. From the graph, the volume of enzyme extract corresponding to 50% inhibition of the reaction was read and considered as one enzyme unit (Beauchamp & Fridovich, 1971) and the results have been expressed as units (U) of SOD activity/mg protein. Protein
content in extracts was estimated by the dye binding method (Bradford, 1976) using bovine serum albumin as the standard.

b) **Catalase**

The catalase (CAT) activity was determined by the method of Aebi (1984). The 45µl homogenate was mixed with 495µl of 0.05M phosphate buffer (pH 7.0) and 500µl of 0.03M H₂O₂ was incubated at room temperature for 2 min. The CAT activity was calculated by the change of the absorbance at 240 nm for 3 min. One unit (U) of catalase was defined as the amount of enzyme required to decompose 1µmol of H₂O₂ per min, at 25°C and pH 7.0. Results are expressed as units (U) of CAT activity/mg protein.

c) **Glutathione peroxidase**

Glutathione peroxidase (GPx) activity was determined as described by Mohandas et al., (1984). Briefly, 0.1 ml of liver homogenate was mixed with 0.8 ml of 100mM potassium phosphate buffer (1mM EDTA, 1mM NaN₃, 0.2mM NADPH, 1 unit/ml GR, and 1mM GSH, pH7.0) and incubated for 5 min at room temperature. Thereafter, the reaction was initiated after adding of 0.1ml of 2.5 mM hydrogen peroxide (H₂O₂). GPx activity was calculated by the change of the absorbance at 340 nm for 3 min. GPx activity is expressed as nmol/min/mg protein.

d) **Glutathione S-transferase**

Glutathione S-transferase (GST) activity was determined as described by Warholm et al., (1985). Briefly, 0.01ml of liver homogenate was mixed with 0.89 ml of 100 mM sodium phosphate buffer (pH 6.5), 0.05ml of 20mM CDNB and 0.05ml of
20mM GSH. GST activity was calculated by the change of the absorbance at 340nm for 3min. GST activity is expressed as n mol/min/mg protein.

iii) *In vivo* lipid peroxidation (LPO) assay

Melanodialdehyde (MDA) is one of lipid peroxidation product determined by the method of Ohkawa *et al.*, (1979). In brief, 0.5 ml of the 10% homogenate was mixed with 100 µl of FeCl$_3$ 0.2 mM, 2 ml reaction mixture (0.25N HCl containing 15% TCA, 0.30% TBA and 0.05% BHA), heated at 80°C for 1h cooled and then centrifuged at 1,500 rpm. The supernatant was collected. Lipid peroxidation products were estimated by measuring the concentration of thiobarbituric acid reaction substances (TBARS) in fluorescence at 530 nm.

iv) Histopathology of liver tissue

The liver tissue was washed with normal saline and kept in 10% formaldehyde buffer for 18h. The tissues were dehydrated in graded (50–100 %) ethanol, followed by clearing in xylene. Paraffin (56–58°C) embedding was done at 58 ± 1°C for 4 h, followed by paraffin block preparation. Paraffin sections of 5 µm were taken using a rotary microtome. The sections were de-paraffinised with alcohol xylene series, stained with haematoxylin–eosin, mounted in DPX with a cover slip and histological changes were observed under microscope (Galigher & Kayloff, 1971).

v) Statistical analysis

Results are expressed as mean ± S.E.M. The statistical analysis was carried out using one way ANOVA followed by Tukey’s *t*-test. The differences in values at *p* < 0.05
or p < 0.01 were considered as statistically significant. Statistical analysis was performed by ezANOVA 0.98.

3.5.5. Evaluation of anticancer activity

The leaves and stem bark of *C. bonducella* chloroform extracts and the isolated compound have been studied for *in vitro* cytotoxicity using human cancerous cell lines. The study was conducted with the help of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai, India. The cell lines used for this study are: human pancreatic cancer cell line MIA-PA-CA-2, human ovarian cell lineSK-OV-3, human hepatoma cell line HEPG2 and Jurkat by employing the sulforhodamine- B (SRB) assay.

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100µL at plating densities depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5%, CO₂, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.

After 24 hrs, a 96 well plate containing 5 x 10³ cells/well was fixed in situ with TCA. Experimental drugs were initially solubilized in dimethyl sulfoxide at the concentration of 100 mg/ml and diluted to 1 mg/ml using sterilized distilled water and stored at frozen condition prior to use. At the time of drug addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test sample. Aliquots of 10 µl of these
different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e. 10, 20, 40 and 80 µg/ml.

After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (Final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded and the plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. The bounded stain was subsequently eluted with 10mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percentage of growth was calculated on a plate-by-plate basis for test wells relative to control wells. The growth percent was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells x 100.

Using the six absorbance measurements [time zero (Tz), control growth (C) and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated using the formulae: \[
\frac{(Ti-Tz)}{(C-Tz)} \times 100 \]
for concentrations for which Ti >/ =Tz (Ti-Tz) positive or zero \[
\frac{(Ti-Tz)}{Tz} \times 100 \]
for concentrations for which Ti< Tz. (Ti-Tz) negative.
The dose response parameters were calculated for each test. Growth inhibition of 50% (GI50) was calculated from \[ \frac{(T_i-T_z)}{(C-T_z)} \times 100 = 50 \], which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from \( T_i = T_z \). The \( LC_{50} \) (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from \[ \frac{(T_i-T_z)}{T_z} \times 100 = 50 \]. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested. The LC50, TGI and GI50 parameters were calculated and they were compared with the positive control drug doxorubicin (Adriamycin, ADR).

### 3.5.6. Diabetic wound healing activity

Alloxan monohydrate was dissolved in saline and administered intraperitonially into fasted rats at a dose of 120 mg/kg body wt. The solution was fresh and prepared just prior to the administration. After 72 h, rats with blood glucose level (BGL) greater than 200 mg/dl and less than 400 mg/dl were selected and observed for consistent hyperglycaemia (fasting blood glucose level greater than 200 mg/dl and lesser than 400 mg/dl) up to 5 days. Such animals were used for experiment (Kesari et al., 2006). The blood glucose was measured on 1\textsuperscript{st}, 8\textsuperscript{th}, 16\textsuperscript{th} and 21\textsuperscript{st} days after treatment. Blood samples were drawn from the tip of the tail vein of the overnight fasted rats. Glucose
concentrations were determined with an Optium Xceed Glucose Monitor (MediSense, UK).

i) **Preparation of ointments**

Formulations of extract and its constituents were prepared to evaluate its efficacy, in comparison with povidone–iodine ointment. Paraffin wax was used as ointment base. Two types of topical formulations were prepared, 5% w/w (crude extracts ointment formulation) and 0.25% w/w (isolated constituents ointment formulation). The ointment base was mixed with extracts/isolated constituents and stirred to get the homogeneous ointment preparation. The drug formulations were freshly prepared on every fifth day.

ii) **Grouping of animals**

The excision, incision and dead space wound models were used to evaluate the wound healing activity of leaves, stem bark extracts and their isolated constituents of *C. bonducella*. The rats were divided into 8 groups, each containing six animals, for excision and incision wound models. Fifty milligrams of formulated ointments were applied topically to each animal once a day. The animals of Group-I received ointment base (control). Groups-II: Leaves chloroform extract ointment (5% w/w); Group-III: Leaves ethanol extract ointment (5% w/w); Group-IV: Stem bark chloroform extract ointment (5% w/w); Group-V: Stem bark ethanol extract ointment (5% w/w); Group-VI: LC3 ointment (0.25% w/w); Group-VII: SC2 ointment (0.25% w/w); Group-VIII were treated with a 5% w/w povidone–iodine ointment.

For the dead space wound model, 7 groups, each containing six animals, were used. The animals of Group-I: Control animals were treated with vehicle (1% DMSO).
Groups-II: Leaves chloroform extract (300 mg/kg b.wt.); Group-III: Leaves ethanol extract (300 mg/kg b.wt.); Group-IV: Stem bark chloroform extract (300 mg/kg b.wt.); Group-V: Stem bark ethanol extract (300 mg/kg b.wt.); Group-VI: LC3 (60 mg/kg b.wt.); Group-VII: SC2 (50 mg/kg b.wt.). The animals were anaesthetized with diethyl ether prior to and during infliction of the wound. All the animals were closely observed for any infection, so that the infected animals can be excluded from the study.

iii) Excision wound model

The animals were anaesthetized prior to and during the creation of experimental wounds, with diethyl ether. Rats were then inflicted with excision wound as described by Morton & Malone (1972). The dorsal fur of the animals was shaved with electric clipper and full thickness of excision wound of 500 mm\(^2\) was created along the marking using toothed forceps, a surgical blade and pointed scissors. The entire wound was left open. All groups of animals were treated in the similar manner as mentioned above. The healing of wound was assessed by tracing the wound on 1\(^{st}\), 8\(^{th}\) and 16\(^{th}\) post-wounding days using transparency paper and a marker, and the recorded wound areas were measured graphically.

The rate of wound contraction was measured as percentage reduction of wound size. Progressive decrease in the wound size was monitored periodically using transparency paper and a marker, and the wound area was assessed graphically to monitor the percentage of wound closure, which indicates the formation of new epithelial tissue to cover the wound. Wound contraction was expressed as reduction in percentage of the original wound size.
The percentage (%) wound = \frac{(\text{Wound area on day ‘0’} - \text{Wound area on day ‘n’})}{\text{Wound area on day ‘0’}}

iv) Incision wound model

The test rats were anesthetized with diethyl ether prior to and during the creation of experimental wounds. The dorsal fur of the animals was shaved with electric clipper and two para-vertebra along incision of 6 cm length were made through the skin at a distance of about 1.5 cm from the midline on each side of the depilated back of the animals as described earlier (Ehrlich & Hunt, 1968). After incision, the parted skin was stitched together at intervals of one centimeter using surgical thread (No.000) and curved needle (No. 11). The wounds were then left undressed. All groups of animals were treated as described above. Sutures were removed on eighth post-wounding day and the treatment was continued. The skin breaking strength of healed wound was measured on the 10\textsuperscript{th} day by the method of Lee (1968). The breaking strength is the strength of a healing wound, which is measured by the amount of force required to disrupt it.

a) Determination of wound breaking strength

The sutures were removed on the eighth post-wounding day and the breaking strength was measured on 10\textsuperscript{th} day. After removal of skin sutures, on 10\textsuperscript{th} post-operative day gradually increasing weight was applied to one side of the wound while the other side was fixed in tensiometer. The weight that completely separated the wound from the incision line is considered to be the breaking strength. The mean breaking strength on the two para-vertebral incisions on both sides of the animals was taken as the measure of the breaking strength of individual animal.
v) **Dead space wound model**

Dead space wounds were created by subcutaneous implantation of sterile cylindrical grass pith (2.5 x 0.3 cm), on either side of the lumber region on ventral surface of each rat (Turner, 1965). On the 10th post-wounding day, animals were sacrificed under diethyl ether anesthesia, and the granulation (wound) tissues formed on the grass piths were excised.

a) **Hydroxyproline estimation**

After recording the wet weight, the granulation tissues were dried at 60°C for 12 h in an oven to obtain constant dry weight. Simultaneously, the dried tissue was hydrolyzed with 6N HCl (5.0 ml) for 24 h at 110°C, and then neutralized (pH - 7). The neutralized hydrolysate was used for hydroxyproline estimation as described by Neuman & Logan (1950). About 200 µl of hydrolysate was mixed with 0.01M copper sulfate solution (1ml) followed by 2.5N NaOH (1ml) and 6% H₂O₂ (1ml). Samples were then kept at 80°C (for 5min) on a shaking incubator, cooled and mixed with 4ml of 3N H₂SO₄ with agitation. Finally, 2ml of 5% p-dimethyl aminobenzaldehyde was added to the mixture, incubated (70°C for 16 min), cooled (20°C) and the absorbance was measured at 540 nm in a colorimeter. The amount of hydroxyproline present in the sample was calculated from a standard curve prepared with pure L-hydroxyproline.

b) **Hexosamine estimation**

To estimate the hexosamine levels, the weighed granulation tissues were hydrolyzed in 6 N HCl for 8 h at 98°C, neutralized to pH 7 with 4 N NaOH, and diluted with Milli-Q water. 200 µl of test sample was mixed with 1 ml of acetylacetone solution (the reagent was prepared by dissolving 1 ml of acetyl acetone in 50 ml of 0.5N sodium
carbonate solution) and heated to 96°C for 40 min. The mixture was cooled, and 96% ethanol was added, followed by the addition of 1ml of p-dimethylaminobenzaldehyde solution (0.8g p-dimethylaminobenzaldehyde was dissolved in 30 ml alcohol + 30 ml conc. HCl). The solution was thoroughly mixed and kept at room temperature for 1 h, and the absorbance was measured at 530nm using a double-beam UV/VIS spectrophotometer. The amount of hexosamine was determined via comparisons with a standard curve (Pauly & Ludwig, 1922).

c) **Uronic acid estimation**

Uronic acid in the wound tissue was estimated by carbazole method with slight modifications as described by Bitter & Muir (1962). 5 ml of sodium tetraborate-sulfuric acid reagent (0.025 M sodium tetraborate was dissolved in conc. sulfuric acid) was placed in tubes cooled to 4°C and 1 ml of the test sample or standard (containing 20- 200 µ moles uronic acid) was layered on to the surface. Tubes were closed with stoppers and the contents mixed with cooling so that the temperature of the mixture does not exceed room temperature. The tubes were then heated in a boiling water bath for 10 min and cooled to room temperature. 0.2 ml of carbazole reagent (0.125% in absolute ethanol) was added. The resulting solution was heated for 15 min in a boiling water bath and cooled, the absorbance was read at 530 nm.

3. 5.7. Evaluation of antimicrobial activity

Preparation of nutrient agar media for microbial production: Bacteriological peptone-10 g, Beef extract-3 g, Sodium chloride-5 g and agar-15 g were dissolved in 1000 ml of distilled water and pH was adjusted to 7.2 to 7.5 and sterilized the media for
30 min at 15 lbs pressure. Antibacterial activity was conducted by using the method of Mackie & McCartney (1996).

i) **Microorganisms tested**

Strains of most common pathogenic gram positive bacteria namely, *Pseudomonas aeruginosa, Bacillus Subtilis* and gram negative bacteria namely, *E-coli* were obtained from National Chemical Laboratory, Pune and Microbial type culture collection and gene bank, Chandigarh, India and four clinically isolated pathogenic fungi such as *Aspergillus niger, Rhizopus tolonifer and Fosarium oxysporum* obtained from Shivamogga Institute of Medical Sciences, Shivamogga were used as test organisms. Before testing, the suspensions were transferred to LB broth and cultured overnight at 37°C. Inocula were prepared by adjusting the turbidity of the medium to match the 0.5 McFarland standards. Dilutions of this suspension in 0.1% peptone (w/v) solution in sterile water were inoculated on LB agar, to check the viability of the preparations. The fungal stocks cultures were stored on Brain heart infusion (BHI, Merck) culture media (pH 6.5).

ii) **Antimicrobial assays**

Antimicrobial activity was tested by employing agar-well diffusion method (Mukherjee *et al.*, 1995; Nair *et al.*, 2005) and was used to assess the antimicrobial activity of the test samples. Sterilized LB agar (tryptone 10 g/1, yeast extract 5 g/1, sodium chloride 10 g/1, agar-agar 15 g/1, pH 7.2) medium was poured into sterilized petri dishes (90 mm diameter). LB broth containing 100 µl of 24 h incubated cultures of the respective strains was spread separately on the agar medium. Wells were created using a sterilized cork borer under aseptic conditions.
In order to identify the antifungal activity of extracts against fungal pathogens an agar diffusion assay was performed in BHI culture media (pH 6.5). Fungal cells were obtained by centrifugation at 1500 x g, 4°C for 15 min and diluted in PBS, pH 7.2. The final concentration of each strain was 106 cells/ml. Cultures were grown for 3 days at 37°C. One hundred µl of fungal spores were spread on BHI agar plates and wells were made using a sterilized cork borer and 50 µl of test compounds were loaded into each well. The plates were refrigerated for 2 h in order to stop fungal growth and facilitate diffusion of the substances.

The chloroform (50 mg/ml) and ethanol extract (50 mg/ml) and its constituents (5mg/ml and 10 mg/ml), reference antibacterial agent ciprofloxacin (1 mg/ml; Cipla, Mumbai) and antifungal agent amphotericin B (1 mg/ml; Medico Remedies Pvt. Ltd, Mumbai) were loaded in the corresponding wells (100 µg per 100 µl of sterilized distilled water). As a control, the wells were loaded with the same volume of sterile distilled water. Plates were then incubated at 37°C for 48 h. At the end of the incubation period, inhibition zones formed on the medium were determined and data was statistically evaluated by Tukey’s pair-wise comparison test.

iii) Minimum inhibitory concentration (MIC) of extract and isolated constituents

Minimal inhibitory concentration (MIC) values were determined by broth dilution method. Serial dilutions (final volume of 1 ml), chloroform and methanol extract and the constituent of C. bonducella (0.5 to 0.05 mg/ml) were performed with 0.9% saline. Following this, 9 ml of nutrient broth was added. Broths were inoculated with 100 µl of each bacterial suspension (5 x 10⁴ CFU) and incubated for 24 h at 37°C. Ciprofloxacin was used as the positive control and 0.9% saline as negative control. After 24 h, bacterial
growth was assayed by measuring absorbance at 625 nm. MIC was defined as the lowest concentration in mg of stem bark of *C. bonducella* and constituent of calli to restrict the growth to < 0.05 absorbance at 595 nm (National Committee for Clinical Laboratory Standards, 2005).

The *in vitro* fungicidal activity (MFC) was determined described by Espinel-Ingroff *et al.*, (2002). After 72 h of incubation, 20 µl was subcultured from each well that showed no visible growth (growth inhibition of over 98%), from the last positive well (growth similar to that for the growth control well), and from the growth control (extract-free medium) onto PDA plates. The plates were incubated at 27°C until growth was seen in the growth control subculture. The minimum fungicidal concentration has regarded as the lowest extract concentration that did not yield any fungal growth on the solid medium used.