SUMMARY AND CONCLUSION

Herbal drugs are playing an important role in catering the health of worldwide population and there is a resurgence of interest in the herbal medicines for the treatment of various ailments like hepatitis, epilepsy, insomnia etc., for which there is no specific allopathic medical care. It is believed that herbal drugs are relatively safe and exhibit a remarkable efficacy in the treatment of chronic ailments. The therapeutic properties of the medicinal plants are due to the presence of active principles, which has to be extracted and screened for medicinal properties. Hence, many pharmaceutical companies collect the medicinal plants in bulk from the forests for the preparation of drugs. In addition, many anthropogenic factors like urbanization of land, over-exploitation of plants, uncontrolled grazing, frequent forest fire and the pollution stress are responsible for the depletion of medicinal plants from the natural habitat and rendering them as rare and threatened species. There has been a growing awareness of the imminent danger to plant life and naturalists are evincing interest in biodiversity and its conservation through \textit{in vivo} and \textit{in vitro} propagation. The plant biotechnology offers quick and efficient methods to exploit medicinal plants meaningfully to meet the measuring demands of the pharmaceutical industries and to reduce \textit{in situ} harvesting pressure from natural forest resources.

In the present investigation, a threatened medicinal plant of the Western Ghats \textit{Caesalpinia bonducella} has been selected. Medicinal property of seeds has known from the ancient treaties like Charaka Samhitha and it has been used in the preparation many
ayurvedic formulations. Because of high medicinal value, seeds of this species have been rigorously explored for its phytoconstituents and pharmacological properties. Due to destruction of habitat and over exploitation for its medicinal purposes this species is at the verge of threatening in most of the tropical countries. In this investigation a sincere attempt was made in the *ex situ* conservation by standardizing micropropagation protocols by culturing seedling and mature plant explants. Different explants like cotyledon, hypocotyl, stem, leaf, root, immature flower buds and anther were subjected to *in vitro* studies to explore their regenerative potentialities. The traditional medical claim of this species has authenticated with the evaluation of prophylactic effect, diabetic wound healing property, anti cancerous and antifungal activities of the stem bark, leaves, leaf calli extracts and their isolated constituents using pharmacological models.

### 6.1. Evaluation of germination potency of the seeds

The seeds of *C. bonducella* are very hard and found to remain dormant in the soil for many years. In the preliminary investigation an attempt has been made to break the dormancy of *C. bonducella* seeds using various parameters such as hot water treatment, mechanical stress treatment, acid treatment and hormonal treatment.

The results revealed that the seeds *C. bonducella* subject to boiling water treatment for 10 min recorded 86% germination after 45\(^{th}\) day of sowing. The hot water treatment reduced the impermeability of the seed coat and facilitated the uniform water uptake of the seeds and it has also evidenced the increase in weight of the seeds. However, in the control group of *C. bonducella* seeds remained dormant even after 12 months of sowing. Due to the presence of hard seed coat of *C. bonducella* may be
impermeable for water and after the treatment with strong acids and bases made the seed coat permeable to water. In concentrated Sulfuric acid and in punctured seeds stress 30% of the seeds were germinated and marked reduction in the final weight of the seeds has observed. Among the three concentrations of Gibberellic acid treatment 44% of germination observed at 5mg/l. The percentage of germination increased in the seeds pretreated with hot water and cultured on MS media fortified with 5mg/l GA$_3$. With in 30 days of culture 92% of the seeds germinated on the media and it is considered as the ideal method to break the dormancy of the seeds of _C. bonducella._

6.2. Micropropagation of plantlets

Micropropagation protocol has been standardized for this threatened woody liane of the Western Ghats by culturing cotyledon, hypocotyl, leaves, stem, root, flower bud and anther explants. Regeneration plantlet was achieved in both direct and indirect method. In the culture of seedling explants like, cotyledon, hypocotyl and root, synergetic effect of Benzyleaminopurine (BAP) and Thidiazuron (TDZ) have provoked shoot organogenesis directly from the explants. Among them, frequency of shoot organogenesis was highest in cotyledon explant culture. A mean of 41.80 ± 5.43 shoots per explant were organized at the concentration of 5 mg/l BAP and 0.2 mg/l TDZ. In hypocotyl culture a mean of 5.20±1.14 shoots per explant were organized directly at the concentration of 4 mg/l BAP and 0.5mg/1 TDZ. MS medium supplemented with 4 mg/l BAP and 0.5 mg/l IBA, induced a mean of 3.40 ± 1.07 shoots directly from the surface of excised root explant. On the contrary, regeneration of plantlets via callogenesis was achieved from the hypocotyl and root explant culture.
In indirect organogenesis callus induction was optimized from the hypocotyl explant at the concentration of 3 mg/l 2,4-D and 0.3 mg/l 6-benzyladenine (BAP). Subculturing of the callus onto the differentiating media supplemented with 1-6 mg/l BAP and 0.1–1 mg/l indole-3-butyric acid (IBA) resulted the development of small green protuberances formed over the entire surface of the callus which later developed into small shoots. 4 mg/l BAP with 0.5 mg/l IBA was the optimal hormonal combination for multiple shoot bud differentiation from the hypocotyl callus with a mean of 3.40 ± 0.97 shoots/explant and a mean shoot length of 7.09 ± 0.23 per callus was achieved. In root explant culture, callogenic frequency was optimized (96.66%) at the concentration of 2 mg/l 2,4-D and 0.2mg/l BAP. An average, 15.30 ± 5.25 shoots were differentiated from the root callus at the concentration of 4 mg/l BAP and 0.5 mg/l IBA.

Among the mature explant culture, organogenic potentialities of stem explant was more and plantlet regeneration was achieved both directly from the explant and indirectly through the stem calli. A mean of 3.10 ± 0.88 shoots per explant were organized directly at the concentration of 4 mg/l BAP and 0.5 mg/l IBA. Initially, small knot like structures were appeared all over the surface of the stem explant. Later they developed in to shoot buds. Aseptic isolation and sub culturing of these shoot bud on to the same media revealed growth of buds in to shoots. In indirect organogenesis, callus induction was optimized at 2 mg/l 2,4-D and 0.2 mg/l 6 BAP in 96.66% of all explants. After culturing callus for 15 days on MS medium with a small green protuberances formed over the entire surface of the callus which later developed into small shoots. 4 mg/l BAP with 0.2 mg/l IBA was the optimal hormonal combination for multiple shoot organogenesis with a mean of 36.6 ± 1.17 shoots/explant and a mean shoot length of 7.09 ± 0.23 per callus has grew up in to shoots.
In leaf explant culture plant regeneration was achieved only through the callus. The leaf explants of *C. bonducella*, induced luxuriant callus on MS media supplemented with 1.0 mg/l 2, 4-D and 0.5 mg/l Kinetin. The Shoot bud differentiation was achieved on the differentiating media with a range of 2.0 - 6.0 mg/l BAP and 0.5- 2.0 mg/l IAA. At optimal concentration (4.0 mg/l BAP and 0.5 mg/l IAA) a mean of 15.30±5.25 shoot buds per callus mass was observed.

Flower bud explants were collected in the month of December from an elite plant. Floral callus was initiated on MS media at the growth regulator levels of 0.5 to 3 mg/l 2,4-D and 0.1 to 0.9 mg/ l BAP. Optimal callogenesis was noticed from the flower bud explants at the concentration of 0.5 mg/ l BAP and 2 mg/l 2,4-D. callogenesis was initiated from the thalamus region of the flower, gradually it proceeded towards the non accessory and accessory floral parts of the flower. The primary floral callus fleshy creamy white and nodular, while the shoot differentiating callus was pale blackish interspersed with greenish loci. The primary calli subcultured on to the shooting media which contain MS basal salts supplemented with a range of 2 to 5 mg/ l BAP and 0.3 to 0.9 IBA and high frequency shoot induction was noticed at 3 mg/ l BAP and 0.5 IBA.

Anther 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 0.4 mg/l Kn and Optimal callogenesis was noticed at the concentration of 0.2 mg/l Kn and 2 mg/l 2,4-D. During callogenesis, fleshy pale yellowish mass of callus was sprouted from the anther lobes. The theca and the connective tissues of the anther lobe turns to brown, while fleshy mass of the callus grew in to whitish nodular mass. Differentiation of slender shoot bud noticed from this nodular mass at the concentration of 2.5 mg/l BAP and 0.7 mg/l IBA.
The well grown shoots organised either directly or indirectly were transferred to rooting media. Rooting was achieved when shoots attained the height of 3 to 4 cm with 2 to 4 pinnately compound leaves on MS half strength medium fortified with 0.6 mg/l Indole-3-butyric acid (IBA). Regenerated plants were successfully acclimatized in polythene bags filled with garden soil, saw dust, and cattle dung manure in the ratio 1:1:2 for a period of 4 to 5 weeks at 70 to 80% relative humidity in greenhouse condition.

6.3. Mass production of calli for the evaluation of secondary metabolites

One of the objectives of this investigation is to explore the mass production of the calli for harvesting of the bioactive constituents. Therefore, calli were incubated and maintained at optimal concentrations of growth regulators. When compared to leaf culture, callus induction efficiency was more in stem explants. The leaf calli were multiplied in bulk amount on the MS medium supplemented with optimized concentrations of growth regulators. The maximum callus growth was observed at BAP (0.5mg/l) and Kn (1mg/l) and it was fleshy, pale yellowish, spongy, and loosely arranged. The stem calli was mass multiplied on the MS media containing 3 mg/l 2,4-D and 1 mg/l BAP with 5ml/l of coconut milk. Initially cultures were maintained in bottles and in successive subculture they were transformed in to 20cm diameter Petri plates for mass multiplication. The fresh weight of the total mass of the calli was evaluated between the day one of inoculation and upto 45 days of incubation. The total dry weight of the calli mass was evaluated after complete drying of the callus at 50°C in a hot air oven.
6.4. Phytochemical evaluation

The yield of petroleum ether (grayish jelly), chloroform (dark brown dry solid in nature) and ethanol crude (dark brown dry waxy in nature) extracts for 1 kg of the powdered stem bark plant material obtained from Soxhlet extraction was 9.0 g, 16.83 g and 42.5 g, respectively. The yield of petroleum ether (Yellow jelly), chloroform (dark green dry solid in nature) and ethanol crude (dark black dry solid in nature) extracts for 1 kg of the powdered leaves plant material obtained from Soxhlet extraction was 14.0 g, 32.5 g and 87.2 g, respectively. The yield of ethanol crude (dark black dry solid in nature) extracts for 100 g of the powdered leaf and stem calli obtained from Soxhlet extraction was 12.2 g and 9.9 g respectively.

The quantitative estimation of phenolics and flavonoids contents in the leaves, stem bark and their calli extract revealed that the stem bark chloroform extract possess highest content of total phenolics \( (91.15 \pm 1.0 \text{ µg/mg of dry extract of Gallic acid equivalent, GAE}) \) and flavonoids \( (104.1 \pm 1.25 \text{ µg/mg of dry extract of Quercetin equivalent, QE}) \). Whereas, stem bark ethanol extract and stem calli ethanol extract possess moderate amount of phenolics \( (79.15 \pm 0.3 \text{ and } 88.45 \pm 1.0 \text{ µg/mg of dry extract GAE respectively}) \) and flavonoids \( (67 \pm 1.5 \text{ and } 96.3 \pm 2.7 \text{ µg/mg of dry extract QE respectively}) \) content. The leaves ethanol extract comprised comparatively less content of total phenolics \( (27.09 \pm 0.02 \text{ µg/mg of dry extract GAE}) \) and flavonoids \( (12.33 \pm 0.67 \text{ µg/mg of dry extract QE}) \).

HPLC analysis was performed to characterize the phenolic acids and flavonoids present in pharmacologically very active extract of leaves (chloroform extract) and stem
bark (chloroform extract). HPLC analysis of chloroform extracts of leaves and stem bark revealed the presence of gallic acid, ellagic acid. In addition the leaf extract showed two unknown phenolic acids with retention time of 2.69 and 3.44 min and the stem bark extract possess two unknown phenolic acids with retention time of 1.09, and 4.18 min respectively.

The HPLC-UV spectral peaks of chloroform extract of leaves showed the peaks for presence of flavonoid compounds, rutin, quercetin, myricetin, Kaempferol at 350 nm with the analysis of retention time of standard flavonoids. The stem bark extract showed peaks for the presence of flavonoid compounds, rutin, quercetin, and five unknown peaks. Whereas, the HPLC analysis of stem calli extract revealed the presence of Gallic acid, Coumaric acid, Rutin, Quercetin and Luteolin.

The leaves chloroform extract and stem bark chloroform extract were therapeutically more active and hence, both the extracts were subjected for the isolation of phytochemicals. In the present investigation, five phytoconstituents were isolated from the leaf and stem bark extracts of C. bonducella using silicagel adsorbent column chromatography. Isolated compounds were subjected to spectral characterization viz., IR, $^1$HNMR and MASS spectral analysis. From the leaves chloroform extract, Compound LC1: (Eicosane); Compound LC2: (1-Triacontanol) and Compound LC3:(β – Sitosterol) were isolated using solvent system hexane: chloroform in the ratio of 8:2. These three phytoconstituents were also isolated from the *in vitro* grown leaf calli for comparative analysis. The concentration of 1-Triacontanol was enhanced in leaf calli extract compared to intact natural plant extract. From the stem bark Chloroform extract two terpenoids compounds *viz.*, Compound- SC1: ((E)-pentadecyl dec-5-enoate) and
Compound SC2: Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate were isolated. SC1 and SC2 were also isolated from the stem calli extract. However, the concentration of SC2 is comparatively more in stem calli extract.

6.5. Pharmacological evaluation

The traditional practitioners of the Western Ghats are using the leaves of *C. bonduc*ella to cure liver disorders and jaundice. The stem bark has used to cure diabetic wounds and microbial infections. This investigation also supported the traditional medicinal claim of *C. bonduc*ella with the evaluation of prophylactic effect, diabetic wound healing property, anti cancerous and antifungal activity of the stem bark, leaves, leaf calli extracts and their isolated constituents using pharmacological models. The prophylactic activity of extracts and the constituents were evaluated by performing *in vitro* and *in vivo* antioxidant activity assays.

Different *in vitro* assays have been conducted in this investigation includes, DPPH radical scavenging assay, superoxide radical scavenging assay, nitric oxide radical scavenging assay, hydroxyl radical scavenging assay, metal chelating assay, lipid peroxidation inhibition assay, total antioxidant capacity assay and total reductive capability assay. The stem bark chloroform extract and its isolates SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) have exhibited remarkable results in the radical scavenging activity *viz.*, DPPH radical, hydroxyl radical (OH), superoxide radical (O$_2^-$) and nitric oxide (NO) free radicals; highest metal ion chelating activity, reductive capability and inhibition of lipid peroxidation activity. The leaves chloroform extract and its isolate oleanolic acid has shown moderate activity, and leaves chloroform extract has shown least activity in
comparison with the other extracts. Compounds LC3: (β – Sitosterol) has shown least antioxidant activity.

In acute toxicity studies, results revealed that the LD$_{50}$ value of chloroform and ethanol extracts of leaves and stem bark has found to be 3,000 mg/kg b.w. similarly, LD$_{50}$ value of the isolated constituents, LC3: (β-Sitosterol) and SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) were 80 and 180 mg/kg b.wt respectively. One tenth of these LD$_{50}$ doses has considered as safer dose for oral drug administration in the pharmacological models.

Estimation of liver function marker enzymes and oxidative stress marker enzymes of the liver tissue was conducted for the evaluation of in vivo antioxidant activity. CCl$_4$ induced hepatotoxicity has been employed to investigate prophylactic effect of the extracts and the compounds using rat models. As oxidative stress plays a central role in liver pathologies and their progression, the use of antioxidants have been proposed as therapeutic agents, as well as drug co-adjuvants, to counteract liver damage. In the present study intoxication of CCl$_4$ to the rats showed significant increase in total bilirubin, triglyceride, total cholesterol, Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP) activities and decrease in total protein content indicating the hepatic damage.

The animals treated with stem bark chloroform extract reduced the higher levels of liver function markers nearer to the normalcy. Among the isolated phytoconstituents, SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) was more significant by reducing the serum markers level towards normalcy. Similarly,
in the assay of oxidative stress enzyme activity of the liver homogenates, the stem bark chloroform extract administered animals showed significant amelioration effect by elevating the decreased levels of SOD, CAT, GPx and GST levels. The increased level of MDA was also restored. The restoration of levels of oxidative stress marker enzymes was also significant in tested phytochemicals, SC2. The levels of these oxidative stress enzyme markers were nearly equal to that of the standard drug silymarin treated group.

Histological profile of the control animals showed normal hepatocytes. The sections of the animal treated with CCl₄ exhibited severe intense centrilobular necrosis and vacuolization. The sections of liver taken from the animals treated with standard drug silymarin showed the hepatic architecture was well protected as compared to the observations made in the control animals liver section. Normal hepatic architecture, absence of necrosis and few fatty lobules were noticed in the liver sections of the animals treated with stem bark chloroform extract and its isolated compounds viz., and SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) against the toxicant CCl₄. The hepatoprotective activity of the above herbal drugs may be due to its potent antioxidant properties.

In the present investigation, leaves and stem bark extracts and the phytoconstituents LC3 and SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) have been used for in vitro cytotoxicity on the following human cancer cell lines namely: Human hepatoma cell line HEPG2, Human Ovarian Cancer Cell Line SK-OV-3 and Human Pancreatic Cancer Cell Line MIA-PA-CA-2. The anticancerous activity was evaluated by employing the sulforhodamine-B (SRB)
assay. The study was carried out with the help of Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai. The chloroform extract of leaves and stem bark chloroform extract were did not show cytotoxicity against all tested cancerous cell lines.

Wound healing activity was evaluated in Alloxan induced diabetic rats. The plasma glucose level was measured in normal and experimental rats on day 1, 8, 16 and 21 of drug treatment. Alloxan treated diabetic rats showed significant increase in the levels of blood glucose (318.5 ± 3.5 mg/dl) when compared to normal rats (86.5 ± 2.5 mg/dl) on 21st day. Among the crude extracts tested, the stem bark chloroform extract has shown highest anti-hyperglycemic activity by reducing blood glucose level to 204.5 ± 4.5 mg/dl and least hyperglycemic activity (286 ± 2.0 mg/dl) by stem bark ethanol extract at the end of the experiment. The three different wound models viz., excision, incision and dead space wound models have been used to screen the healing property of different crude extracts of leaves and stem bark, and their isolated compounds. The commercial ointment povidone iodine was used as a reference standard to assess the healing effect of the various extracts and the constituents.

In above said models significant wound healing activity was observed in the animals treated with the stem bark chloroform extract and its constituent SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate). Considerable decrease in the period of epithelialization, increase in the wound contraction rate, increase in the tensile strength and restoration in the levels hydroxyproline, hexosamine
and uronic acid content of the wound tissue were observed in these groups of animals. Whereas, moderate and least wound healing activity was observed in the animals treated with the leaves ethanol extract.

Histological study of the granulation tissue provides further evidence on the wound healing efficacy of the extracts and the constituents. The section of the granulation tissue of the untreated animals showed monocytes and fibroblasts. Incomplete healing was evidenced with lesser epithelialization, fibrosis and collagen formation. The sections of the granulation tissues of the animals treated with stem bark chloroform extract showed complete epithelialization, fibrosis and collagen formation. Whereas, high deposition of collagen and significant reduction of macrophages infiltration has been noticed in histology of wound section treated with SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate).

The antibacterial and antifungal activity of the leaves and stem bark extracts, and isolated constituents showed varying magnitudes of inhibition patterns with standard positive control depending on the susceptibility of the tested microorganism. Among the leaves and stem bark extracts tested, the leaves chloroform extract showed a significant level of inhibition against all bacterial strains followed by stem bark chloroform extract. Leaves chloroform extract showed highest inhibition zone against \textit{Pseudomonas aeruginosa} (16.10 ±1.10 mm) and least against \textit{E. coli} (12.1 ± 0.82 mm). Stem bark chloroform extract exhibits maximum inhibitory activity against \textit{Pseudomonas aeruginosa} (18.20 ± 0.35 mm) and poor activity against \textit{E. coli} (16.80 ± 0.44 mm). Stem
bark ethanol extract showed comparatively least antibacterial activity against all bacterial strains. Similarly, leaf chloroform extract also exhibited good antifungal activity against tested fungal strains.

In antifungal assay, the zone of inhibition of stem bark chloroform extract, leaves chloroform extract of *C. bonducella* was evaluated. Among the three fungal isolates cultured for antifungal assay, the zone of inhibition of leaves chloroform extracts were found to be maximum on *Fusarium oxysporum* (7.0 ± 1.30). whereas stem bark chloroform extract showed maximum inhibition in *Rhizopus stolonifer* (8.1 ± 0.05). The isolated constituent SC2: (Methyl (4E)-5-{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxyphenyl}pent-4-enoate) showed maximum inhibition zone against *Aspergillus niger* (7.2 ± 1.2), *Fusarium oxysporum* (7.0 ± 1.3) and *Rhizopus stolonifer* (6.8 ± 0.22). The isolated constituent LC2 showed less inhibition activity in all the fungal strains tested.

*Caesalpinia bonducella* traditionally used mainly for the treatment of liver disorders, to heal wounds, tumors, elephantiasis recorded in Ayurvedic system of medicine. Due to unscientific and over collection of plant materials for medicinal purposes the population of this species is depleting drastically. In the present investigation, the protocol has been developed for the in vitro regeneration of *C. bonducella* from different explants and also method has been standardized for mass production of calli. This provides the eco-friendly system for harvesting of therapeutically active phytomedicine from the *C. bonducella* without harming its natural population. The results of this investigations supported the ethnomedical claims of *Caesalpinia bonducella* as a potent antioxidant and diabetic wound healing plant.
6.6. Conclusion

Mass multiplication of medicinally important threatened plants through tissue culture technique suggests *ex situ* alternative method conservation. Most of the medicinal plant resources are being threatened and are on the verge of extinction due to the high medicinal value, non scientific exploitation and destruction of natural habitat. In this study an efficient *in vitro* regeneration protocol has been standardized for a threatened medicinal plant *C. bonducella* through seedling and mature plant explant culture and regeneration was achieved directly and through the respected calli.

Seed dormancy is a curse in the improvement of population of threatened/endangered plants. The woody legume seeds are very hard with extended dormancy period. Development of an efficient protocol to break the dormancy of seeds will helpful in maintenance of the population and it may provide source for the industries for the pharmaceutical exploitation of *C. bonducella*.

The therapeutic properties of the medicinal plants are due to the presence of active principle, which has to be extracted and screened for medicinal properties. The main aim of medicinal plant explant culture is to instinct the cells to biosynthesize the therapeutic constituents continuously under controlled condition. So that, the medicine men / industries, need not depend upon the natural population and season of the extraction of phytoconstituents.
The traditional claim indicated that the leaves and stem bark of *C. bonducella* are the potential source of drug for jaundice and diabetic wound healing. This investigation supported the traditional claim that stem bark chloroform extract and its isolated constituent that is Methyl (4E)-5-\{2-[(1E)-buta-1,3-dien-1-yl]-4,6-dihydroxy phenyl\} pent-4-enoate showed significant activity on diabetic wound healing model and this thesis can serve to guide the future studies by pointing out promising therapies for diabetic wound healing property of *C. bonducella*. Further clinical trials are essential to confirm the therapeutic efficacy of these bioassay guided phytoconstituents.