DISCUSSION
V. DISCUSSION

The supreme importance of plant wealth for the sustenance of life on the planet earth and the survival of mankind cannot be emphasized enough. Deforestation and changing environmental conditions have been causing a threat to many species thereby some species have already become extinct and some others are threatened to extinction. There has been a growing awareness of the imminent danger to plant life and naturalists are evincing interest in biodiversity and its conservation through in vivo and in vitro propagation.

1. In vitro studies

The in vitro regeneration is an efficient means of ex situ conservation of plant diversity (Krogstrup et al., 1992; Fay, 1994) because with this technology many endangered species can be quickly propagated and preserved from a minimum of plant material and with little impact on wild population. Moreover, this technique has the unique advantage of propagating the desired taxon, independent of season, reproductive barriers, germination hurdles and so on.

Many investigators were successful in deriving the protocols for rapid multiplication of endangered medicinal plants, such as Gymnema elegans (Komalavalli and Rao, 1997); Enicostemma axillare (Sudhersan, 1998); Triphyophyllum axillare (Bringmann and Rischer, 2001); Embelia ribes (Shankarmurthy et al., 2004); Celastrus paniculatus (Maruthi et al., 2004); Andrographis alata (Nagaraja et al., 2005) etc. The increasing demand for medicinally valuable natural products such as steroids, alkaloids, glycosides, flavonoids etc., has also resulted in increased attention of plant tissue culture (Dziezak, 1986).

In the present investigation also protocols are standardized for the in vitro conservation of two medicinal plants belongs to the family Ranunculaceae, viz., C. gouriana and N. zeylanica.
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Choice of the explant

The importance of choosing the ideal explant and following thorough sterilization procedure for the successful establishment of *in vitro* cultures has been emphasized by Torres (1989). However, taking explants from healthy *in vitro* plants involves in reducing the risk of sterilization procedure.

Media selection

Selection of an appropriate basal medium for multiple shoots production was the main objective of this experiment. Several basal media formulations have been examined for their ability to sustain growth and development of excised explants in various plant species by different workers (Shankarmurthy, 2007 and Vidya, 2007).

Keeping in view of the above works, four different media- MS, LS, B5 and Nitsch media were used along with varied concentration and combinations of auxins and cytokinins. Among all the media tested, MS medium was the appropriate one for induction of maximum number of multiple shoots the stem explants of both the species. Nitsch’s media showed poor response in terms of callus formation, but LS and B5 resulted in callus formation. Therefore, MS medium was chosen as the basal medium for all the future experiments.

The effect of different growth regulators in combinations was also studied using BAP and FAP as cytokinin and NAA, IAA, IBA and 2, 4-D as auxins. Generally, most of the plant species require an auxin for dedifferentiation. The ratio of auxin and cytokinin is an important with respect to the response in the culture system. For callus initiation, the requisite ratio of auxins to cytokinins is high, while the *vice versa* is true for induction of regeneration from cultures (George, 1993). Activated charcoal is generally used to overcome the phenolics, secreted by the explants in to the media. It was found that, in *C. gouriana* there was no problem of phenolics but in *N. zeylanica* showed the problem of phenolics
which was overcome by the addition of PVP and changing pH in the culture medium and the addition of charcoal decreased the response in terms of shoot number. This decrease in shoot number in presence of charcoal may be due to the interference of charcoal in the uptake of nutrients as well as growth hormones.

**In vitro studies on Clematis gouriana Roxb. and Naravelia zeylanica (L.) DC.**

In many species cytokinin alone or in combination with lower concentration of auxin provoked direct organogenesis from the explants (Bansal and Pandey, 1993). The reason for effectiveness of the FAP may lie in the ability to stimulate the plant tissue to metabolize the natural endogenous hormones or could induce the production of natural hormone system for the induction of shoot organogenesis (Blakoshey and Lenton, 1987). In the present study also decisive factors controlling adventitious shoot organogenesis from the leaf explant was the balance of cytokinin and auxin ratio. Direct organogenesis has the unique advantage of maintaining the genetic stability of a desired taxon.

The experiment was conducted, based on the reports on shoot induction from callus, as reported by Lee *et al.*, (1982) Ferreira and handro (1988) and Nualbunruang (1990). In one of the earlier experiments, it was noticed that FAP induced compact callus growth. Therefore, various concentration of FAP along with the combination of auxin- IAA, IBA, NAA and 2,4-D were tried. Results suggested that FAP at low concentration with 2, 4-D induced profuse callus and response decreased with increased concentrations.

2, 4-D in combination with FAP gave good results. The concepts that high auxin and low cytokinin leading to undifferentiated cells was evident here, 2, 4-D induced callus was nodular and greenish in color, with faster rate of morphogenesis. In the majority of the plant species the synthetic hormone 2, 4-D interacted with endogenous hormones of the explants and stimulated the cells to proliferate into callus mass (Narayanaswamy, 1994 and Razdan, 2003).
As observed in the culture of *Guizotia abyssinica* (Gangopadhyay and Mukhergee, 2002), in *C. gouriana* also proliferation of callus observed at the range of 2.0 to 3.0 mg/l 2, 4-D and 0.2 to 0.6 mg/l FAP. The rate of induction and development of luxuriant callus mass depends on different concentrations and combinations of 2, 4-D and FAP and it also varies with the different plant part (explant) used for culture. The combination of 2, 4-D with FAP at the range of 1.0 to 4.0 mg/l 2, 4-D and 0.25 mg/l to 1.0 mg/l FAP respectively induced better proliferation of callus in case of stem explant and it was optimized at 3.0 mg/l 2, 4-D and 0.4 mg/l FAP.

In *N. zeylanica* BAP was found to be superior cytokinin. The effect of BAP on organogenesis of shoot is reported by many investigators (Jyothi and Dhar, 1996; Singh *et al.*, 2002; Maruthi *et al.*, 2004). The reason for effectiveness of the BAP may lie in the ability to stimulate the plant tissue to metabolize the natural endogenous hormones or could induce the production of natural hormone system for the induction of shoot organogenesis. The leaf explants of *N. zeylanica* were capable of undergoing morphogenic responses. Within three week of incubation, the leaf explants became swollen and showed sprouting of shoot buds from directly along the edge of the leaf explants without intervening callus. Similar mode of organogenesis of the shoot buds directly along the edge of the leaf explant was reported on *Coffea bengalensis* (Mishra and Sreenath, 2003). In many species the synergetic effect of higher concentration of cytokinin with lower concentration of auxin in balance induced better shoot organogenic response than the medium supplemented with cytokinin alone (Bansal and Pandey, 1993; Chandrasekhar Reddy *et al.*, 1995; Susan *et al.*, 1998; Xie and Hong, 2001; Suchita and Susan 2004; Krishna and Shanthamma, 2004). The same observation was noticed in the explants of *N. zeylanica* where 2.5 mg/l BAP and 0.5 mg/l IBA could induce 13.5 ± 0.30 shoots.
The stem explants of *N. zeylanica* cultured on the media containing 4.0 mg/l BAP and 0.6 mg/l NAA showed the best multiplication and adventitious multiple shoot induction from the nodal region of the stem explants. At this optimal concentration 15.1±1.45 shoots per explant differentiated. The increase in NAA concentration with a decrease in BAP level induced calli induction and diminished the average number of shoots per explant. A similar type interaction of BAP with NAA was reported on multiple shoot induction from the nodal segments viz., *Mimosa tenuiflora* (Villarreal Ma and Rojas, 1996); *Canavalia virosa* (Kathiravan and Ignacimuthu, 1995); *Ochreinauclea missionis* (Naomita and Ravishankar Rai 2004); *Decalepis hamiltonii* (Anitha and Pullaiah, 2002); *Wedelia chinensis* (Martin et al., 1995); *Solanum nigrum* (Jabeen et al., 2005); *Psoralea corylifolia* (Anis and Faisal 2005); *Pseudoxytenanthera stocksii* (Sanjaya et al., 2000) and *Mecardonia tenella* (Liliana et al., 2006) etc.

The regeneration of shoot from callus is a re-differentiation process whose physiology is least explored. The potential genetic variability associated with this system may assume importance for genetic improvement and selection strategies. Callus culture also provides excellent tool for biochemical investigation. Callus growth and development are influenced by a complex relationship between the explants used, the constituents of the medium and the proper environmental conditions (Brar and Khush, 1994). As *N. zeylanica* has high polyphenols, PVP was added to the medium and changing the pH -5.8 to 6.2 in culture medium was adapted in order to reduce the browning of the explants and also the production of secondary metabolite.

The alteration in the ratio of auxins and cytokinins leads to varied type of morphogenesis (Skoog and Miller, 1957). In the present experiment on *N. zeylanica*, the interaction of higher levels of BAP with lower levels of NAA in provoking the caulogenic potency of the callus. The reports of many of the medicinally important species Sainfarid JaiVval, (2000) and Ramulu et al., (2002) also support our results for differentiation of plantlets from the combination of BAP and NAA hormones.
During caulogenesis, the concentration of BAP and NAA varies with the various callus developed from different source of the explants used for culture. In *N. zeylanica* stem callus culture, differentiation of shoot buds were observed on the medium supplemented with BAP and NAA at the range of 4.5 mg/l to 5.5 mg/l BAP and 0.2 to 0.6 mg/l NAA respectively. At an optimum concentration (5.0 mg/l BAP and 0.4 mg/l NAA) high frequency shoot bud (15.0 ± 0.39) differentiation were noticed. The callus derived from the stem explants devoid of leaf primordia were cultured on MS medium, explant required similar concentration of BAP (4.0 to 6.0 mg/l BAP), but the NAA concentration increased from 0.1 mg/l up to 0.9 mg/l when compared to leaf callus culture. Though, the BAP at same concentration with little increase in NAA concentration (0.7 mg/l), caulogenesis from stem calli was noticed. As reported in case of *Enicostemma axillare* frequent isolation and subculture of the shoot buds also enhanced the multiplication rate of the shoot buds (Sudersan, 1998). Reports of auxin and cytokinin combinations supporting organogenic differentiation have been well documented in several species viz., *Aristolochia bracteolata* (Remashree et. al., 1994), *Catalpa ovata* (Lisowska and Wysokinska, 2000); *Pothomorphe umbellate* (Pereira et. al., 2000); *Hypericum perforatum* (Pretto and Santarem, 2000); *Embelia ribes* (Shankarmurthy et. al., 2004); *Centella asiatica* (Shashikala et. al., 2005) etc. In all the cases of leaf and stem cultures the hormone 2, 4-D in combination with FAP was favored in the induction of callus. But the optimum concentration of the hormones used in combination was differing with different explants. In the same way the cytokinin BAP and auxin NAA was also responsible for the induction of shoot buds in the explants like leaf and stem commonly on the MS medium. High concentrations of BAP favored maximum number of shoot buds in case of leaf and stem explant.

In both the plants *in vitro* rooting of microshoots was obtained on MS media pretreated with IBA at the concentration of 0.1 and 0.5 mg/l induced root initials from the excised cut ends of the shoots. The similar type of results was also
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reported in case of Sesbania bispinosa (Sinha and Mallick, 1991); Dalbergia latifolia (Raghava Swamy et. al., 1962); Swainsona salsula (Yang et. al., 2001) and Celastrus paniculatus (Maruthi et. al., 2004).

Morphological Variation was screened after success of tissue culture depends on the establishment of in vitro regenerated plantlets under natural conditions. In case of C. gouriana survival rate of direct organogenesis was 80% and in calli regenerants it was 66% without morphological variations whereas in N. zeylanica it was 96% and in indirect organogenesis it was 72% with morphological variation was observed. The morphology of the two month-old regenerants derived from direct organogenesis was similar with the in vivo plants. The callus regenerants showed some variations like abnormal growth, reduced height, a slender stem and curled leaves, which was screened by RAPD markers.

Evaluation of Somoclonal variation using RAPD markers

Selection, domestication and cultivation of plants are based on the genetic diversity of the species. Therefore, information on distribution, preservation, variation and relations is extremely important for several purposes. Studies of genetic diversity in plants have greatly enhanced our understanding of mode of speciation, adaptation, and population dynamics. Such studies have important applications in in situ and ex situ conservation strategies as well as in plant breeding (Bussell, 1999; Nybom and Bartish, 2000). Genetic diversity of a species depends on factors like phyletic group, life form, geographic range, regional distribution, breeding system, seed dispersal mechanism, mode of reproduction and successional status (Hamrick and Godt, 1989; Bhat, Babrekar & Lakhanpaul, 1999). Several techniques, including morphological, biochemical and molecular methods, have been used to measure genetic diversity in plant species. The most widely used PCR-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and recently
simple sequence repeats (SSRs) or microsatellites (Staub, Speiper, Gupta, and Varshney, 1996; Gupta and Varshney, 2000). In vitro regeneration usually results in high genetic and phenotypic variability in individuals derived from cultures, which is called somatic variation. Somatic variation can be beneficial in medicinal plant improvement especially on traits for which somaclonal mutants can be enriched during in vitro culture, including resistance to disease pathotoxins, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites.

The results were scored as patterns of bands obtained from in vitro micropropagated plants and compared with plants maintained in the field. Only bands that were consistently reproduced across amplifications were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. In C. gouriana the OPB-17 and OPB-19 primers produced amplification products that were polymorphic across all micropropagated plants. The rest of the other primers did not yield good amplified products. The size of the polymorphic DNA fragments, produced by these two primers ranged from 0.3 to 1.0 Kb. The Dendrogram generated by scoring of the gel profile provides one major cluster and a minor cluster, the major cluster involves Cl-1, Cl-5, Cl-4 and Cl-6 and in minor cluster forms Cl-2 and Cl-3 there is a little difference between Cl-4 and Cl-6 and it has formed one group and there is no much genetic variation between Cl-1 and Cl-5 and the group Cl-4 and Cl-6. Thus they have formed one major group similarly, in minor cluster Cl-2 and Cl-3 formed one cluster and it might be due to their genetic similarity. The linkage distance among the individuals is 7.5.

In N. zeylanica the regenerants were amplified with six decamers out that, five informative primers were selected and were used to evaluate the degree of
polymorphism within all the micropropagated plants. The selected primers generated distinctive products in the range of 0.1–1.0 Kb. Maximum number of bands were produced by the primer OPF-17 and OPF-19. The results scored from gel profile are drawn in the dendrogram amplified with OPF-17 primer formed two major clusters. Cluster one consists of Direct 1 (D1), Direct 2 (D2), Direct 3 (D3), Indirect 1 (IND1), Indirect 2 (IND2), Indirect 3 (IND3) and Indirect 5 (IND5) and cluster two consists of Direct 4 (D4), Direct 5 (D5), Direct 6 (D6), Indirect 4 (IND4), Indirect 6 (IND6) and In vivo. Due to similarity in banding pattern in D1 they have clustered together with D2, the D1 and D2 are similar with D3 these are clustered with D3 it lacks one band and remaining bands are similar with that of the in vivo plant it has connected with the in vivo plant. The cluster one formed four major groups among these groups genetic variation was observed by the presence or absence of single bands. In Cluster two it has formed two groups with direct, indirect and in vivo plants they have showed maximum similarity which could be observed in gel profile. The linkage distance is 6.3. The results are drawn in the dendrogram amplified with OPF-19 primer formed one major clusters and one minor cluster, in major cluster involves D1, D2 and D3 formed one group and IND1, IND2, IND3 and IND5 forms a group and IND4 and IND6 forms another group. In cluster two forms D4, D5 and D6 and in vivo plant, these analyses shows that direct organogenesis regenerants are almost similar to the in vivo plants.

When the results of both the primers were compiled and a new dendrogram was constructed which forms two major clusters. The cluster-1 forms all the direct organogenesis plants and the cluster - 2 forms all the indirect organogenesis plants with the linkage distance of 14. The regenerants derived from direct organogenesis were related to in vivo plants which indicate that they are deviated from their parental characteristics. The variation differentiating the callus regenerants from in vivo plants may be due to cytodifferentiation influenced by exogenously
supplemented growth regulators. The present study of DNA profiling in *N. zeylanica* species clearly showed that it was possible to analyze the RAPD patterns for correlating clonal variation and their genetic distance by which one could predict the variation within the species to a great extent.

The DNA amplification products, which represent one allele per locus, could result from changes in either the sequence of the primer binding site or changes which alter the size and prevent the successful amplification of target DNA. In our study, the amplified products exhibited monomorphisms among all the *in vitro* plants and were similar to those from control plants. The method is simple and the results are reproducible. Because only micro-amounts of material are necessary, this approach can be used to assess tissue at several stages of *in vitro* culture. Large sample sizes can be handled rapidly, and the technique lends itself to automation (Williams *et al.*, 1990; Welsh and McClelland, 1990; Hedrick, 1992). Rani *et al.* (1995) found RAPD variations among 23 micropropagated *Populus deltoides* plants originating from the same clone and morphologically similar. Bouman *et al.* (1992) and Bouman and Kuijpers (1994) also found intraclonal RAPD polymorphism amongst micropropagated *Begonia* spp. but at a lower frequency than phenotypic variations and without any correlation with the phenotype.

Somaclonal variation is a common phenomenon in plant cell cultures, characterized by phenotypic variation of either genetic or epigenetic origin, may be an explanation for the wide range of inhibitor activity found. Accordingly, somaclonal variation can be either an advantage (variability increase) or disadvantage (loss of stability of cell lines), depending on the aim of the study. However, it has become apparent that, rather than being an unexplainable aberration in an otherwise uniform cell proliferation process, the appearance of variants in cell cultures may be a routine occurrence for certain types of plants or specific explant sources. Since all cells of an organism are derived from a single
cell, it has been assumed that plants derived from cells of a donor plant would yield identical individuals. Recently, quantitative analyses of Velutinol - A and its glycosylated form MV-8612 revealed the occurrence of higher content in cell culture extracts when compared to the *in vivo* plant (Maraschin, 1998). Several studies have shown that somaclonal variation can be assessed by analysis of phenotype, chromosome number and structure, proteins or direct DNA evaluation of plants (De Klerk, 1990). The types of variation that are frequently observed may differ from species to species, and it is often difficult to determine the genetic nature of the observed variation (Saunders *et al.*, 1992). However, one should keep in mind that the correspondence between changes at the phenotypic and cytological/molecular level should be considered, since good correlation might not be found between the extent of mutations (molecular level) and phenotypic changes (De Klerk, 1990).

In contrast, analysis of the occurrence of variants in plant cell cultures concerning biochemical phenotype has been undertaken to a lesser extent. It has been recognized that this phenomenon may provide a source for useful variation, which can form the basis for the development of plant cell lines with characteristics of interest even on an industrial scale. As an example, we may mention the production of shikonin from *Lithospermum erythrorhizon* (Fujita, 1988) of berberine from *Coptis japonica* (Fujita *et al.*, 1987), and of ginsenosides from *Panax ginseng* (Ushiyama, 1991). Thus, the information about somaclonal variability can be considered fundamental to studies concerning the production of secondary metabolites (Petiard *et al.*, 1985).

**Callus Culture for secondary metabolite production**

Medicinal plants are the most important source of life saving drugs for the majority of the world’s population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. *In-vitro*
regeneration holds tremendous potential for the production of high-quality plant-based medicine. Natural products are naturally derived metabolites and/or by products from microorganisms, plants or animals (Baker et al., 2000). In the field of traditional medicine, natural products have been exploited for human use for thousands of years, and plants have been the main source of compounds used for medicine. As natural products gain increasing importance and attention from chemists and pharmacologists, their discovery from new sources will continue to be essential in order to provide novel lead compounds which the synthetic chemist can modify. This is the major reason for embarking on research projects in the field of natural products. Secondary metabolites are molecules that are not directly necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. Secondary metabolites are often involved in key interactions between plants and their abiotic and biotic environments that influence those (Facchini et al., 2000). Plants produce secondary metabolites as defenses against fungi, bacteria, insects and viruses. They also produce them as colourful pigments to attract insects for pollination. The exact nature of the role of many secondary metabolites is not known although they have been identified and extracted.

Plants are the traditional source of many chemicals used as pharmaceuticals. Most valuable phytochemicals are products of plant secondary metabolism. The production of secondary metabolites in vitro can be possible through plant cell culture (Barz, 1981; Deus, 1982). Successful establishment of cell lines capable of producing high yields of secondary compounds in cell suspension cultures has been reported by (Zenk, 1978). The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on environmental conditions (Stafford, 1986). Strategies for improving secondary products in suspension cultures, using different media for
different species, have been reported by Robins, 1994. The production of solasodine from calli of *Solanum eleagnifolium*, and pyrrolizidine alkaloids from root cultures of *Senecio* sp. are examples (Nigra, 1987; Toppel, 1987). Scragg *et al.*, (1992) isolated quinoline alkaloids in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana*. Enhanced indole alkaloid biosynthesis in the suspension culture of *Catharanthus roseus* has also been reported (Zhao, 2001). The possible use of plant cell culture for the specific biotransformation of natural compounds has been demonstrated (Cheetham, 1995; Scragg, 1997; Klings and Berger, 1998; Ravishankar and Ramachandra Rao, 2000). Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations. So far, the manipulation of culture media, culture conditions and phytohormone levels have permit commercial production of those phytochemicals useful in medicine and industry. The secondary metabolites produced are stored either intracellularly (in the vacuole or in other organelles), or extracellular (bound to the cell wall or released into the medium; Brodelius, 1990). In some cases the secondary products can be enhanced by changing the composition of the medium. Berlin *et al.*, (1988) investigated that the lowered concentration of inorganic phosphate in the medium, resulted in an increased production of alkaloids in *Thalictrum rugosum*. The pH of the medium can also influence the enhancement of secondary metabolites. Majerus and Pareilleux (1986) observed a sharp increase in the enhancement of alkaloids in *Catharanthus roseus* when the pH of the culture medium was changed from 9.0 to 4.3. The effect of pH also had a profound influence on shoot multiplication and callogenesis. At normal pH (5.8) exudation of phenolics from the organogenic explants or the calli continued until the medium and the explants turned black, node and subsequent callus formation. In contrast, at pH 6.0-6.2 normal growth and caulogenic response was observed and also the production of secondary metabolites was slightly increased when compared to normal pH.
The earlier investigator did not attempt to evaluate the biosynthetic potency of the callus for secondary metabolite production. In the present study calli induced from the leaf explants of *C. gouriana* and *N. zeylanica* were subjected for the evaluation of secondary metabolites.

In case of *C. gouriana* the combination of higher concentration of 2, 4-D and lower concentration of FAP induced whitish hard nodular mass in both the leaf and stem explant culture. But these growth regulators influenced the varying amount of calli in leaf explants. The luxuriant proliferation was observed at the concentration of 2.5 mg/l 2, 4-D and 0.4 mg/l FAP (55.12 ± 2.04 g fresh weight and 1.37 ± 0.23 g dry weight). The effect of 2, 4-D and FAP on callus initiation was also reported by Tefera (1998) and Mersinger *et al.*, (1988).

In *N. zeylanica* among the different concentrations of 2, 4-D tested with BAP for callus mass production, the interaction of 0.3 mg/l 2, 4-D with 1.0 mg/l BAP yielded significant amount of calli (63.05 ± 3.26 g fresh weight and 1.90 ± 0.13 g dry weights). This showed that the action of growth regulators in *in vitro* condition is highly specific, it may also be due to the synergetic effect of BAP with the endogenous growth regulators of the explants. The effect of growth regulators on callus growth of different plant species was studied in several research reports. *Thymus vulgaris* (Tamura *et al.*, 1995); *Nigella sativa* (Youssef *et al.*, 1998); *Gypsophila paniculata* khat callus (Hamid *et al.*, 1999); *Mikania glomerata*, *Cephaelis ipecacuanha* and *Maytenus aquifolia* (Kapchina and Stoyanova, 2003); *Zataria multiflora* (Mohagheghzadeh *et al.*, 2004); *Gymnema sylvestre* (Gopi and Vatsala, 2006).

Successful establishment of cell lines for the production of secondary metabolites has been reported in various medicinal plant species. The production of solasodine from calli of *Solanum eleagnifolium*, (Nigra *et al.*, 1987) and pyrrolizidine alkaloids from root calli of *Senecio* sp. (Toppel *et al.*, 1987).
Cephaelin and emetine were isolated from callus cultures of *Cephaelis ipecacuanha* (Jha et al., 1988). Callus culture also provides an excellent tool for biochemical investigation. Callus growth and development are influenced by a complex relationship between the explants used, the constituents of the medium and the proper environmental conditions (Brar and Khush, 1994). Scragg (1992) isolated quinoline alkaloids in significant quantities from globular cell callus culture of *Cinchona ledgeriana*. Enhanced indole alkaloid biosynthesis in the callus culture of *Catharanthus roseus* has also been reported (Zhao et al., 2001).

In the present investigation, a triterpene ursolic acid was isolated from the leaf and leaf calli of *C. gouriana* and alkaloid Berberine was isolated from methanol extract of leaf and leaf calli *N. zeylanica*. The production of phytochemical constituent was enhanced in callus as compared to *in vivo* plant, this may be due to the presence of nutritional requirement present in the media. Further, in case of *in vivo* condition the production of secondary metabolite is influenced by stress and stimuli, only at adverse condition of the normal metabolic reactions synthesis of secondary metabolite takes place. Whereas, in *in vitro* condition the biosynthetic pathway of the secondary metabolite can be altered by supplementing nutritional requirements and growth regulators. Similarly, Ravishankar and Grewal (1991) reported that the influence of media constituents and nutrient stress influenced the production of diosgenin from callus cultures of *Dioscorea deltoidea*. Parisi et al., (2002) obtained high yields of triterpenes from the callus tissue culture of garlic (*Allium sativum* L).

2. **Phytochemical screening**

The phytochemistry achieved very significant progress during the 19th century. In spite of tremendous developments in the field of allopathy during the 20th century, plants still remain as one of the major source of drugs in modern as well as traditional systems of medicine throughout the world. The crude medicinal
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herbs have long been cultivated and traded in many countries around the world. The drugs are either supplied in dried or powdered form. The medicinal herb is a biosynthetic laboratory as it contains number of medicinally important secondary metabolites such as alkaloids, glycosides, steroids, flavonoids, volatile oils etc. These compounds exert therapeutic effect and account for medicinal property of the medicinal herbs due to the occurrence of active principles, which has to be extracted and screened for medicinal properties.

Natural products provide unique chemical diversity, distinct from that found in synthetic or combinatorial chemical libraries currently available. It is important to note however, that chemists working predominantly in the fields of either natural products or combinatorial chemistry that can demonstrate the beautiful chemical diversity of natural products. The importance and the value of natural products in this regard can be assessed using three criteria: (1) The rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semisynthetic and total synthetic modification, (2) The number of diseases treated or prevented by these substances and (3) Their frequency of use in the treatment of diseases.

Synthetic drugs are perceived to have certain disadvantages in relation to compounds derived from biological systems. Synthetic drugs are viewed as insufficiently complex and as having limited structural rigidity. Synthetic compounds by definition are not “natural,” they cannot a priori be considered either biologically relevant or compatible without testing. Whereas, natural products have significant value, by the definition, they are biologically compatible and relevant to cellular systems. Many are structurally rigid making them inherently stable and exhibit extremely broad chemical diversity (Stephen, 2000). Another adverse effect of synthetic drugs is that they may render the individuals to get drug-addicts during prolonged treatment procedures. Sometimes their side effects are severe than the curing.
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There is increasing evidence that many synthetic drug therapies simply suppress symptoms and ignore the underlying disease process. In contrast, many natural products, including nutritional supplements, glandular products, and herbal medicines, appear to address the cause of many diseases and yield superior clinical results.

The pharmaceutical companies that create synthetic drugs are brilliant. The drugs used for blood pressure, cholesterol, hepatoprotective, muscle and joint pain drugs have increased life span, but along with these they have hazardous adverse effects as well. The problem is that the synthetic drugs which manufactured by the pharmaceutical companies to control blood pressure, cholesterol and muscle and joint pain are not completely natural in their biochemical profile. It is not that the body cannot digest these compounds, but they put more stress on the liver, heart and kidneys than substances found in nature. In order to patent their designer synthetic drugs, the pharmaceutical companies must alter the natural chemical structure. These altered synthetic chemicals have been linked to many serious side-effects. For example, the family of statin drugs, which has been shown to lower cholesterol levels, has side effects which include abdominal pain, allergic reactions, back pain, changes in eyesight, constipation, diarrhea, dry eyes, dry skin, hair loss, headache, heartburn, leg cramps, muscle aching or weakness, neuromuscular degeneration, transient memory loss (read about this at www.spacedoc.net) and in rare situations rhabdomyolysis (a muscle condition that can cause kidney failure). With the advances made in molecular genetics, the modification of natural products is now possible via genetic, rather than strictly chemical or any other synthetic routes (Stephen, 2000).
i) **Isolation of active constituents**

A. **Isolation and Characterization of the active constituents from methanol extract of the leaves of *clematis gouriana***

In the present study the Ursolic acid was isolated from the methanol extract of *C. gouriana* following the method of Suresh Chandra and Sastry (1989). They have isolated ursolic acid from the leaves of *D. cordifolia*. Ursolic acid inhibited 12-o-teradecanoyal-phorbol-13-acetate induced Epstein-Barr virus activation (Ohigashi *et. al.*, 1986). It also showed potent inhibitory activity against HIV-I protease (Singh *et. al.*, 1994), suppressed the tumor promoter induced inflammation (Hirota *et. al.*, 1990) and increased the blood sugar concentration, glycogen and ATP contents in muscles, heart and uterus (Golovina *et. al.*, 1976). Mankani and Krishna, 2004 have isolated ursolic acid from the stem bark of *Diospyros cordifolia* and comparatively screened the hepatoprotective activity of the isolated constituents and the crude extracts. The IR, $^1$HNMR and MASS spectral analysis of the isolated constituents confirmed the structure of ursolic acid. The spectral data of this compound was also compared with the data of the earlier investigators.

There are few reports of phytochemical and pharmacological screening on *C. gouriana* and *N. zeylanica*. Nevertheless, the species of *Clematis*, Clemontanoside-C, a new hedragenin-based saponin isolate from the stem of *Clematis montana* (Thapliyal and Bahuguna, 1993) and from the aerial part of *Clematis tibetana*, two new hederagenin, 28-O-bisdesmosides called lematibetosides A and C. A new gypsogenin 3, 28-O-bisdesmoside called clematibetoside B, were isolated together with ten known saponin (Kawata *et. al.*, 2001). Protoanmonin has been isolated from the Australian ‘Headache Vine’ *Clematis glycinoides* (Southwell and Tucker, 1993). In *N. zeylanica* only alkaloid berberine was detected from the methanol extract (Praveendhar and
Ashalatha, 2003). The Extract from flowering plants of *N. zeylanica* yielded three simple benzenamides, 3, 4-methylenedioxybenzamide, 4-methoxybenzamide and 4-hydroxy-3-methoxybenzamide (Jaroszewski et al., 2005). In the present investigation a protocol was followed for the isolation of phytochemical constituents from the leaves *C. gouriana* and *N. zeylanica*. All the compounds were isolated and purified after repeated column chromatography of the fractions, in various solvent systems. The compounds were characterized with the help of mechanistic spectral data.

In the present study, the petroleum ether extract and chloroform extract were subjected to phytochemical analysis and column chromatography but no active constituents were observed. The methanol extract of *C. gouriana* using TLC and column chromatography elution technique using the solvents methanol: chloroform in the ratio of 8.5:1.5, which yielded a brown colored crystalline compound which were labeled as CGM and CGM'. The yield of these compounds were 450 mg per 20 g of crude extract (*in vivo* leaves) and 250 mg per 1.15 g of crude extract (leaf calli) respectively. Both the compounds showed the positive results for Salkowaski and Liebermann-Burchard tests and it was found to be a triterpenoid. On TLC the compounds CGM and CGM' exhibited the same spot with same Rf value and the melting point of this compound was found to be 289°C. The characterization of the compound was done by the following spectral studies.

The IR (KBr) spectrum of the compound showed bands at 3411 cm⁻¹ due to O-H stretching; 2931 cm⁻¹ due to C-H stretching of CH₃; 2862 cm⁻¹ due to C-H stretching of CH₂; 1714 cm⁻¹ due to C=O stretching (of COOH); 1382 cm⁻¹ due to O-H stretching; 1045 cm⁻¹ due to C-O stretching.

The ¹H-NMR (DMSO) spectrum showed the presence 1H (O-H of COOH) at of δ-11.0; at δ-2.0, 1H (O-H of alcohol); δ-1.44, 2H (cyclohexane); δ-0.88, 3H
(methyl) and in MASS spectrum the molecular ion peak was observed at m/z 457 which indicated that the molecular weight of the compound is 457. From these data it was concluded that the compound was identified as Ursolic acid.

In the present study, the petroleum ether extract of \textit{N. zeylanica} was subjected to column chromatography elution technique. A mixture of triterpene and a phytosterol were isolated from petroleum ether eluted fractions which were then separated by preparative TLC using the solvent system benzene: methanol in a ratio 8.5:1.5. β-sitosterol are the abundant plant sterols present richly in the leaves and bark. There are some similar reports on isolation of taraxerol from the medicinal plants extracts using column chromatography elution technique. For example, Byung-Sun \textit{et al.} (1984) isolated taraxerol from the stem bark of \textit{Styrax japonica}. In their study they chromatographed methanolic extract on Silica gel column with a stepwise gradient elution of chloroform and methanol to yield the respective fractions. The initial fractions eluted with chloroform yielded taraxerol with a yield of about 87 mg per 28 g of extract. The percentage yield of taraxerol from the stem bark of \textit{S. japonica} by this method is more or less equal to that of the method used in the present study to isolate taraxerol from petroleum ether extract of \textit{N. zeylanica}. Lie-Chwen \textit{et al.} (2001) isolated taraxerol using the same method as mentioned above. They isolated taraxerol form the ethanolic extract of stem bark of \textit{Ventilago leiocarpa}. In our research laboratory Shankar and Krishna (2007) and Khadeer Ahamed \textit{et al.} (2007) have isolated taraxerol from the petroleum ether extract of the leaves of \textit{Embelia ribes} and \textit{Grevia tiliaefolia} respectively.

There are some reports on the isolation of this phytosterol from the other plant species. Waffo \textit{et al.}, (2006) isolated this phytosterol from the stem bark of \textit{Garcinia afzelii}. In this study the petroleum ether was subjected to the Silica gel column chromatography. The fractions eluted with the solvent system hexane: methanol (9: 1) yielded β-sistosterol (7.0 mg/7g) and stigmasterol (11.0 mg/7g).
Lim et al., (2005) isolated these phytosterols from the thorns of *Gleditsia sinensis*. The fractions obtained from the hexane: ethanol (4:1) of methanolic extract yielded a mixture of phytosterols which were separated by preparative TLC dichloromethane: methanol (50: 1). In this method the yield of β-sitosterol and stigmasterol was 35 and 15 mg per 3 g of the extract. Giang et al., (2005) reported the isolation of phytosterols from the rhizomes of *Alpinia pinnanensis*. A mixture of stigmasterol and β-sitosterol (82.6 mg/9g) was obtained from the n-hexane-soluble fraction of methanolic extract, but they did not report the separation of these two phytosterols from one another. Comparatively, in the present investigation it is found that the yield of these phytosterol from the leaves of *N. zeylanica* is much higher. The yield of β-sitosterol from the leaves of *N. zeylanica* is 56 mg/ 5 g.

The detailed mechanistic spectral studies were used for the characterization and structural elucidation of isolated constituents. The characteristic spectra for the phytosterol isolated from the petroleum ether extract are discussed below.

**Characterization of taraxerol (NZP-1)**

The infrared spectrum revealed the presence of a hydroxyl group and a double bond between two carbon atoms in the regions 3421.6 cm$^{-1}$ and 1597.5 cm$^{-1}$ respectively.

The $^1$H NMR spectrum of Code-NZP-1 contained resonances corresponding to eight methyl groups in the region δ 0.60 to δ 1.09, all as singlets. The ethylenic proton (H-15) resonated as a double doublet at δ 5.63 and the signal of one exchangeable hydroxy group was observed as a doublet at δ 3.322. According to the fact that naturally occurring polyoxygenated triterpenoids in general contain an oxygen function at position 3 (Agarwal and Rastogi, 1974) the single hydroxy groups was placed at that position.
The observation of the base peak at $m/z$ 203 (96%) in the mass spectrum and the characteristic double doublet signal of the single olefinic proton in the $^1H$ NMR spectrum suggested the compound Code-NZP-1 to be a $\Delta^{14}$-pentacyclic triterpenoid (Ageta and Arai, 1983). The FAB spectrum indicated a molecular ion at $m/z$ 427 [M$^+$] confirming to the molecular formula $C_{30}H_{50}O$, as established by FAB-mass spectrometer ($m/z$ found: 427 [M$^+$]).

The pentacyclic triterpenoids such as friedelin, lupeol and betulin are the constituents which occur most abundantly in the stem bark of higher plants. The earlier workers, Badami et al., (2002; 2004) attempted to determine these triterpenes qualitatively in the bark of *G. tiliaefolia* using HPLC technique. While, in the present investigation we carried out both quantitative isolation of this triterpene from the leaves of *N. zeylanica* and qualitative determination and identification of these constituents by mechanistic spectral studies.

**Characterization of $\beta$-sitosterol (NZP-2)**

The IR spectrum of Code-NZP-2 showed absorption band for hydroxyl group at the region of 3430.1 cm$^{-1}$ and trisubstituted double bond at the regions 2938.9 and 1637.8 cm$^{-1}$. The $^1H$ NMR spectrum revealed the presence of six tertiary methyl groups at $\delta$ 0.64 to 1.006, along with a multiplet at $\delta$ 1.44 which was assigned to methine protons. In the electron impact mass spectrum of Code-NZP-2, besides molecular ion peak at $m/z$ 414, the major fragment ion peaks were recorded at $m/z$ 397 (M-Me$^+$). Other abundant ion peaks were observed at $m/z$ 329 (M-C$_7$H$_{17}$H$_2$O)$^+$, and 303 (M-C$_7$H$_9$H$_2$O)$^+$ which were characteristic for sterol with double bond at C-5 (Nargis Akhtar, 1992). The presence of ion peaks at $m/z$ 273 and 255 corresponded to (M-side chain) and (M-side chain-H$_2$O)$^+$, respectively. Therefore, compound Code-NZP-2 was characterized as stigma-5-en-3-ol or 24-ethylcholest-5-en-3-ol which is commonly known as $\beta$-sitosterol. This compound is widely distributed in plants and considered as the most common sterol of higher plant.
Characterization of Berberine (NZM)

The compound code NZM eluted from methanol extract gave positive tests alkaloids. IR, ¹H NMR and MASS spectroscopic studies confirmed the structure of the compound. The structural elucidation and molecular formula of compound Code- NZM was established by the following spectroscopic data.

The IR spectrum showed a band due to NH at the vibration frequency 3429.7 cm⁻¹ and 1597.6 cm⁻¹ due to C=C stretching. The ¹H NMR spectrum of Code- NZM showed absorption at δ 0.837 to δ 0.992 due to the presence of methyl groups. The FAB⁺-MS indicated a molecular ion peak at m/z 339 [M⁺] corresponding to the molecular formula C₂₀H₂₀NO₄.

Various secondary metabolites present in plants are responsible for the curative properties against various ailments. C. gouriana and N. zeylanica were being traditionally used for jaundice, wound, epilepsy, chest pain, treating worms etc. but there are no reports on the clinical evaluation of these plants. The therapeutic efficacy of these two species might be either due to the presence of a single constituent in higher concentration or might be due to the synergistic effect of different phytocconstituents. Therefore to ascertain this aspect, the crude extracts and the isolated constituents of C. gouriana and N. zeylanica were subjected for the evaluation of the pharmacological activities such as Wound healing activity, Antioxidant activity, antimicrobial activity and Anticancer activity.

3. Pharmacological investigation

Clematis gouriana and N. zeylanica are important medicinal plants which have extensively and frequently been used by the traditional healers. Its medicinal significances have been recorded in the traditional literatures. C. gouriana is used in the Indian system of medicine ‘Ayurveda’ this plant is used to alleviate malarial fever and headache. Root and stem paste is applied externally for psoriasis, itches
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and skin allergy (Manjunatha et al., 2004). The traditional medicine practitioners residing in the vicinity of Bhadra Wild Life Sanctuary, India are using the leaf and stem juices for treating infectious old wounds, psoriasis, dermatitis, blood diseases, leprosy, wound healing, and cardiac disorders.

*Naravelia zeylanica* is useful in the treatment of pitta, helminthiasis, dermatopathy, leprosy, rheumatalgia, odontalgia, colic inflammation, wounds and ulcers (Praveendhar and Ashalatha, 2003). The leaf paste of *N. zeylanica* is consumed to treat Chest pain. (John De Britto and Mahesh, 2007). The root and stems have a strong penetrating smell (Warrier et al., 1995). In the Indian system of medicine, ‘Ayurveda’, the plant is used to relive malarial fever and headache while root and stem paste is applied externally for psoriasis, itches and skin allergy (Harsha et al., 2002). In Kerala, India *N. zeylanica* is used as a source of drug for intestinal worms, skin disease, leprosy, and toothache (Sivarajan and Balachandran 1958). The traditional medicine practitioners residing in the vicinity of Bhadra Wild Life Sanctuary, Karnataka, India are using the leaf and stem juices for treating psoriasis and dermatitis. Many pharmaceutical industries in India (Hindustan Liver Ltd., Mumbai; Himalayan Drug House, Bangalore) are engaged in the production of skin ointments from this plant. Biosystematically, this species holds much importance because only two stoe climbing species are reported in the genus *Naravelia* (Manjunath et al., 2004). So, in view of the high medicinal value of these species, the present investigation was undertaken, wound healing activity, Antioxidant activity, Antimicrobial activity and Anti cancer activity.

i) **Wound healing activity**

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of
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the wound undergoes shrinkage. Wound healing proceeds via an overlapping pattern of events including haemostasis, inflammation, proliferation, and tissue remodeling (Douglas and Alan, 2003). The normal healing response begins the moment the tissue is injured.

Tissue injury initiates a response that first clears the wound of devitalized tissue and foreign material, setting the stage for subsequent tissue healing and regeneration. The initial vascular response involves a brief and transient period of vasoconstriction and haemostasis. Around 5-10 minutes period of intense vasoconstriction is followed by active vasodilation accompanied by an increase in capillary permeability. Platelets aggregated within a fibrin clot secrete a variety of growth factors and cytokines that set the stage for an orderly series of events leading to tissue repair.

The second phase of wound healing, the inflammatory phase, presents itself as erythema, oedema, and warmth, and is often associated with pain. The inflammatory response increases vascular permeability, resulting in migration of neutrophils and monocytes into the surrounding tissue. The neutrophils engulf debris and microorganisms, providing the first line of defense against infection. Neutrophil migration ceases after the first few days post-injury if the wound is not contaminated. In the late inflammatory phase, monocytes converted in the tissue to macrophages, which digest and kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils. Macrophages begin the transition from wound inflammation to wound repair by secreting a variety of chemotactic and growth factors that stimulate cell migration, proliferation, and formation of the tissue matrix.

The subsequent proliferative phase is dominated by the formation of granulation tissue and epithelialization. Its duration is dependent on the size of the wound. Around the blood capillary loops is an area of rapidly dividing fibroblasts.
This is known as granulation tissue. As the granulation tissue is beginning to form, the body utilizes another strategy in minimizing the risks posed by a wound i.e. the process of wound contraction. Contraction is thought to be mediated by myofibroblasts located around the edges of the wound. By a physical contractile process, the edges of the wound are brought closer together, at the same time stretching the normal skin beyond the wound edge. Chemotactic and growth factors released from platelets and macrophages stimulate the migration and activation of wound fibroblasts that produce a variety of substances essential to wound repair, including glycosaminoglycans (mainly hyaluronic acid, chondroitin-4-sulfate, dermatan sulfate, and heparan sulfate) and collagen (Stadelmann et al., 1998). Collagen is secreted into the intercellular matrix, where it undergoes maturation (cross-linking and coiling) into strong fibers oriented so as to allow stretchability while providing tensile strength. Collagen levels rise continually for approximately three weeks. The amount of collagen secreted during this period determines the tensile strength of the wound.

The final phase of wound healing is wound remodeling, including a reorganization of new collagen fibers, forming a more organized lattice structure that progressively continues to increase wound tensile strength.

We have presented a multiscale modeling framework which allows us to analyze the effects of different factors on wound healing, such as contraction of cutaneous wound, its tensile strength, collagen alignment, the hydroxyproline content and the strength of granuloma tissue and scar formation during dermal wound healing. Therefore, in order to examine the above mentioned parameters, three different types of wounds were inflicted on the experimental rats to assess the healing efficacy of various extracts and the isolated constituents of *C. gouriana* and *N. zeylanica*. The standard drug Nitrofurazone is used as a standard reference to assess the healing effect of the drug and the constituents against the controls.
The results of the present study clearly indicated that methanolic extract and its constituents of *C. gouriana* and *N. zeylanica* enhanced healing of all the three types of cutaneous wounds. Application of ointment base prepared from methanolic extract displayed significant wound healing activity. The healing time required for complete epithelialization of the excision wound was found to be much earlier (18th post wounding day) and it was on par with that of the standard reference drug Nitrofurazone. While in placebo treated group animals the duration of epithelialization was delayed by 4 days. The application of isolated constituents also exhibited significant wound contraction when compare to the control group of animals. The complete epithelialization in this group occurred on 19th post wounding day. On the other hand the berberine treated animals showed moderate healing activity, the period of epithelialization was longer than the other crude extracts. Among the constituent, ursolic acid and berberine isolated from the methanolic extracts exhibited potent wound healing activity. As the rate of wound contraction was faster in these groups of animals and the complete epithelialization of the excision wound was observed on 18th day. The constituents isolated from methanol extract of *C. gouriana*, Ursolic acid showed epithelialization on 18th post wound day whereas, the berberine showed insignificant activity since the period of epithelialization was extended up to 22 days.

The newly formed tissue is known as granulation tissue and the conversion of granulation tissue into fibrous scar tissue is known as cicatrization. The breaking strength of the granulation tissue increases proportionately with the collagen deposition. The tensile strength of the wound is determined by the rate of collagen synthesis and maturation process involving inter and intramolecular crosslinking of collagen fibrils. The breaking strength is the tensile strength of a healing wound and it can be measured practically by the minimum amount of force required to disarticulate it. In the beginning a wound will be having little
breaking strength because the clot alone will be holding the edges together. Thereafter breaking strength increases proportionately as collagen deposition increases and cross linkages are formed between collagen fibers. By the 8th to 10th day there is sufficient restoration of breaking strength and stitches can be removed. The breaking strength of a wound is a point of practical importance in surgery.

In the present study, using a linear resutured incision model the wound breaking strength was determined on 10th post wounding day. By treating the wounded animals with either methanolic extract or its constituents, we observed consistent and significant stimulation of wound healing. The tensile strength of the resutured incision wound was increased significantly in methanolic extract treated groups. The result is comparable to that of standard drug Nitrofurazone. A moderate increase in the tensile strength was noticed in the methanol extract treated animals. The isolated constituents, again Ursolic acid found to be most effective in increasing the tensile strength of cutaneous incision wound. The effect of Ursolic acid was more or less equal to the animals treated with Nitrofurazone. Followed by these constituents, the constituents isolated from methanol extract namely Berberine exhibited moderate healing activity.

Dead space wound model provides an opportunity to study the effect on the granulation and collagenation of the healing process. Such wound models have been employed for the quantitative studies on wound healing such as granuloma breaking strength and hydroxyproline content (Patil and Kulkarni, 1984). The granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema, and new small blood vessels. Collagen, the major component which strengthens and supports extra cellular tissue. The increase in the granulation tissue weight suggests higher protein content (Azad, 2002) and increase in the hydroxyproline content of the granulation tissue indicating increased collagen turnover (Gupta and Gupta, 1985), and its
measurement could be used as an index for collagen turnover (Madhura and Sushma, 2003). Gain in granuloma breaking strength indicates increased collagen maturation by increased cross-linking. Collagen mainly composed of the amino acid, hydroxyproline, and so the estimation of hydroxyproline content gives the net rate of synthesis and deposition of collagen in wound healing (Kumar et al., 2006).

The presence of the foreign body in the subcutaneous area initiates the formation of granulation tissue around it. Initially new blood vessels are formed accompanied by lymphatics. These arise from the preserved lymphatics at the margins of the wound. In the initial three days of the injury the intercellular spaces are filled with proteinous fluid. Later the fluid becomes gelatinous and shows increasing quantities of mucopolysaccharides which are either produced locally by fibroblasts or mast cells or come from the blood. The intercellular fibers are laid down in the wound fluid from the 4th day onwards and the concentration of mucopolysaccharides starts declining. At first these are fine thread like, later they coarsen and thicken. In the beginning collagen fibers run parallel and in one plane but soon their arrangement are remodeled to suit local mechanical stresses. Finally it forms a tough membrane of laminated collagen, which is the essential material for healing of the wound. By this time fibroblasts decrease in number and appear as shrunken in conspicuous fusiform cells in between rows of collagen fibers. The concentration of mucopolysaccharides becomes normal or even low. The wound has now acquired significant tensile strength (Somen Das, 2001).

The healing efficacy of extracts and isolated constituents on the dead space wound models were evaluated by the assessing the weight of granuloma tissue, by the estimating it's breaking strength and hydroxyproline content of the granuloma tissue. Among the treated animals the response was shown to be the best in methanolic extracts and its constituents, Ursolic acid and Berberine supplemented animals. The result was assessed by the increase in granulation tissue weight, its
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breaking strength and the hydroxyproline content. Both the methanolic extract and its constituents significantly augmented the breaking strength of granuloma tissue harvested on the 8\textsuperscript{th} day, increased the weight of granuloma tissue and hydroxyproline content of the tissue. This may be due to the enhanced collagen maturation by increased cross-linking of collagen fibers. The increased weight of both wet and dry granuloma tissue also revealed the presence of higher hydroxyproline content. The constituent Ursolic acid displayed comparably best healing activity while the other constituents were moderate in their effect on dead space wound. Again the constituent Berberine found to be impotent in healing all tested models of wound.

The histological inspection of the granuloma tissue showed the infiltration of fibroblasts and monocytes in the subcutis was significantly greater in the untreated animals. Furthermore, there was significantly decreased epithelialization and lesser collagen regeneration, indicates the incomplete wound healing. The sections of the granuloma tissue harvested from the animals supplemented with the methanolic extracts and its constituents, provide further evidences on their wound healing efficacy. The sections of granuloma obtained from methanolic extract treated animals showed complete epithelialization, fibrosis, few macrophages and significantly increased collagen formation. In case of animals treated with Berberine, moderate deposition of collagen and as compare to the control few macrophages were noticed. The sections of the granuloma tissues of the animals treated with Ursolic acid showed lesser monocytes, fibroblasts and increased collagen deposition. This fact suggests their potent wound healing property. Comparatively moderate collagen formation was observed in the animals treated with Berberine.

There are reports that the plants having antioxidant property would also enhance wound healing activity (Shirwaikar et. al., 2003). Hence, the better wound healing activity was observed in methanolic extracts of \textit{C. gouriana} and
The methanolic extract contains a triterpenoid, which are strong antioxidants. This fact may convince the better wound healing potency of the methanolic extract. On the other hand, in this study, significant wound healing activity was recorded in extracts. This, in fact, is due to the extract’s richness in triterpenoids. Triterpenoids are also known to promote the wound-healing process mainly due to their astringent property (Scortichini and Pia Rossi, 1991), which seems to be responsible for wound contraction and increased rate of epithelialization. Indeed, we also reported the bactericidal activity of triterpenoids namely, lupeol and taraxerol isolated from Grewia tiliaefolia (Khadeer Ahamed et al., 2007). In the present study also methanol extracts and its triterpenoidal compounds exert the wound healing activity by, at least partly, due to their antibacterial property. Similarly, other studies also reported that tannins (Ya et al., 1988) and triterpenoids (Scortichini and Pia Rossi, 1991) are known to promote the wound healing process, mainly due to their astringent and antimicrobial property. These active constituents promote the process of wound healing by increasing the viability of collagen fibrils, by increasing the strength of collagen fibers either by increasing the circulation or by preventing the cell damage or by promoting the DNA synthesis (Getie et al., 2002).


In the present study, the methanolic extracts found to be the significant in healing all three wound models. The percentage of wound closer and period of epithelialization for methanolic extracts was 95.41% (18.23 days) and 93.76%
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(19.79 days) respectively. The skin braking strength in incision model was significantly increased to 578.06 and 410.54 g respectively. The effect of intraperitoneal administration of methanolic extracts on dry weight and tensile strength of granuloma tissue was (27.58mg/g and 594.32g) and (20.36 mg/g and 418.68g). The constituents, Ursolic acid and berberine found to be the very significant wound healing agents.

ii) Screening of Caseinolytic activity by proteases

The gels loaded with methanol extracts and isolated compound treated tissue sample of C. gouriana showed bands distributed in the molecular mass range from 68 kDa to 43 kDa and N. zeylanica, only the methanol extracts treated sample showed protease activity band at ranges from 29 kDa to 18.4 kDa. The extracts of C. gouriana and N. zeylanica found to respectively enhance the activity of a high molecular weight and a low molecular weight matrix degrading enzymes as evidenced by casein substrate gel assay. The varied intensity of protein bands in SDS-PAGE suggests the variations in protein composition of tissue samples. The samples differed greatly in proteolytic activity in casein-SDS-PAGE zymogram. The activity bands seen for methanol extract of C. gouriana While, the isolated constituents did not show similar activity-banding pattern. The proteolytic activity in general is implicated in tissue necrosis and hemorrhage and responsible for the clinical manifestations of wound healing. The Hemorrhagic metalloproteases that caused local tissue destruction and damaged dermis of blood vessels. This resulted in hemorrhage and defects in platelets adhesion and wound healing (Feitosa et al., 1998; Veiga et al., 2000a, b, 2001a, b; da Silveira et al., 2002; Zanetti et al., 2002).

iii) In vitro Anti-oxidant activity

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention
of many experimental works. It has been proved that these mechanisms may be
important in the pathogenesis of certain diseases and ageing. There are many
reports that support the use of antioxidant supplementation in reducing the level of
oxidative stress and in slowing or preventing the development of complications
associated with diseases (Rose, 1982). Many synthetic antioxidant components
have shown toxic and/or mutagenic effects, which have shifted the attention
towards the naturally occurring antioxidants. Numerous plant constituents have
proven to show free radical scavenging or antioxidants activity (Aruoma, 1997).

a) DPPH Radical Scavenging Activity

The present study revealed that, DPPH is reduced in the presence of an
antioxidant molecule, giving rise to uncoloured methanol solutions. Ursolic acid
showed higher DPPH scavenging activities and positively correlated with total
phenolic content. Siriwardhana et al., (2003) have also reported higher DPPH
scavenging activities for a water and methanol extract of *Hizikia fusiformis* (a
brown alga), while ethanol, chloroform and ethyl acetate extracts also indicated
strong inhibition activities over 50%. Therefore the authors sample showed
significant activity in DPPH scavenging when compared standard ascorbic acid.

b) Superoxide anion scavenging activity

Higher superoxide anion scavenging activity was observed in Ursolic acid.
The superoxide anion derived from dissolved oxygen by Phenazine
methosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The
decrease the absorbance at 560 nm with the plant extract thus indicates the
consumption of superoxide anion in the reaction mixture. In the PMS/NADH-
NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH
coupling reaction reduces NBT. The decrease of absorbance at 560 nm with
antioxidants thus indicates the consumption of superoxide anion in the reaction
mixture. Addition of various concentrations of methanolic extracts and isolated
constituents as well as curcumin (standard) in above coupling reaction showed decrease in absorbance. The antioxidant property of curcumin is generally attributed to its phenolic nature (Toda, 1988), Sreejayan and Rao et al., (1997) have earlier observed that for superoxide and DPPH scavenging activity of curcumin.

c) **Hydroxyl radical (OH) Scavenging activity**

Ferric EDTA was incubated with H₂O₂ and ascorbic acid at pH -7.4, hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2- ribose into fragments that on heating with TBA at low pH form a pink chromogen (Aruoma, 1989; Halliwell, 1987). When methanolic extracts and isolated constituents of both the plants and vitamin E were added to the reaction mixture they removed hydroxyl radicals and prevented the degradation of 2-deoxy-2- ribose as mentioned above. The observed IC50 values of the methanolic extracts and isolated constituents and Vitamin E were analogous to the reported values of Sen et al., (2002). The above results showed that the extract and the constituent’s ability to act as OH radical scavenging agents.

d) **Reducing power assay**

The reducing ability of a compound generally depends on the presence of reductones (Pin-Der-Duh, 1998), which has been exhibit antioxidative potential by breaking the free radical chain and donating a hydrogen atom (Gordon, 1990). The methanolic extracts and isolated constituents being reductants in the methanol extract of *C. gouriana* might have caused the reduction of the Fe³⁺/ferricyanide complex to the ferrous form. Therefore, the Fe²⁺ can be monitored by measuring the formation of Perl’s Prussian blue at 700 nm. The activity was almost equivalent to that of BHT, methanolic extracts and isolated constituents showed higher activities indicating that more hydrophilic phenolics are present in those fractions which affect those interesting values in reducing capacities. Also the
reducing ability of methanolic extracts and isolated constituents was dose dependent and significantly higher than the control. Guo et al., (2004) have reported that the aqueous and methanol extracts of stem and leaf of broccoli showed higher reducing power at the concentration of 4 mg/ml. Kuda et al. (2005) have reported that crude fucoidan and crude alginate showed the reducing abilities (absorbance less than 1.0) at the concentration 10mg/ml. At the concentration of 0.8 mg/ml chitosan, the highest absorbance value reached by a purified sample was 0.26 (Xing et al., 2005).

iv) In vivo antioxidant activity

In recent years, attention has been focused on the role of biotransformation of chemicals into highly reactive metabolites that initiate cellular toxicity. Many compounds, including clinically useful drugs, can cause cellular damage through metabolic activation of the chemical to highly reactive species such as free radicals, carbenes and nitrenes. CCl₄ has probably been studied more extensively both biochemically and pathologically than any other hepatotoxin. CCl₄ hepatotoxicity depends on the reductive dehalogenation of CCl₄ catalysed by Cyt 450 in the liver cell endoplasmic reticulum leading to the generation of an unstable complex CCl₃⁺ radical. This trichloromethyl radical has been shown to be a highly reactive species, capable of attacking microsomal lipids leading to its peroxidation. This also covalently binds to microsomal lipids and proteins initiating secondary biochemical processes which is the ultimate cause for the unfolding of the panorama of pathological consequences of CCl₄ metabolism. This lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl₄ (Cotran et al., 1994; Kaplowitz et al., 1986), by encouraging the auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane, thus altering the permeability of the liver cell membranes (Handa and Sharma, 1990).
Further, oxidative stress, the consequence of an imbalance of prooxidants and antioxidants in the organism, is also gaining recognition as a key phenomenon in chronic illnesses like inflammation and heart diseases, hypertension and some forms of cancer (Oh et al., 2002). ROS produced through mechanism of signaling leads to deleterious effects. Hydrogen peroxide (ROS) has reported as an important mediator of signaling oriented to the activation of transcription factors which are sensible to redox cycle and activators of responsible genes of cancerigenic cells growth and of some inflammatory processes. Oxidative stress results in toxicity when the rate at which the ROS are generated exceeds the cell capacity for their removal. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. MDA is one of the end products in the lipid peroxidation process (Kurata et al., 1993). The data obtained in our study clearly shows an increase in the MDA level in serum and liver of rats treated with CCl₄ suggesting enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals.

Silymarin is known to have hepatoprotective and anticarcinogenic effects (Kang et al., 2004). Silymarin possesses a hydroxyl group at C5 in addition to the carbonyl group at C4, which may form a chelate with ferrous iron. This chelation can raise the activity to the level of most active scavengers, possibly by site specific scavenging. Likewise the constituents gulonic acid γ-lactone and isoascorbic acid also have a hydroxyl group and carbonyl moiety in their structures, which may probably responsible for their antioxidant activities and so the hepatoprotective activities. Treatment with methanolic extracts of leaf of C. gouriana and N. zeylanica and isolated constituents Ursolic acid and Berberine is seen to significantly reverse these changes in a dose dependent manner. Methanolic extracts of leaf C. gouriana and N. zeylanica at 500 mg/kg
significantly inhibited the formation of MDA levels in CCl₄ treated group. Among the isolated constituents Ursolic acid and Berberine inhibited the formation of MDA levels as compared with the standard drug silymarin (50 mg/kg). CCl₄ metabolism begins with the trichloromethyl free radical (CCl₃•) by the action of the mixed function of the cytochrome P450 oxygenase system. This free radical, which is initially formed as relatively unreactive, reacts very rapidly with oxygen to yield a highly reactive trichloromethyl peroxy radical (CCl₃OO•). Both radicals are capable of binding to proteins or lipids, or abstracting a hydrogen atom from an unsaturated lipid, thus, initiating lipid peroxidation (Williams and Burk, 1990; Lee and Jeong, 2002). Lipid peroxidation may cause peroxidative tissue damage in inflammation, cancer, aging, ulcer, cirrhosis, and atherosclerosis. Therefore, inhibition of the cytochrome P450-dependent oxygenase activity could cause a reduction in the level of toxic reactive metabolites and a decrease in tissue injury. On the other hand, an elevation of plasma AST and ALT activities could be regarded as a sign of damage to the liver cell membrane. In this study, ALT and AST activities and liver MDA levels significantly decreased in rats treated with Ursolic acid and Berberine. The elevation of MDA levels, which is one of the end products of lipid peroxidation in the liver tissue, and the reduction of hepatic GSH levels are important indicators in CCl₄-intoxicated rats. In this study, it was ascertained that MDA levels have been suppressed (compared with the control group) and CCl₄ induced depletion of GSH was prevented (compared with the control group) by the compounds Ursolic acid and Berberine. It is concluded that pretreatment with Ursolic acid and Berberine fraction decreases the CCl₄ induced elevation in biochemical parameters (liver MDA, plasma MDA, AST and ALT). These findings suggested that these two fractions were effective in bringing about functional improvement of hepatocytes. The healing effect of this fraction was also confirmed by histological observations.
During hepatic injury, superoxide radicals generate at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages liver. Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by CCl₄. The reduced levels of parameters of SOD and CAT, in CCl₄ treated rats were significantly increased by treatment with plant extracts evidently shows the antioxidant property of the extract against oxygen free radicals (Badami \textit{et al.}, 2005; Rai \textit{et al.}, 2006). Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes such as SOD and CAT system (Proctor and McGinness, 1986). Catalase (CAT) is a key component of the antioxidant defense system. Inhibition of this protective mechanism results in enhanced sensitivity to free radical induced cellular damage. The reduction in the activity of CAT may, therefore, result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Sampathkumar \textit{et al.}, 2005). Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by CCl₄. In our study administration of crude extracts and isolated constituents Ursolic acid and Berberine increases the CAT level in CCl₄ induced liver damage to rats thus preventing the accumulation of excessive free radicals and protects the liver from CCl₄ intoxication. The methanolic extract of leaf of \textit{C. gouriana} and \textit{N. zeylanica} (500 mg/kg, respectively) and isolated constituents Ursolic acid and Berberine (100mg/kg) significantly ($p<0.001$) restored the enzyme activity to the normal levels. The data was compared with the standard drug silymarin (50 mg/kg). The SOD converts superoxide radicals ($O_2^-$) into $H_2O_2$ and $O_2$, thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. The present study reveals that there is an increase of SOD activity in a dose dependent manner suggesting that the methanolic extracts of leaf \textit{C. gouriana} and \textit{N. zeylanica} (500 mg/kg, respectively) and isolated constituents Ursolic acid (75mg/kg), Berberine (100mg/kg) have an
efficient protective effect in response to ROS. When compared to standard drug silymarin at 100 mg/kg significantly ($p < 0.001$) restores the SOD activity in CCl$_4$ treated groups. The SOD and CAT activities were brought to near normal after pretreatment with extract in CCl$_4$-treated rats evidently shows the antioxidant property of the extract against oxygen free radicals.

In this study, rats treated with single dose of CCl$_4$ developed a significant hepatic damage and oxidative stress, which was observed from a substantial increase in the activities of serum, AST, ALT and ALP. This is indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Mukherjee, 2005). The rise in serum levels of AST and ALT also attributed to the damaged structural integrity of the liver because these are cytoplasmic in location and are released into circulation after cellular damage (Ahmed and Khater, 2001). The disturbance in the transport function of hepatocytes resulted in leakage of enzymes from cells due to altered permeability of membranes. The levels of the serum marker enzymes was significantly increased in the animals treated with CCl$_4$. Concomitant administration of the methanolic extracts C. gouriana and N. zeylanica with CCl$_4$ showed significant reduction in the serum enzyme levels. The isolated constituents Ursolic acid and Berberine were effective in reducing the toxic effect of CCl$_4$ by controlling the levels of the serum marker enzymes AST and ALT. This effect was comparable to that of the standard drug silymarin. Reduction in the levels of AST, ALT and ALP towards the respective normal values by plant extract (500 mg/kg) and isolated constituents is an indication of the stabilization of plasma membranes as well as repair of hepatic tissue damage caused by CCl$_4$. This effect is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Maiti et al., 2005).

Similarly, Krishna and Shanthamma (2004); Krishna et al., (2005); Vidya et al., (2007) reported the hepatoprotective activity of root extracts of Boerhaavia
erecta, Diospyros cordifolia and Clerodendrum serratum respectively. The 50% ethanol extract at the dose of 100 mg/100g body weight (b.w) significantly reversed the toxicity induced by the CCl₄. It was evidenced by the decreased level of serum bilirubin (1.35 mg/dl), significantly elevated concentration of total proteins (6.68 mg/dl) in serum and the depletion in the levels of serum markers such as, AST (11.53 IU/L), ALT (13.82 IU/L) and ALP (12.73 IU/L).

The administration of methanolic extracts C. gouriana and N. zeylanica protect the liver from the toxic effects of CCl₄ by restoring the levels of serum bilirubin (1.27 mg/dl), serum protein (6.52 mg) and subsequent decrease in the levels of serum hepatic enzymes, like AST (334.02 IU/L), ALT (124.28 IU/L) and ALP (435.53 IU/L). The hepatoprotective activity of methanolic extract may probably due to the presence of bioactive compound Ursolic acid and Berberine. The constituent exhibited significant hepatoprotective activity at the dose of 10 mg/kg bw.

The histopathological studies of the liver showed swelling and necrosis in hepatocytes in CCl₄ treated rats in comparison to normal control rats. Administration of different extracts and isolated constituents of the plants did not exhibit a significant recovery of hepatocytes in different sections of the liver, wherein the methanolic extracts of C. gouriana and N. zeylanica and their active constituents Ursolic acid and Berberine showed almost complete normalization of the tissues as neither fatty accumulation nor necrosis was observed. The central vein appeared clearly indicating a potent antihepatotoxic activity. The liver sections of the rats treated with CCl₄ toxicant showed CCl₄ induced steatosis and hydropic degeneration of the liver tissue, intense centrolobular necrosis and vacuolization. Microscopical examination revealed loss of architecture with inflammatory collections in the central zone in CCl₄-induced rats. In contrast to this, the severe hepatic lesions induced by CCl₄ were remarkably reduced by the prior administration of the methanolic extracts and its constituents. These
observations were in good agreement with the results of the biochemical tests. The observation of a significant corrective effect of Ursolic acid and Berberine on biochemical parameters was supported by histological examination. The rats treated with silymarin along with CCl₄ toxicant showed sign of protection against this toxicant to considerable extent as evident from formation of normal hepatic cords and absence of necrosis and vacuoles. Liver sections from rats treated concomitantly with methanolic extracts of leaf of C. gouriana showed subnormal morphology without appreciable histological abnormalities. The compound Berberine failed to protect the liver against CCl₄ induced liver injury, as it exhibited severe intense centrolobular necrosis, vacuolization and macrovesicular fatty change.

A number of scientific reports indicated that, some plants are found to have hepatoprotective activity due to presence of flavonoid, triterpenoid, tannin and steroids (Banskota et al., 2000; Takeoka and Dao, 2003; De Feudis et al., 2003). Presence of those compounds in the petroleum ether and methanolic extracts may be responsible for the protective effects on CCl₄-induced liver damage in rats. There are several other methanolic/ethanolic extract of plants which are reported to possess antioxidant potential, e.g. Emblica officinalis (Bhattacharya et al., 2000), Withania somnifera (Bhattacharya et al., 2001), Mangifera indica (Anitha and Vijayalakshmi, 2003), Bacopa monniera (Rohini et al., 2004), and Vitis vinifera (Yilmaz and Toledo, 2004).

v) Antimicrobial activity

For a long period of time, plants have been a valuable source of products to treat a wide range of medical problems, including ailments caused by microbial infection. Numerous studies have been carried out in different parts of the globe to extract plant products for screening antimicrobial activity (Essawi and Srour, 2000; Ravikumar et al., 2005). Plants produce highly bioactive molecules that
allow them to interact with other organisms in their environment. Many of these substances are important in the defense against herbivores and contribute to the resistance to disease (Cowan, 1999). Due to the increasing prevalence of antibiotic-resistant pathogens in hospital and homes, deliberate search is in progress for alternative treatments to combat further spread of antibiotic-resistant pathogens (Olukoya et al., 2003).

Antimicrobial drugs exert their action by interfering with either the structure or the metabolic pathways of bacteria. The molecular mechanisms of action of specific antibiotics and synthetic antibacterials are considered in more detail when individual groups of antibacterial agents (Oreste, 2003). Ciprofloxacin has a high bactericidal activity against gram-negative bacteria (Wolfson and Hooper, 1985; Zeiler, 1985). Another important characteristic of ciprofloxacin is a large volume of distribution and a high capacity of tissue penetration (Schlenkhoff et al., 1986; Wise et al., 1984).

*Clematis gouriana* and *N. zeylanica* is used as a traditional medicine to treat microbial infections. These traditional claims have been supported by the current bioassay results, which have shown activity against human pathogenic bacteria. A remarkable antimicrobial activity of the *C. gouriana* and *N. zeylanica* were observed against both gram-negative bacteria (*P. aeruginosa* and *K. pneumonia*), gram-positive bacteria (*S. aureus*) and dermatophytes. The isolated constituents, Ursolic acid and Berberine exhibits strong antimicrobial activity against the selected bacterial and fungal strains.

In most of the medicinal plants exhibits antimicrobial activity, which attack Gram-positive strains while few are active against Gram-negative bacteria (Meng et al., 2000; Scrinivasan et al., 2001). The researcher found that the antimicrobial extracts of the herbal plants is mostly on gram positive bacteria (Herrera et al., 1996; Kelmanson et al., 2000). Interestingly, our current finding shows
antibacterial activity on two gram-negative pathogenic bacteria and a gram-
positive and their medical isolates of different clinical originates. This is of
considerable interest since conventional antibiotics are generally more active
against gram-positive than gram-negative bacteria. Generally the Gram-positive
bacteria should be more susceptible having only an outer peptidoglycan layer
which is not an effective permeability barrier (Scherrer and Gerhardt, 1971).
Where as, the Gram-negative bacteria possess an outer phospholipidic membrane
carrying the structural lipopolysaccharide components. This makes the cell wall
impermeable to drug constituents. Because of the presence of multilayered
peptidoglycan and a phospholipidic bilayer wall most of the Gram-negative
bacteria showed multi drug resistant characteristics. In spite of these barriers the
constituents of C. gouriana and N. zeylanica were effective in controlling the
growth of these pathogenic strains.

A good activity of the isolated compounds indicates that those compounds
alone are solely responsible for antimicrobial activity of the C. gouriana and
N. zeylanica. The crude extracts tested against Gram-negative bacteria, the least
polar one showed the greatest activity, suggesting a correlation between polarity
and antibacterial activity of Gram-negative bacteria. One of the antimalarial
studies of lupeol (Fotie et al., 2006; Ziegler et al., 2004) concluded that the
antiplasmodial activity of this type of compounds was indirect, exclusively due to
stomatocytic transformation of the host cell membrane and not to toxic effects via
action on a drug target within the parasite (Ziegler et al., 2004). Usually the targets
of antibiotics would be DNA replication, protein synthesis or the rate limiting
enzymes. To accomplish this task the antibiotics should be hydrophilic in nature in
order to invade the bacterial cell. However, in case of hydrophobic drugs, a certain
degree of lipophilicity might determine the toxicity by the interaction with the
membrane constituents and their arrangement (Tomas-Barberan et al., 1990). An
important characteristic of non polar compounds like oils and fats is their
hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Knobloch et al., 1986; Sikkema et al., 1994). Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (Denyer and Hugo, 1991).

The study is purely a preliminary one, we cannot be certain of the reason for the efficient activity of the isolated compounds, since the drug-pathogen interaction studies are still to be confirmed by profound investigation. However, our findings ratify the bactericidal and fungicidal property of *C. gouriana* and *N. zeylanica* and provide a supportive scientific evidence for its medicinal use. From our research laboratory, several investigators have reported the bactericidal activity of some medicinal plants and their bioactive constituents on the same bacterial isolates for e.g. Harish et al., (2007) reported the antibacterial activity of Celapanin isolated from the leaves of *Celastrus paniculatus*. The activity against clinical isolates of two gram negative bacterial species namely, *P. aeruginosa* and *K. pneumonia*. Celapanin at the concentration of 100μg/100 μl showed significant inhibition against all strains of both bacteria. The zones of inhibition were range between 7.8 to 13.10 mm in diameter for the strains of *P. aeruginosa* and 9.40 to 13.26 mm in diameter for the strains of *K. pneumonia*.

Prabuseenivasan et al., (2006) reported the antibacterial activities of plant essential oils against the strains of *P. aeruginosa* and *K. pneumonia*. Out of 21 essential oils tested, 19 oils showed antibacterial activity against all the strains. Particularly, Cinnamon oil showed promising inhibitory activity with the zone of inhibition about 27.5 mm in diameter for the strains of *P. aeruginosa* and 29.8 mm in diameter for the strains of *K. pneumonia* at the concentration 50μl. In their study they used Streptomycin as reference drug for the comparison.
Zakaria et al., (2007) studied the antimicrobial activity stem bark extracts of *Terminalia brownii* against the clinical isolates of *P. aeruginosa* and *K. pneumonia*. Aqueous extracts exhibited the significant activity against both bacterial strains. The extract at the concentration 5.0 mg/disc showed 8.7 mm zone of inhibition for *P. aeruginosa* and 9.3 mm for the strains of *K. pneumonia*. The results of extract were compared with that of standard reference drug gentamicin.

The antimicrobial activity of the methanol extracts displayed better zone of growth inhibition against all the clinical isolates of *P. aeruginosa* and *K. pneumonia*. The constituent, Ursolic acid, at the concentration of 1.0 mg/ml, found to be very significant as it inhibited the growth of both gram positive and gram negative bacterial pathogens with the zone of inhibition range from 16.73 to 21.30 mm for *K. pneumonia*, 18.33 to 21.50 mm for the isolates of *P. aeruginosa*, *S. aureus* 19.70 to 23.63 mm and for antifungal activity among the dermatophytes only *Trichophyton rubrum* (10.16 mm) and *Candida albicans* (9.96 mm) respectively.

**vi) Evaluation of Anti cancer activity**

*In vitro* cytotoxicity was screened against three human cancer cell lines and one normal cell line (lung carcinoma cell line COR-L23, the human breast adenocarcinoma cell line MCF-7 and human colon adenocarcinoma cell line LS-174T and normal human keratinocytes SVK-14) of 11 Thai medicinal plant species (Arunporn Itharat et al., 2004), 76 ethanolic extracts of medicinal herbs from the Jordanian flora, belonging to 67 species and 34 families, were evaluated for their antiproliferative activity on a breast cancer cell line (MCF7). (Rana Abu-Dahab and Fatma Aiif, 2007), *In vitro* cytotoxic activity of *Lantana camara* Linn was screened by Raghu et al., 2004. The *in vitro* antiproliferative activity of crude methanol extracts of three traditional Korean medicinal plants: *Achyranthes fauriei*, *Epimedium koreanum* Nakai and *Scutellaria baicalensis* were screened on
four human cancer cell lines: lung cancer cells (Lu1), colon cancer cells (Col2), oral epidermoid carcinomas (KB) and hormone-dependent prostate cancer cells (LNCaP), (Hye Hyun Yoo et al 2007). Anthrapyrazoles are potent cytotoxic agents that intercalate into DNA, causing DNA strand breaks, inhibition of DNA synthesis and topoisomerase II was investigated the *in vitro* cytotoxic activity of two anthrapyrazole analogues (AP-10 and AP-11) in human prostate (DU-145) and testicular (NTERA-2) carcinoma cells ( Cuevas ME and Seilheimer K, 2008).

In present investigation anticancer property of the above said plants were evaluated with the help of Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai. The crude extracts and isolated constituents of *C. gouriana* and *N. zeylanica* have been evaluated for their *in vitro* cytotoxicity on Human Colon Cancer cell Line HT29 from Human Colon and Human Breast Cancer cell Line MCF7 from Human breast by employing the sulforhodamine B (SRB) assay. The results showed that the methanol extracts and isolated constituents of both plants were not significantly active in the assay system used. According to NCI, USA guidelines for extracts, GI50<20 μg/ml is considered as active but the extracts and isolated constituents of both plants were showed the negative result. But it may possess anticancer property on other cell lines and other assay system.