Chapter: 7

General discussion

In drug discovery and development, medicinal plants have consistently been considered the leading source of lead molecules and pharmaceuticals, employed in the treatment of various human diseases due to their high chemical diversity and broad biological functionality (Jaroszewski, 2005). Medicinal plants contain bioactive ingredients called ‘phytochemicals’ that can reduce oxidative stress and modulate harmful biological pathways, and are, therefore, used in ameliorating different diseases. In recent years, several researchers have focused on the efficacy of medicinal plants (Abesundara et al., 2004; Onal et al., 2005; Yuhao et al., 2005) on the most prevalent metabolic disorder called diabetes mellitus. Most of the synthesized drugs are quite effective but induce several long lasting and severe side effects (Klepser and Klepser, 1999). Herbal medicines, on the other hand, produce much less side effects in the treatment of diabetes as compared to synthetic drugs (Klepser and Klepser, 1999; Anon, 2002).
Costus pictus is now well known as an antidiabetic plant with several medicament potentials. It is the plant material for the present research work presented in this thesis. Chapter 1 describes the overall importance of this study with particular reference to C. pictus with important antidiabetic properties. Diabetes mellitus is a major endocrine disorder (Burke et al., 2003) responsible for renal failure, blindness or diabetic cataract (Thylefors, 1990), poor metabolic control, increased risk of cardiovascular diseases, including atherosclerosis and AGE (advanced glycation end) products. The earliest recorded attempt to treat diabetes mellitus using phytochemicals dates back to more than 3,500 years. Nowadays, insulin and other oral blood-glucose lowering agents are used in the clinical management of diabetes mellitus (Bailey and Day, 1989). The prevalence of this disease continues to rise worldwide and so far, limited help can be offered to prevent and/or delay the onset of secondary complications. Thus, the search for new antidiabetic drugs with novel mechanisms of action continues, and poses a huge challenge. Therefore, it was considered to initiate a study on this emerging popular antidiabetic plant, C. pictus, for possible future source of molecules of importance and systematic use in the treatment of diabetes.

There are no reports on the cultivation as well as availability of C. pictus in north India. Therefore, it was thought relevant to make some attempts to grow this miraculous plant in north India, primarily to check its survival and growth. The plants were collected from three different locations of Kerala and planted at Nainital, Almora and Sitapur. It could not be acclimatized under outdoor conditions and failed to survive in the north Indian high mountain ranges like Nainital and Almora, but exhibited good growth and survival in the plains, e.g., at Sitapur. It could not tolerate the low winter temperature in the hills. The rhizome pieces that were planted in DSB Campus of Kumaon University, Nainital did not show any signs of growth due to very low temperature during winter months. But all three accessions (clones) maintained in the Institute green house at GBPHIED, Kosi-Katarmal, showed excellent survival and growth.

India is famous for its varied climatic and diverse edaphic conditions and the tropical plant, C. pictus, is well suited for the prevailing climatic conditions, except for places with severe winters. The results of growth parameters clearly show that C. pictus grows well in areas with full sun, as well as under slight shade, with well draining loamy
soil. However, during winter months (October to March) the plant shows minimal growth, and this may be considered as a resting (dormant) period for the plant under winter conditions prevalent even at Sitapur. The plant showed good increment in growth parameters, such as the number of clumps per plant, height of clump (stems) during April to October. The plant is subjected to severe winter conditions in north India and perennates through underground parts for some duration in the year, the period of growth is confined from spring to autumn, through the summer and rainy months, i.e., April to November. It would therefore, seem fair to conclude that the warmer conditions favour plant growth, and it is not possible to cultivate it in low temperature areas like Nainital and Almora under natural field conditions.

The morphological analysis of three clones collected from three locations in Kerala did not show any significant difference in their vegetative as well as reproductive characters. In all 54 morphological attributes and growth parameters were recorded for the 3 clones (4 plants per clone) collected from three different locations of Kerala. From this it is concluded that the 3 clones (or accessions) are perhaps one and the same. The plant having been recently introduction in 2004 in Kerala (Merina, 2005), there is little possibility of any variation occurring in morphological parameters in such a short time frame. Further, the findings seem to open up the the possibilities of its cultivation in different, warmer and humid parts of India so as to exploit its utility as a source of antidiabetic biomolecules.

The third chapter contains details of work done to evaluate the propagation protocols suited for the multiplication of *C. pictus*, by using 6 plant growth regulators (PGRs). *C. pictus* regenerates in nature by rhizome segments as well as stem cuttings. The improved vegetative multiplication can help in promoting large scale propagation of this species. Plant growth regulators are important during the whole process of growth and development of plants (Davies, 1987). The current study was thus attempted to evaluate the impact of PGRs for mass propagation of *C. pictus*. In the study, selected plant growth regulators of different classes, like the auxins, cytokinins, gibberellins, abscisic acid etc. (chemicals) did not elucidate much differences on various parameters; somewhat stimulatory influence was observed on shoot characteristics by IBA (auxin) and BAP (cytokinin) treatments at both the concentrations (10μM, 100μM) used. The
IBA and BAP treatments exhibited maximum values in per cent sprouting of axillary buds, number of clumps (shoots) and the overall shoot length. The effect of tested plant growth regulators/chemicals was found to be negligible in terms of differences on leaf characteristics. IBA (10µM), BAP (100µM) and KNO₃ (5mM) produced maximum number of leaves, while GA₃ and ABA (10µM) were less effective. The results of average rhizome weight, diameter and number of roots, etc. did not show much difference among variously treated groups of plants. The IBA (10µM), BAP (10µM) and KNO₃ at two concentrations could stimulate the growth of rhizome and roots compared to untreated control group of plants, as also seen in some shoot and leaf related parameters. The influence of plant growth regulators on biomass of C. pictus was also assessed and reported in this thesis. Results showed that KNO₃ had maximum stimulatory effect on biomass (above and below ground and on total biomass) production, while minimal biomass production was seen in ABA treated group.

In the overall response of selected plant growth regulators and chemicals on the growth of C. pictus, slight stimulatory effect was observed with KNO₃. IBA and BAP. On the other hand, minimal growth was observed in GA₃, ABA and Bavistin (a synthetic fungicide with reported auxin like activity in some cases) treated groups of plants. This negative impact on growth might be due to the inhibitory effect of these plant growth regulators on the vegetative growth of C. pictus. These preliminary findings should pave the way for future work on improvement, conservation and mass propagation of C. pictus, to fulfill the ever increasing demand for commercial level cultivation of this important antidiabetic plant.

The investigations on molecular and physiological characteristics of C. pictus have also been evaluated during the study (Chapter: 4). Since C. pictus is a newly introduced plant, references regarding molecular analysis work on the plant are not available. In the study, C. pictus plants, originally collected from three different parts of Kerala, and grown for the first time in north India, were profiled using RAPD markers to assess its genetic diversity. RAPD analysis clearly revealed that molecular differences did not exist between the samples of C. pictus, taken from tree different clones, grown and maintained in the green house at GBPIHED, Kosi- Katarmal, Almora. The similarity value within plants was 0.96 to 1.0 ranges. The very high similarity observed between the
analyzed plants may be due to propagation by vegetative (clonal) means. Analyses based on RAPD tools have shown that this method is capable of revealing appreciable levels of polymorphism in plants. Further, literature studies indicate that if assay conditions are carefully controlled, the RAPD analyses do provide a cheap, rapid and effective means to evaluate the genetic diversity among a large number of plant populations and help devise sampling strategies to complement classical morpho-agronomic descriptors (Mishra et al., 2006). The results of present investigation using RAPD markers for studying the genetic diversity and relationship of *C. pictus* plants selected from different agroclimatic zones of Kerala, India may serve as reference information for germplasm conservation of *C. pictus*.

Based on the reported biological activities of the plant, *C. pictus*, Chapter 5 describes investigations on active extracts (leaf, stem and rhizome) made with various solvents, in order to screen major phytochemicals, and towards separation and tentative identification of the active principles for improved exploitation of the plant in antidiabetic therapeutics. Phytochemical screening was performed using standard procedures (Harborne, 1998; Trease and Evans, 2002; Sofowara, 1993) for extraction, separation and attempts towards identification of the plant constituents. The moisture content was more than 85% in all the materials, *i.e.*, leaf, stem and rhizome, so considerable difference was observed in the fresh and dry weight of *C. pictus* samples. This is a common characteristic shared by all the members of Costaceae and Zingiberaceae (Specht and Stevenson, 2006).

The two extraction methods (maceration and hot continuous extraction) were tried with three samples (rhizome, stem and leaf) using 4 solvents. Maximum yield of extract was obtained from leaf samples with methanol solvent through maceration. Plant extracts are the most attractive source of new drugs and have shown promising results for treatment of diabetes. The preliminary phytochemical qualitative tests for steroid, phenols etc. were conducted using different extracts prepared through 2 extraction methods. The results of phytochemical tests and TLC of various extracts revealed great similarity in chemical constituents. However, methanolic leaf extract of *C. pictus* showed maximum number and concentration of secondary metabolites. This result is in broad agreement with previous studies (Jothisel et al., 2007; Gireesh et al., 2009; Kumawat and Shimpi,
where methanolic leaf extracts were reported to show maximum secondary metabolites. Therefore, leaf methanol extract (maceration) was used for further analysis regarding separation and putative identification of compounds. The HPLC chromatogram indicated the presence of two major compounds in the crude methanol extract of leaves. Among 13 peaks seen in the HPLC chromatogram, the purity of two major peaks was 42.27% and 39.80%, respectively, and these values indicated that, of the analyzed fraction of the extract approximately 82% consisted of these two compounds. Therefore, further investigations carried out for the separation of two major compounds and attempts made for their identification.

Based on initial column chromatography the major fraction (2a, 2b), obtained was analysed further with HPLC and TLC. The HPLC chromatogram of the fraction showed one peak of 99.7530% purity and it matched with the results of TLC. The colour of the spots under UV spectrophotometer and the identification test with ammonia reagent indicated the presence of glycoside (Harborne, 1998). Therefore, this indicated that the major phytochemical found in crude methanolic leaf extract is a glycoside.

The same fraction from column chromatography, after further purification, was subjected to GC-MS (Wilson and Walker, 2000) and the fragmentation pattern of the sample and a reference (marker) compound showed partial similarity in their molecular fragmentation. β- L- Arabinopyranose methyl glycoside was used as reference compound. Since the spectral graph of sample showed some similarity in the molecular fragmentation with reference compound, it is assumed that the glycoside in the sample could possibly be a compound similar to β - L- Arabinopyranose methyl glycoside. This or a similar compound may be a possible candidate for the antidiabetic activity found in the methanol fraction of the leaf extract of C. plectron. However, much work remains to be done in this regard.

The above findings are consistent with the results obtained in the oral glucose tolerance test and antidiabetic activity in streptozotocin induced mice presented in Chapter: 6. M₂ (Methanol extract prepared after the removing the hexane and ethyl acetate fractions) at 500mg/kg body weight and MC (Methanol extract) also 500mg/kg body weight were found to show maximum activity in both tests. M₂ denotes the
methanol extract that was prepared after the fractionation with hexane and ethyl acetate solvents. The same fraction would appear to have been collected as 2a and 2b in the initial, bulk column chromatography. The above observations would appear to lead to conclusion that at least a major phytochemical found in *C. pictus* could be a glycoside (possibly similar to β- L- Arabinopyranose methyl glycoside); this may provide some lead towards the identity of the active antidiabetic compound of *C. pictus*.

From the available literature on *C. pictus*, there is no report on comparative and systematic study regarding the antidiabetic activity of variously prepared extracts and fractions. Therefore, investigations were carried out to assess the antidiabetic (hypoglycaemic) activity of various extracts and fractions obtained from *C. pictus* leaves in albino mice, through OGTT (Oral glucose tolerance test) and in streptozotocin induced hyperglycemic mice. Based on OGTT highly significant (*p* < 0.001) and promising antihyperglycaemic effects were obtained from 13 extracts made with different solvents (in two doses) on glucose fed mice. The response of 10 extracts (except fresh juice (FJ), water crude (WC) and hexane crude (HA) extracts) on glucose lowering was comparable to control. The results of oral glucose tolerance test revealed the potency of *C. pictus* leaf extracts in the reduction of elevated blood glucose levels to near normal value in fasted animals. The results of OGTT showed that the improved effect on glucose tolerance was found to start within 15 minutes of oral administration of the extract. Although gradual reduction in glucose levels was observed, the maximum reduction was recorded only after 120 minutes of extract administration. The methanol extracts (MC, M2), at 500 mg/kg body weight caused the most pronounced effect (*p* < 0.001), comparable to control group in the oral glucose tolerance test. The ethyl acetate extract (500 mg/kg body weight) also exerted significant influence (*p* < 0.001) in lowering of blood glucose level on glucose fed mice. MEA (Methanol: Ethyl acetate) extract at 500 mg/kg body weight substantially reduced the glucose level after 90 minutes of oral administration. The effect of these three differently prepared extracts was dose and time dependant.

Methanol and ethyl acetate extracts at 200 mg/kg b.w and 500 mg/kg body weight produced antidiabetic effect on streptozotocin injected mice, and the effect was
significant at p< 0.001 over the diabetic control group. Even though the antihyperglycaemic effect of C. pictus leaf extracts was studied, none of the studies done earlier reported results on streptozotocin induced mice. Moreover, no single study could be found on evaluation of the effect of various extracts and fractions on OGTT in mice, and subsequent assessment of antidiabetic activity of the most promising ones in streptozotocin induced mice. The magnitude of reduction in blood glucose level in treated diabetic mice was found to be time and dose dependent. The ethyl acetate extract exhibited optimal antidiabetic effect in normal mice at 500 mg/kg body weight with no contraindications. However, methanol extract exerted faster antidiabetic effect at 500 mg/kg body weight by reducing blood glucose level upto 55.5%, in the 3nd week after extract administration. The methanol extract was found to show maximum effect in both oral glucose tolerance test and in antidiabetic experiments. Body weight, which is another important parameter in diabetes, was also found to increase with the treatment of methanol and ethyl acetate extract. During the 21-days of experimental period, the body weight was reduced in diabetic mice, whereas, there was a significant gain of body weight in treated mice (p< 0.001). The study also reports the effect of C. pictus extracts on important biochemical parameters with respect to diabetes in streptozotocin induced mice model. Serum glucose, triglycerides, total and direct bilirubin, creatinine and the liver enzymes ALP, ALT, ASP and total protein levels were compared in control and treated groups (p<0.001). The administration of 500 mg/kg body weight of methanol and ethyl acetate extracts of C. pictus leaf were able to bring back these biochemical parameters near to normal level in treated mice. The overall results of antidiabetic experiments suggest that C. pictus leaf extract has antihyperglycemic effect and it protects the liver from the complications of the diabetes by improving body weight and the level of biochemical parameters in blood serum. However, longer duration studies on chronic models are necessary to elucidate the exact mechanism of action, so as to develop it as a potent antidiabetic drug.

By way of conclusion, the present investigations have provided more and significant insight on the insulin plant, C. pictus. The hypoglycaemic (blood sugar lowering) property of this plant is of huge potential benefit and should be exploited in combating the global war against diabetes mellitus. This plant could be easily grown even
in pots through rhizome segments and stem cuttings. It could be grown in home gardens, like in Kerala, and the diabetic patients can use it as an easy and cheap remedy for their diabetic ailments. The putative glycoside compound discussed earlier could be responsible for imparting the hypoglycaemic activity of this plant. This is an important lead and need to be followed with concerted and focused work. The success lies on the people’s trust in herbal medicines. Laboratory tests should necessarily benefit the common man. This study would throw some light regarding a possible low cost drug which could be made use even by the poorest among the poor people. Nature has provided abundant plant wealth for all living creatures and this is one such precious plant from Mexico. The large scale cultivation of this insulin plant may be encouraged for developing a natural drug for diabetic patients.

Future research needs to be continued based on some leads obtained from these investigations. It is important to remember that this plant is used by the local people, and, therefore, further pharmacological investigations and clinical trials must be carried out and results taken into account before its commercial use as a source of therapeutic agent can be recommended. This study also draws attention towards need for further biochemical investigations of the plant constituents to identify active principles, and to assess the efficacy of various plant constituents in the treatment of diabetes, and also to elucidate the underlying mechanism. It is hoped that this information would be helpful in future, as reference, for furthering research leading to all important applications on this miraculous plant, namely Costus pictus.