Chapter 6: SUMMARY AND CONCLUSION
Besides the nourishing capacity of plants, their recuperating activities advantage humankind as a good reservoir of bioactive compounds, no less. However, the need for effective herbal therapies against oxidative damage related to diabetes, are still sort for and this led to the screening of several medicinal plants. Chosen after a vast literature survey, the famous, commonly found plant, *R. communis*, the ‘castor oil plant’ demonstrated satisfactory results on exploration and evaluation as a potent anti-hyperglycemic and capable anti-oxidative agent at *in vitro, in vivo* and molecular level. Moreover, principle compounds were also isolated and purified successfully from the most effective part of the plant. However, the findings can be summarized as follows:

**Phytochemical screening:** Flavanoids and Phenols have known to be of utmost anti-oxidative, free radical scavenging nature, similarly Alkaloids are renowned to be of anti-diabetic nature. Thus, the plant extracts exhibiting these phyto-constituents could be expected to combat the diabetes induced oxidative stress which was the area of concern for this study.

The results revealed that the leaf extract RCL showed high amount of Alkaloids and Flavonoids; The stem extract, RCS exhibited low Flavonoids and Alkaloids. The root extract, RCR displayed Alkaloids, Flavonoids, Phenols, Steroids and Tannins in moderate amounts. Alkaloids, Flavonoids, Glycosides, Phenols in moderate amount in RCF or the flower extract. RCP, the pod extract which showcased Alkaloids, Flavonoids, Glycosides, Terpenoids moderately and Resins, Saponins in low amount.

**Anti-diabetic activity:** All the extracts showed some amount of diabetic enzyme inhibition which increased with increasing concentrations. The best estimated IC$_{50}$ values of $\alpha$-glucosidase by RCL, was 348 $\mu$g/ml. The IC$_{50}$ values for control Acarbose was 230.71 $\mu$g/ml. Lower IC$_{50}$ values of RCL RCR reveal it as antidiabetic with greater potential as compared to other extracts.

While, the IC$_{50}$ values of $\alpha$-amylase calculated the control used, Acarbose was 325.50 $\mu$g/ml and that for RCL was 320 $\mu$g/ml (lower than that of Acarbose).

Thus, it was concluded that RCL, RCL displayed high *in vitro* anti-hyperglycemic activity comparable to the standard, Acarbose.
Anti-oxidative activity: The ABTS radical inhibition percentages were found to be increasing with the increasing concentrations of all the extracts. From the results it is evident that the plant’s leaf (185.2 μg/ml) and root (197.4 μg/ml) extract show most efficient inhibition which is comparable to that of standard Ascorbic acid.

Considering the DPPH radical scavenging activity, a similar trend of increase in percent inhibition with concentrated extracts was noticed. Besides, all the extracts showed higher DPPH scavenging ability than EDTA; the best being RCL with lowest IC\textsubscript{50} value 154.6 μg/ml. RCP, on the other hand, clearly fails to scavenge free radicals with an IC\textsubscript{50} of 1490 μg/ml.

The metal chelating behavior of the various extracts showcased the lowest IC\textsubscript{50} value of RCL: 77 μg/ml after EDTA: 162.82 μg/ml thus, proving it as potent metal ion scavengers. Whereas, RCP shows an exceptionally poor metal ion scavenging ability with the highest IC\textsubscript{50}, of 589.7 μg/ml.

From the observations based on in vitro SOD activity, it can be said that both RCL and RCS showed highest specific activities, being 18.26±8.17 μ/mg and 17.20±8.994 μ/mg respectively at concentration 1000 μg/ml. Antagonistically RCP showed the lowest SOD activity of 5.2± 3.4 at 1000 μg/ml concentration.

Talking about the in vitro Catalase activities of all hydro-ethanolic extracts it can be stated that the leaf and the stem extracts exhibit the highest potential for CAT with 16.87± 3.5 μ/mg and 15.98± 7.3 μ/mg respectively at 1000 μg/ml concentration. The activity of RCP at the same concentration was 6.7± 3.46 μ/mg.

The GPx enzyme activity of each extract of \textit{R. communis} is depicted that the lowest specific activity values were recorded in case of RCP with 116±2.1 μ/mg at 1000 μg/ml and the highest in case of RCL and RCS being 180±5.3 μ/mg and 162±3.9 μ/mg at the same concentration.

The \textit{in vitro} results therefore suggested that owing to the trend of poor performance of RCP in various free radical scavenging assays and anti-hyperglycemic protocols it could not be expected to curb diabetes or diabetes caused oxidative stress. So it was not
included for the further *in vivo* study. On the other hand, miraculous exhibition of antioxidative ability and anti-hyperglycemic potential, RCL, RCS and RCR could be expected to be the best treatments.

Further, the study budged towards the *in vivo* activity antioxidant activity of *R. communis*.

**Effect on body weight and morphology:** Alloxan administration intra-peritonially (150mg/ kg BW) resulted in a moderate polyuria, polydypsia and polyphagia along with some aggressive behavior and fur fall. There was a significant decrease in the body weights of Alloxan treated mice. The 45 day treatment in GT, RCLT, RCFT, RCST seemed helpful in significantly (*P*<0.05) increasing the lowered levels body weights but however failed to restore their normal values. On the other hand, RCRT showed its adverse effect by further depreciating the mean body weight of the animals to only 22.0 ± 1.6 which is 16.28% decrease. All the treatments were found successful in rectifying other symptoms to a certain extent after the experimental duration. However, these symptoms persisted till the end in animals of group DC.

**Anti-hyperglycemic effect:** Diabetes caused by alloxan treatment produced a significant increase in the blood glucose concentration. Glibenclamide and crude extracts resulted in a considerable decline in the increased fasting blood glucose level. Different treatments followed different recovery patterns on FBG levels till the end of experiment. RCRT showed an exceptional a hypoglycaemic effect by displaying rapid decrease in FBG levels, the lowest being 68 ± 17.98 (61.37%) on the 45th day. RCLT also showcased the most powerful anti-hyperglycaemic activity by bringing down the outraged FBG levels to 89.92 ± 8.73 (51.6 %) which was comparable to the effect of GT 95.7 ± 18.1 (53.8 %) on the 45th day.

**Anti-dyslipidemic effect:** The effect of various treatments on the dyslipidemia observed in diabetic mice; it was unraveled that extracts of all parts were efficient in managing the disturbed lipid profile due to alloxan induction. From the data it could be inferred that treatment with all the four crude extracts and glibenclamide was efficient to certain extent in lowering the elevated levels of lipid profile parameters with the only exception of
RCRT which exerts exceptionally adverse effects. The results clearly state that among all extracts of plant, leaf and stem almost normalized the lipid profile parameters.

**Effect on proteins:** Serum total proteins, albumin and globulin content were significantly decreased post alloxan administration. These values were however motivated in all the treated groups after the 45 days treatment. The best treatments being Glibenclamide and RCL.

**Effect on hepatic Glycogen:** After alloxan treatment, a marked decrement of 57.9% was noticed in DC. However, the various treatments lead to a commendable relief to the lowered glycogen levels. All the treatments studied were capable of not only restoring but improving the total hepatic glycogen content. The most positive being the standard drug, glibenclamide (80.2% higher); followed by RCRT (77.2% rise) and the next being RCLT (76.2% more)

**Effect on lipid peroxidation:** Alloxan treatment resulted in a significant (P<0.05) amount of hideous turnover of lipid peroxidation in all the organs. This hike was however reduced after the subsequent administration of various treatments. The RCLT showed the most potent anti-lipid peroxidative ability by significantly (P<0.05) dropping down the elevated levels to normalcy.

- Anti-oxidative effect

The effect of all the extracts of *R. communis* on SOD activity in alloxan induced mice, resulted in elevating the lowered SOD activity in all three organs, RCLT being the most successful. While treatment with glibenclamide, not only failed to normalize rather further lowered the SOD activity in liver and kidney. All the treatments failed to curb the fall in pancreatic SOD level except for RCLT.

The CAT activity saw a quite evident, marked decline in all the organs after alloxan administration. All the extracts except RCRT displayed not only a restorative but significantly beneficial effect on the CAT level in all the three studied tissues, RCLT was again proved most capable
Status of **GPx activity** in DC declined in the three organs studied. Glibenclamide administration led to further decrease in kidney. The 45 days treatment with herbal extracts proved motivational. Leaves did the maximum benefit to the GPx content.

A picture of **GSH content** elucidated that Alloxan treatment also lowered amount of hepatic, renal and pancreatic GSH. Treatment with glibenclamide and the four herbal extracts provided some relief to the ablated GSH values. Treatment with RCRT was unsuccessful in elevating the GSH content, while treatment with RCLT was most useful.

**Histopathological studies**: revealed that alloxan administration resulted in deformities in all the three selected tissues. In **liver**, it led to abrasion such as dilation in sinusoids, infiltration in portal triad, granular cytoplasm degeneration and neutrophilic infiltration. However, treatment with various extracts resulted in normalizing all deformities. Among all, herbal treatment with leaves, flowers and stem were successfully capable in recovering the anomalies caused by alloxan. **Kidneys** of diabetic group revealed shrinking of the glomerulus and necrotic epithelium. However, all extracts had the ability to restore the abnormalities in diabetic mice with exceptions in case of root treated group.

In **pancreatic tissue**, alloxan administration led to destruction of islets of langerhans. However, all the extracts were more or less comparable in normalizing the pancreatic damage.

Therefore it was accomplished that *R. communis* removed almost all the deformities both biochemically as well as histopathologically and its most efficient part for controlling diabetes and related oxidative stress could be its leaves. The hydro-ethanolic extract of *R. communis* leaves was thus further studied for its active compound and its anti-oxidative ability at RNA level.

**Isolation of active compounds from the best treatment**: The TLC analysis of the leaf extract with Ethyl acetate: Acetone: Formic acid (9: 6: 1) unraveled the presence of six different compounds: C1, C2, C3, C4, C5 and C6. Two of these, namely, C1 (R_F 0.46) and C4 (R_F 0.83) were estimated to be alkaloids owing to their response to Dragendorf’s reagent.

Further, the study budged to their distinct isolation using Column chromatography analysis with the same solvent system oozing out 120 fractions. Fraction number 112-115
resulted into distinct, single, prominent spots with $R_F$ value similar to C4 isolated from TLC, while fractions 45-47 lead to development of bold, single spots with $R_F$ value same as C1 on confirmatory TLC. All the spots obtained from both the set of fractions showed positive response to Dragendorf’s treatment. Thus these two compounds (C1, C4) out of the six isolated from TLC were verified to be of alkaloidal origin.

**Evaluation of compound isolated for anti-diabetic and antioxidative ability in vivo:**

The isolated alkaloids C1 and C4 were administered to Alloxan treated diabetic mice for 45 days at 50mg/ kg BW and were compared with the efficiency of the crude leaf extract and that of standard drug, Glibenclamide for their anti-hyperglycemic, anti-dyslipidemic and anti-oxidative abilities. In the end it was concluded that both the compounds were able to almost normalize the morphological status of alloxan treated mice after a brief stage of polyuria, polydypsia and polyphagia. However, C4 was capable of completely restoring the loss in body weights of the animals unlike Glibenclamide or the crude leaf extract.

- Amelioration of the increased FBG levels was better by C1 instead of C4 and Glibenclamide but the anti-hyperglycemic potency of the crude extract was slightly higher.
- Restitution of total serum proteins, serum albumin and serum globulin content was best by Glibenclamide followed by crude leaf extract followed by compound C1.
- Both the isolated compounds C1, and C4 displayed motivational anti-dyslipidemic effect similar to that exhibited by the crude leaf extract.
- The increment in the decreased hepatic glycogen content was higher in case of C1 and C4 as compared to that in case of crude extract treatment. C1 being more successful. However, the increment offered by standard drug Glibenclamide was the highest of all.
- Taking into account the SOD activity it was outlined that ablation of SOD activity could not be rectified by Glibenclamide in liver and kidney. C1 however was the most successful in elevating the depleted SOD levels by restoring them approximately in all the organs liver, kidney as well as in pancreas followed by RCLT and C4 treatment.
Summary and Conclusion

- Considering the values representing the effect CAT activity, it was quite evident that after the marked decline in CAT after Alloxan, the treatments RCLT was the most efficient followed by C1, then C4 and lastly Glibenclamide in all the three organs.

- Status of GPx activity unraveled declination in liver, pancreas and kidney after alloxan administration. Glibenclamide administration led to a slight elevation in liver, and pancreas; but a decrease in kidney. The crude leaves extract treatment did the maximum benefit to the GPx content followed by C1, C4 and Glibenclamide respectively.

- GSH content elucidated that Alloxan treatment also lowered amount of hepatic, renal and pancreatic GSH level. Treatment with glibenclamide and treatments RCLT, C1 and C4 provided some relief to the ablated GSH values. GT helped in beneficially elevating the content to almost twice to that of NC in liver, to almost half of the normal content in kidney but could not even normalize the value in pancreas. RCLT and C1 normalized the GSH levels while C4 moved the values to almost normalcy.

Thus, although both the isolated compounds C1 and C4 showed restorative in vivo abilities, comparable to crude extract RCLT and better than standard drug Glibenclamide, but the most potent out of the two was C1. Therefore characterization and identification of C1 was necessary.

Characterization of compound isolated: Characterization C1 was carried out using NMR, GC-MS, FTIR spectroscopic analysis and HPLC studies. It was deduced that the compound isolated from the hydro-ethanolic leaf extract of *R. communis* was **Indole acetic acid**, which is phyto-hormone, chemically a proto-alkaloid of tryptophan origin and was proved to exhibit extensive anti-hyperglycemic and anti-oxidative properties.

Genetic expression analysis of best treatment and standard drug for anti-oxidative potential: The normalized abundance of SOD mRNA to GAPDH mRNA showed that there was higher expression of SOD in case of normal tissues, both liver and kidney. However, with the introduction of diabetes, the expression dipped in kidney as well as in liver. The Leaf extract therapy motivated the expression in both liver and kidney.

Alterations in expression of CAT are different as compared to the other genes as the fold change of mRNA in normal liver is more than that in the calibrator, normal kidney. The
expression of CAT in untreated diabetic kidney and liver was highly reduced. This decrease was very minutely elevated in kidney and liver by Glibenclamide treatment, while the leaf extract motivated a tremendous increment in kidney liver.

Comparative fold change in the expression of the GPx gene revealed GPx mRNA in normal untreated liver was lesser in liver as compared to the calibrators. Glibenclamide treatment only added to the depreciating fold change by depreciating it further. On the other hand, the leaf treatment elevated the reduced fold change value in kidney and in liver.

The variations in the fold change of GSR mRNA also noted the reduction in values in Alloxan affected tissues. Treatment with Glibenclamide only slightly raised it in kidney, further lowered it in liver. The leaf extract exerted some relief by increasing the fold change of GSR mRNA in both kidney and liver tissues.

The study throws light on the development of anti-diabetic drugs from the plant R. communis for which more studies are needed to study the metabolism and drug delivery systems. From the results of this study, it is concluded that various extracts of the plant (except pod extract) and its isolated compounds possess significant anti-hyperglycemic, anti-dyslipidemic and anti-oxidative properties. Hence, apart from controlling hyperglycemia and managing diabetes-induced oxidative stress, these can also be beneficial in the alleviation of other associated diabetic complications including the prevention of the development of atherosclerosis and other coronary artery diseases.

The study for the first time reveals the genetic expression analysis of antioxidant enzyme markers for normal, diabetic, diabetic-Glibenclamide treated and diabetic- R. communis leaf extract treated animals. The efficient antidiabetic, antioxidative and antidyslipidemic nature of the auxin, Indole acetic acid, isolated from the leaves of the extract, had not been known for till date. Conclusions drawn from this study can be useful to trace out metabolic mechanism responsible for producing all these effects. Moreover, these may prove helpful for developing new drugs from these plants for managing diabetes and associated complications.