CHAPTER – 7

Summary and Conclusion
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7.1. SUMMARY

Diabetes mellitus is a metabolic disorder characterized by resistance in the action of insulin, insufficient insulin secretion or both. It is becoming one of the most common diseases of the world. Type II diabetes in young has increased 30 fold over the last 20 years concomitant with increase in obesity. Studies have revealed that all incidences of diabetes in this young age group are 2.5% and alarmingly 25% of their young adults have abnormalities of blood glucose. Being a major heterogeneous endocrine and metabolic disorder, Diabetes mellitus leads to hyperglycaemia and several other complications, such as hyperlipidemia, hypertension and atherosclerosis. And the oxidative stress, caused by hyperglycemia induced free radicals, contributes to the development and progression of diabetes along with various secondary complications.

The treatment for diabetes mellitus would be a drug that not only controls the glycemic level, but also prevents the development of atherosclerosis and hyperlipidemia, hypertension. New drugs and new drug delivery systems for insulin have also been introduced

India has a rich source of indigenous medicinal plants, which are traditionally being used in various health care purposes. Recent research works are involved to establish the medicinal values of the plants on a scientific platform. The Indian indigenous drugs have great importance both from professional and economic point of view. Numerous plants have been used for the treatment of diabetes mellitus in Indian system of medicine and in other olden systems of the world, out of these only a few have been evaluated as per the modern system of medicine. Various extracts of diverse parts of medicinal plants have been shown to possess antidiabetic and hypoglycemic effect. Most of them look to act directly on pancreas (pancreatic effect) and stimulate insulin level in blood. Some have extra pancreatic effect by acting directly on tissues like liver, muscle, etc and alter favorably, the activities of the regulatory enzymes of glycolysis, gluconeogenesis and other pathways.

The research work embodied in the thesis deals with the scientific exploration of two indigenous plants viz., *A. mexicana* and *H. suaveolens* for their possible anti-diabetic and hypoglycemic activity along with their antioxidant properties (both *in-vitro* and *in-vivo*) to validate their folklore claim followed by chromatographic separation, isolation of presence of phytoconstituent among the most potent fraction of both plants and in silico study of isolated
compounds. In the present study ethanolic and aqueous extract of aerial parts of *A. mexicana* and *H. suaveolens* were used initially, for scientific exploration of antidiabetic and antioxidant properties. The dose levels of the each extract were selected based on the results of the acute toxicity study and found as: for *A. mexicana* and *H. suaveolens*, it is 200 & 400 mg/kg b. w. and 250 & 500 mg/kg b. w. respectively. Since both ethanolic and aqueous extracts showed good activity, hence the investigators think it may be more worth full in terms of its BG lowering ability, if we consider hydro-alcoholic extract instead of individual extract. In the above context we have prepared hydro-alcoholic extract of both the plant materials separately and based on the acute study report the test dose levels were fixed as same like that of individual extract as mentioned above. The preliminary study reports in the direction of the antidiabetic activity of all test extracts demonstrate that, the hydro-alcoholic extract is showing better response, in term enforced us to study the hydro-alcoholic extract in a more scientific manner, therefore different fractions were prepared from the hydro-alcoholic extract and were used for more detail study in respect of its anti-diabetic potential. The fractions were made based on the solvent polarity and out of which, we have selected two fractions, i.e., chloroform and aqueous fractions at a dose level of 150 mg/kg, based on its acute toxicity study report.

The antidiabetic and/or hypoglycemic activity study of the extracts at the tested dose levels were assessed on normoglycaemic, glucose loaded and alloxan induced hyperglycemic rats in both single and multi-dose treatment (for 11 days) models. Others associated study parameters are test of; body weight variation, serum lipid profile, Serum biochemical parameters like urea and creatinine, AST, ALT, ALP levels in the 11 days treated diabetic animals using standard experimental procedures.

The antidiabetic and/or hypoglycemic activity study of the fractions at the tested dose level were assessed on normoglycaemic and alloxan induced hyperglycemic rats in both single and multi-dose treatment (for 21 days) models. Others associated study parameters are test of; in-vitro glucose uptake study using isolated rat hemi-diaphragm, body weight variation, food and water intake variation, serum lipids, serum biochemical parameters like SGOT, SGPT and creatinine in the 21 days treated diabetic animals using standard experimental procedures.

The anti-oxidant activity studies were carried out in *in-vitro* models in case of test extracts and in both *in-vitro* and *in-vivo* models in case of fractions of both the plants. The *in-vitro* parameters include determination of the total Phenolic & Flavonoid content; DPPH, hydrogen peroxide free radicals scavenging activities in case of test extracts, where as DPPH, superoxide,
ABTS radical cation, and nitric oxide free radicals scavenging activities in case of test fractions. Similarly, *in-vivo* study was assayed by using the parameters of lipid peroxidation products in the liver, where as antioxidant enzymes, such as superoxide dismutase, catalase, Glutathione-S-transferase, reduced glutathione, total protein level were estimated in the liver, kidney and pancreas and used as marker of the evaluation of antioxidant potential.

The critical analyses of experimental results of the entire study protocol, as illustrated in the thesis describe the following conclusions in support of the anti-diabetic and/or hypoglycemic activity potential of the selected plants; *A. mexicana* and *H. suaveolens*.

- The preliminary phytochemical screening results inferred ethanolic and aqueous extract of *A. mexicana* showed the presence of most of the phytochemicals analyzed such as: alkaloids, carbohydrates, triterpenoids, steroids, tannins, and saponins. Whereas, chloroform and aqueous fractions of *A. mexicana* shows the presence of alkaloids, carbohydrates, triterpenoids, phytosterols, saponins, and tannins except carbohydrates in the aqueous fraction.

- Similarly ethanolic and aqueous extract of *H. suaveolens* showed the presence of the phytochemicals analyzed like: alkaloids, carbohydrates, steroids, phenols, tannins, proteins, terpenoids and flavonoids. Chloroform fraction of *H. suaveolens* shows the presence of glycosides, triterpenoids, steroids, flavonoids, tannins and saponins and its aqueous fraction mainly contains alkaloids, triterpenoids, glycosides, phytosterols, tannins, and phenols.

### 7.1.1. Effect of the extracts/fractions on blood glucose level and other related biochemical parameters in diabetic rats

- Both the plant extracts showed 3-12% and 4-15% fall of fasting blood glucose in normal rats which is not significant enough to interpret hypoglycemic effect of *A. mexicana* and *H. suaveolens* respectively. The results of the antidiabetic study revealed that, ethanolic and aqueous extract of both the plants are capable of reducing the fasting blood glucose level in alloxan induced hyperglycemic rats on acute and sub-acute study methods in a significant extent (*p* < 0.05). The acute and sub-acute study results of antidiabetic activity reveal that the test extracts showed a persistent decrease in blood glucose level till the end of 10 hr in a dose dependent manner in both the plant materials. Furthermore maximal decrease of BGL was noted in aqueous extract at higher dose, reaches 70.25% (*p*<0.01) in
case of *A. mexicana*, whereas at the same time *H. suaveolens* showed maximal decrease in case of aqueous extract at 500 mg/kg dose, reaches 66.72%. In a long term study the result shows the maximal reduction 73.10% for *A. mexicana* and 63.10% for *H. suaveolens* with aqueous extract at high dose level. This suggests the pancreatic and extra pancreatic action of the extracts under investigation. However, in all studies, aqueous extract of both plants are found to more significant in reducing the blood glucose levels than that of ethanolic extract, at the given experimental conditions. The activity is comparable with the standard drug Glibenclamide.

- Since the aqueous and ethanolic extracts of both plants have good effect in the control of glycaemia possibly due to pancreatic and/or extra-pancreatic effect of the extract, hence we select hydro-alcoholic extract instead of individual extract in the same direction of the study. The results of the antidiabetic study of hydro-alcoholic extract of *A. mexicana* and *H. suaveolens* revealed that test extracts are capable of reducing the blood glucose in glucose loaded hyperglycemic rats and alloxan induced hyperglycemic rats on acute study protocols. The single dose study results reveals that there is a significant reduction ($p<0.01$) in blood glucose level from 2h ($p<0.05$) onwards till the end of 10h and registered 71.04 % reduction at the end of 10h at higher dose levels of hydro-alcoholic extract of *A. mexicana*. Similarly, in *H. suaveolens* there is a significant reduction ($p<0.05$) in blood glucose level from 2h ($p<0.05$) onwards till the end of 10h and registered 67.90% reduction at the end of 10h, in animals treated with a higher dose level of hydro-alcoholic extract. The oral glucose tolerance test in the present investigation demonstrated that hydro-alcoholic extract of both plants under investigation at the tested dose levels, are having significant control over the blood glucose levels in the glucose loaded hyperglycemic animals. The so response of the test extract may be due to pancreatic and/or extra pancreatic action contributed by the any of the phytoconstituent present in the test extracts likely to be flavonoids/alkaloids as reported by other investigators.

- While the results of the antidiabetic and hypoglycemic study revealed that, hydro-alcoholic extract, chloroform and aqueous fraction of *A. mexicana* and *H. suaveolens* are endowed with significant reduction of fasting BGL in both normoglycemic and alloxan induced hyperglycemic rats on both acute and sub-acute study procedures. The results of the investigations revealed that treatment with hydro-alcoholic extract and test fractions of
A. mexicana and H. suaveolens produced hypoglycemia in normoglycemic (euglycemic) rats. The test result indicates that, there is a significant reduction (p<0.05) in blood glucose level from 2h (p<0.05) onwards till the end of 10h and registered 16.83%, 17.88% and 16.79% reduction at the end of 10h, in animals treated with 150 mg/kg of the chloroform and aqueous fractions of A. mexicana and similarly 13.77%, 15.56% and 13.94% reduction at the end of 10h, in animals treated with 150 mg/kg of the chloroform and aqueous fractions of H. suaveolens. In long term study hydro-alcoholic extract, chloroform and aqueous fractions of A. mexicana reduces the blood glucose level to an extent of 56.52%, 57.24% and 52.63% at 150 mg/kg body weight, respectively at the end of the 21st day of the study. Similarly hydro-alcoholic extract, chloroform and aqueous fractions of H. suaveolens reduces the blood glucose level to an extent of 51.99%, 52.96% and 51.72% at 150 mg/kg body weight, respectively at the end of the 21st day of the study. This also suggests the pancreatic and extra pancreatic action of the hydro-alcoholic extract and test fractions. However, in all studies, chloroform fraction of both the plants is found to be more significant in reducing the blood glucose levels than that of the aqueous fraction of both plants, at the given experimental conditions. The activity is comparable with the standard drug glibenclamide.

- Hypertriglyceridemia and hypercholesterolemia are two major problems in patient with diabetes mellitus and responsible for vascular complications of diabetes. The ethanol and aqueous extracts of aerial parts of A. mexicana and H. suaveolens at the tested dose levels, registered significant reduction in the level of total cholesterol, triglycerides, in the diabetic rats after 11th days of treatment. The increase in serum levels of urea and creatinine in the untreated diabetic rats as observed in the present study is expected which show renal dysfunction. Administration of test extracts of aerial parts of A. mexicana and H. suaveolens at the tested dose levels, however, produced a significant reduction in the levels of urea and creatinine, thereby conferring protection against impairment due to diabetes. Results are in a good agreement with the support of anti-diabetic potential as well as the safety of the plant extracts.

- Similarly, hydro-alcoholic extract, chloroform and aqueous fractions of A. mexicana and H. suaveolens treated rats showed significant reduction in the level of total cholesterol, triglycerides, in the diabetic rats after 21st days of treatment. While the hydro-alcoholic extract, chloroform and aqueous fractions of A. mexicana and H. suaveolens treated rats
showed a significant decrease in the content of lipid profiles like LDL, VLDL, when compared with diabetic induced rats. Likewise HDL level decreased in alloxan induced diabetic rats when compared with normal rats. On administration of hydro-alcoholic extract, chloroform and aqueous fractions of *A. mexicana* and *H. suaveolens* and glibenclamide to the diabetic rats, HDL level was found to reinstate to normal.

- In the study of body weight variation test in diabetic animals under treatment of ethanol and aqueous extracts of aerial parts of *A. mexicana* and *H. suaveolens* and standard drug, showed that, there is a significant recovery of body weight when compared with solvent treated diabetic rats, at the end of the 11th day of treatment. This may be due to the reversal of gluconeogenesis and glycogenolysis.

- While the hydro-alcoholic extract, chloroform and aqueous fractions of *A. mexicana* and *H. suaveolens* treated rats showed significant recovery in body weight gain when compared to diabetic control rats after 21st days of treatment. This may be due to controlling muscle wasting and an improvement in insulin secretion as well as glycemic control by the hydro-alcoholic extract, and test fractions of both plants. Untreated diabetic rats had severe polyphagia and polydipsia at the end of the third week of the experiment with respective increases in food and fluid intakes. However, in the presence of hydro-alcoholic extract, and test fractions of *A. mexicana* and *H. suaveolens* food and water intake was reduced when compared with diabetic control rats. Likewise, both plants hydro-alcoholic extract, and test fractions registered significant activity in increasing the peripheral glucose uptake by the isolated rat hemi-diaphragm.

- In the untreated diabetic control rats the increased levels of urea and creatinine in serum as observed in the present study is expected. Administration of ethanolic and aqueous extracts of both plants at the tested dose levels, however, produced a significant reduction in the levels of urea and creatinine, thereby conferring protection against impairment of kidney function due to diabetes. Whereas, oral administration of ethanolic and aqueous extracts of both plants at the tested dose levels attenuated the elevated activities of all investigated enzymes in diabetic rats comparable to the control. While, test extracts/fractions of both plants significantly restored AST (Sreum glutamic oxaloacetic transaminase, SGOT), ALT (Serum glutamic pyruvic transaminase, SGPT) hepatic biochemical parameters towards the normal levels. This may be an indication of nontoxic nature and defensive action of the extracts and fractions in reversing liver damage due to
diabetes. The serum urea, creatinine and enzymatic study results are in a good agreement with the support of anti-diabetic and hypoglycemic potential as well as the safety of the plant extracts and fractions.

- Chloroform fraction is showing better activity than hydro-alcoholic extract and aqueous fraction in both plants so, *in vitro* antidiabetic activity of chloroform fraction of both plants has been performed to get the reason of the so observed property and our finding revealed that the chloroform fraction of both the plants, efficiently inhibited the $\alpha$-amylase enzyme as compared to the standard drug acarbose. The observed IC$_{50}$ values for chloroform fraction of both the plants *A. mexicana* and *H. suaveolens* were 48.45$\mu$g/ml and 57.34$\mu$g/ml respectively, which revealed that the chloroform fraction efficiently inhibit the $\alpha$-amylase enzyme as compared to the standard acarbose. *In vitro* inhibition of $\alpha$-amylase by chloroform fraction suggests that antidiabetic effect would be by inhibition of $\alpha$-amylase activity leading to retardation of starch hydrolysis, finally lowering post-prandial hyperglycemia.

### 7.1.2. Anti-oxidant activity study

- High blood glucose level induces the production of free radicals which affect antioxidant defense mechanism, leading to the distraction of cellular functions, oxidative damage to cell membranes and increased susceptibility to lipid peroxidation.

- The experimental results found a marked quantity of phenolics and flavonoids in ethanol and aqueous extracts of aerial parts of *A. mexicana* and *H. suaveolens* contributing their antioxidant potential of the extracts which in turn responsible for antidiabetic activity. The total phenolic content of alcoholic and aqueous extracts of aerial parts of *A. mexicana* is found to 56.75mg and 113.12mg with respect to gallic acid equivalent (GAE)/g respectively, while total flavonoids content is 6.86mg and 8.34mg in relation to, equivalent of quercetin/gm of the dry weight basis. Similarly the total phenolic contents of ethanolic and aqueous extracts of aerial parts of *H. suaveolens* is found to 74.56mg and 89.33mg with respect to gallic acid equivalent (GAE)/g respectively, while total flavonoids content is found to 15.33mg and 28.58mg equivalent of quercetin/gm of the dry weight basis which is quantitatively a greater value. Further, ethanol, aqueous and hydro-alcoholic extracts of both plants significantly scavenge DPPH and Hydrogen
peroxide free radicals in a concentration dependent manner. The 50% inhibition (IC\textsubscript{50}) value for test extracts of \textit{A. mexicana} was found to 45.21, 40.11μg/ml and 32.95μg/ml for ethanolic, aqueous and hydro-alcoholic extracts respectively, while the IC\textsubscript{50} value of ascorbic acid was 18.53μg/ml, which significantly reduces DPPH radical by bleaching. The IC\textsubscript{50} value for test extracts of \textit{H. suaveolens} was found to 30.06, 28.76μg/ml and 23.33μg/ml for ethanolic, aqueous and hydro-alcoholic extracts while the IC\textsubscript{50} value of ascorbic acid was 20.92μg/ml, which significantly reduced DPPH radical by bleaching it. Whereas, ethanolic, aqueous and hydro-alcoholic extracts of aerial parts of \textit{A. mexicana} and \textit{H. suaveolens} significantly scavenge hydrogen peroxide radical. The IC\textsubscript{50} value for \textit{A. mexicana} was found to 56.02, 56.24μg/ml and 51.50μg/ml for ethanolic, aqueous and hydro-alcoholic extracts while, the IC\textsubscript{50} value of ascorbic acid was 21.41μg/ml significantly and concentration dependently scavenges H\textsubscript{2}O\textsubscript{2} radical. The IC\textsubscript{50} value was found to be 178.47 and 176.43μg/ml for ethanolic extracts and aqueous extracts of \textit{H. suaveolens} while the IC\textsubscript{50} value of ascorbic acid was 129.84μg/ml respectively.

- While chloroform and aqueous fractions of \textit{A. mexicana} and \textit{H. suaveolens} significantly scavenge DPPH, Superoxide, ABTS and Nitric oxide free radicals in a concentration dependent manner. The scavenging activity of chloroform fraction of \textit{A. mexicana} and \textit{H. suaveolens} was determined using free radicals of 1, 1-diphenyl 1-2-picryl-hydrazyl (DPPH). Results show that chloroform and aqueous fractions of \textit{A. mexicana} (IC\textsubscript{50} 61.89 and 64.02μg/ml) while test fractions of \textit{H. suaveolens} (IC\textsubscript{50} 57.51 and 70.75 μg/ml) possessed the good antioxidant activity. Standard drug ascorbic acid has 10.05 μg/ml IC\textsubscript{50} value. In ABTS free scavenging model results obtained that chloroform fraction of \textit{A. mexicana} and \textit{H. suaveolens} with IC\textsubscript{50} of 115.29 and 93.16μg/ml, whereas aqueous fraction of both test plants with IC\textsubscript{50} of 117.82 and 98.70μg/ml where ascorbic acid had IC\textsubscript{50} of 7.71μg/ml which is by inhibiting or scavenging the ABTS radicals in a concentration dependant manner. The chloroform fraction of both tested plants with IC\textsubscript{50} value of 82.12μg/ml and 76.3μg/ml and aqueous fraction with IC\textsubscript{50} value of 84.41 and 78.84μg/ml where ascorbic acid had IC\textsubscript{50} value of 7.92μg/ml significantly scavenges nitric oxide free radicals. Similarly, the chloroform and aqueous fractions of both tested plants was found to an effective superoxide anion scavenger to scavenge the superoxide anions as compared to ascorbic acid which is measured in terms of IC\textsubscript{50} (87.76 and 61.36μg/ml for \textit{A. mexicana}; 94.54 and 64.02μg/ml for \textit{H. suaveolens}). Which might contribute to the
reported antioxidant and antidiabetic potential of the chloroform fraction which in turn responsible for antidiabetic activity.

- The significant potential registered by chloroform and aqueous fraction of *A. mexicana* and *H. suaveolens* in reduction of the Liver lipid peroxidation products and elevation of the Liver, kidney and pancreas antioxidant enzymes in the diabetic rats, indicate that, both the fractions of plants are having very good potential to inhibit the oxidative damage of liver, kidney and pancreas tissues in diabetes. The increase in the liver, kidney and pancreas enzymatic antioxidant status might be due to decreased oxidative stress as evidenced by decreased lipid peroxidation in the fractions treated animals.

- The test extracts/fractions of *A. mexicana* and *H. suaveolens* possess radical scavenging ability which in response provides protection against oxidative damage caused by diabetes mellitus. Overall, it is concluded that ethanolic, aqueous and hydro-alcoholic extracts and chloroform, aqueous fractions of hydro-alcoholic extracts of *A. mexicana* and *H. suaveolens* have potent antioxidant activity, contributed by the phyto-constituents like phytosterol, polyphenols and flavonoids found in the test substances.

### 7.1.13. Isolation, Characterization and *In silico* study of the isolated compounds

- Since the test report embodied in the thesis evidence that chloroform fraction of *A. mexicana* and *H. suaveolens* shows comparatively better activity than aqueous fraction, hence it enforced us to isolate compound present in the chloroform fraction.

- Chloroform fraction of aerial parts of *A. mexicana* is done by column chromatographic techniques using different non-polar to polar solvents in different proportions and finally examined using TLC plate to get a single spot. The single spot containing solvent extract was eluted and dried to get the isolate product. The so obtained isolate product was analyzed by UV, IR, $^1$H NMR, $^{13}$C NMR and MASS spectroscopic methods, subsequently the spectral data were interpreted by referring previously reported information of similar spectral data and the isolate product is characterized as stigmasterol containing two double bonds at 5 and 22-position respectively along with a alcoholic group at 3-position of cyclopentanoperhydrophenanthrene. Based on the above characteristic features the IUPAC name of the proposed compound is 3S,8S,9S,10R,13R,14S,17R)-17[(E,2R,5S)-5ethyl-6methylhept-3-2-yl]-10,13-dimethyl,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-
cyclopenta[a]phenanthren-3-ol (stigmasterol). This compound is a new one as found first time from aerial parts of A. mexicana. Various literatures revealed that stigmasterol have a significant potency in the control of BGL in diabetic rats in response to its antioxidant potential.

- Similarly, Chloroform fraction of aerial parts of H. suaveolens led to the isolation of (1R,3aR,5aR,7aR,9S,11aR,13aR)-3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-Z-yl) icosahydro-1-H-cyclopenta[a]chrysene-9-ol (Lupeol). The structure is elucidated by UV, IR, 1H NMR, 13C NMR and MASS spectroscopic techniques and in comparison with the reported literature information of similar structures. Different literatures revealed that lupeol also have a significant potency to control BGL in diabetic rats.

- The 3D structure of the stigmasterol and lupeol isolated from A. mexicana and H. suaveolens respectively were docked with the glycogen synthase kinase-3-β 1Q41 receptor and found a good interaction by establishment of bonds between different site of the receptor and the isolate molecule. The in silico docking interactions prove that the stigmasterol and lupeol can inhibit the glycogen synthase kinase -3- β enzyme.
7.2. CONCLUSION

The antidiabetic, antioxidant, α-amylase deactivation, isolation and in silico docking studies of both plants present *A. mexicana* and *H. suaveolens* as per the plan of the work, in support of scientific validation of folkloric claim, is embodied in the thesis. The studies were carried out in both extracts and fraction by following bioassay guided principle. The results of the present study show that both plants showed potent blood glucose lowering activity, both in the normal as well as alloxan diabetic rats and caused improvement in lipid profile and body weight of the diabetic animals, also have good free radical scavenging activity in both in vitro and in vivo study models. Both the plants also possess good α-amylase inhibitory property. The new isolates found from the test fractions of both the plants were: 3S,8S,9S,10R,13R,14S,17R)-17[(E,2R,5S)-5ethyl-6methylhept-3-2-yl]-10,13-dimethyl,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[al]phenanthren-3-ol (stigmasterol) a stigmasterol compound in *A. mexicana* and (1R,3aR,5aR,7aR,9S,11aR,13aR)-3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-Z-yl)icosahydro-1-H-cyclopenta[a]chrysene-9-ol a lupeol compound in *H. suaveolens* and the isolates have GSK-3-β inhibition property as evident from docking studies. The results and interpretation of batteries of studies in support of scientific validation of the folkloric claim of both these selected plants as embodied in the thesis, endowed with potential antidiabetic activity, probably due to pancreatic or extra-pancreatic effect of test extracts/fractions. Sub-acute studies of both the plant materials caused reversal of the damage of liver seen in diabetic animals and was found to have a high margin of safety and thus these plants seems to have a promising value for the development of a potent phytomedicine for diabetes, though further comprehensive pharmacological investigations are needed to elucidate the exact mechanism of action of the both the plant material.