5 Discussion

Diabetes mellitus as well as metabolic syndrome are metabolic disorders characterized by disturbances in carbohydrate, fat and protein metabolisms. Both the disorders reached every nook and corner of India like epidemic. However till date, there is no study carried out on the prevalence of diabetes based on urban and rural population of Gwalior-Chambal region of northern central India.

A cross sectional study was conducted to determine the prevalence of diabetes and the present study assessed the prevalence of fasting hyperglycemia in urban as well as rural zones covering age groups between 20-79 years and major professions. House to house visits in localities randomly selected, educating the subjects, a day prior to blood sampling and confirmation at the time of blood sampling ensured the blood samples monitored are truly fasting in nature. The T2DM was found significantly higher (11.4%) than pre-diabetes (5.7%). The males recorded relatively higher rate of diabetes (14.3%) than female counter parts (8.9%) and no such gender variation was recorded in case of prediabetes irrespective of their domicile. The recent report by Anjana et al also shows the high prevalence in male population (Anjana et al., 2017). It may be due the physical inactivity and professional stress in male population.

The present study recorded 7.7% diabetes in rural areas, the figure higher than that reported (6.4%) by Anjana et al (2011). Two studies conducted earlier in other rural parts of Madhya Pradesh State reported 33.7% (Joshi et al., 2012) and 14.5% of diabetes (Mustafa and Kushwaha, 2012). The urban diabetes recorded in this study was 12.7% the figure relatively lower than 15.2% reported by Khan et al.(2016) in the neighboring urban Bareilly region. However, it was relatively higher than 11.2% reported by Anjana et al (2017) in 15 states of India.

Older population aged above 60 years recorded the highest rate of diabetes (31%), and the data corroborates with the earlier observations (Corsi and Subramanian, 2012). The high incidence in older age groups could be attributed to poor immunity and lesser physical activity although they are not stressed professionally.
The study recorded interesting observation, high prevalence of hyperglycemia among rural agricultural workers although the rural folk life style is active physically, but may be poor nutritionally. Thus, this study throws light on the importance of nutrition and its possible association to diabetes with the quality of nutrition. Drewnowski & Specter (2004) reported that the people, who eat lesser healthy diets, suffer from the highest rates of T2DM.

Pervasiveness of diabetes was significantly associated with a prior family history as expected. The high incidence of diabetes in those with family history of diabetes further confirms the genetic basis of this metabolic disorder. Arora et al. (2010) and Ahmad et al. (2011) in their studies have also noted significant association between family history and high incidence of diabetes.

The personal habits appear to bear not much significance with regard to diabetes. However, there are studies linking diabetes with smoking (Wu et al., 2016) and alcohol consumption (Carlsson et al., 2003). In urban area, the high incidence of diabetes in vegetarians (13.3%) finds no convincing answers unless the diabetics of this group have a family history coupled with sedentary lifestyle and/or are subjected to stressful life. Another possibility that the vegetables consumed may contain high amounts of pesticide/herbicide residues which might have triggered diabetic circuits in the body. Fast food with processed carbohydrates such as breads, noodles and cornstarch, high calorie drinks and vegetable fat contributes greatly to urban diabetes (Thanopoulou et al., 2003). Meyer et al (2001) found that vegetable fat (saturated fats) intake remained a significant predictor of new diabetes.

More than half of the world’s population still remains undiagnosed for diabetes (Beagley et al., 2014). However about half (49.3%) of diabetic population particularly in rural set ups are diagnosed for the first time and this fact denotes the necessity of periodic diabetic surveys among the population. After the screening, the subjects are instructed to attend the diabetes clinic, at Center for Translation Research, Jiwaji University.
The metabolic syndrome (MetS) is defined as a clustering of abnormalities including abdominal obesity, dyslipidemia, hyperglycemia and hypertension in an individual. Numerous studies around the world have reported that, very high prevalence of MetS in subjects with T2DM irrespective of ethnicity and geographical area (Tan et al., 2013; Pokharel et al., 2014).

Several studies have also been carried out on Indian subjects (Gupta et al., 2004; Mahadik et al., 2007; Kamble et al., 2010; Ravikiran et al., 2010; Nag and Ghosh, 2015; Madan and Narsaria, 2016) by using the standard ATP-III, IDF, WHO criteria and reported the prevalence of 18–35% in urban subjects (Ramachandran et al., 2003; Deepa et al., 2007; Sinha et al., 2013; Pradeepa et al., 2016). Differences in prevalence rate may have arisen due to the diverse cut-off points and sets of criteria used. Harmonized criteria was introduced in 2009 to bring the harmony in the several existing definitions of the MetS (Alberti et al., 2009).

In present study, Harmonized criteria was used to assess the prevalence of MetS in 1190 subjects who attended the diabetes clinic for the first time at Center for Translation Research, Jiwaji University. MetS prevalence rate of 72.7% was observed which is higher than reported in earlier studies using other criteria (Yadav et al., 2013).

The prevalence of MetS in hypertensive population was 91.1% unlike an earlier study on hypertensive population showing only 37% population with MetS (NCEP ATP III ) (Mulè et al., 2005). In hypertensive subjects, the MetS amplifies cardiovascular risk associated with high BP, independent of the effect of several traditional cardiovascular risk factors (Schillaci et al., 2004).

Hypertriglyceridemia is a common lipid abnormality associated with the visceral obesity and T2DM (Subramanian and Chait, 2012). The prevalence of MetS was 79.5% and 96.2% in low HDL and hypertriglyceridemia population respectively.

About 88% subjects with increased waist circumference (abdominal obesity) were seen to suffer from MetS. Waist circumference is believed to be determined by the extent of abdominal obesity, which is linked to visceral adiposity (Lakka et al., 2002; Onat et
al., 2004). Increased intra-abdominal fat deposition contributes to insulin resistance (Seidell et al., 1988). The inability of the fat cell to adequately store excess triglyceride is the first possible step to cause hypertriglyceridemia in insulin resistance condition (Ginsberg, 2000). Some studies also shown the association between body mass index and arterial pressure even in non-obese people (Reisin et al., 1978; Tuck et al., 1981).

The prevalence of MetS among T2DM subjects is high, because T2DM is an entity of the MetS. South East Asians are at a higher risk with respect to diabetes & CVD and the numbers are steadily increasing (Enas et al., 2007). However in this study, the prevalence of MetS noted were 83.5%, 80.5% and 39.6% in diabetic, prediabetic and normal subjects respectively. The frequency of MetS in Asian Indians varies according to the region, the urbanization, lifestyle patterns, and socio-economic factors. Earlier studies reported that about one third of the urban population in India’s major cities have MetS (Misra and Khurana, 2009).

Almost all (99%) of the people had at least one abnormal biochemical or anthropometric parameter leading to MetS in this population. This study observed high prevalence of MetS in female population. Earlier, MetS was reported to be more common in females than in males in Chennai, Tamil Nadu (Ramachandran et al., 2003) as well as in Wardha, Maharashtra India (Kamble et al., 2010).

Indians have an increased risk for developing T2DM and related metabolic abnormalities compared to other ethnic groups (McKeigue et al., 1991; Mohan et al., 1986 and Abate et al., 2001). However, the risk of normal subjects developing MetS, ultimately leads to diabetes with CVD is a much feared possibility. Therefore, remedial measures starting from identifying potential MetS subjects, advising proper medication and life-style changes to managing the prevalence of MetS in patients is imperative. Continued surveillance efforts would provide us with an opportunity to develop detection, and management strategies for MetS.

Ancient ayurvedic scriptures suggest that physicians in India have effectively managed diabetes with a balance of therapeutic diets, herbs, and plant food sources.
Discussion

For various reasons, the popularity of complementary medicine has increased in recent years. The WHO has also recommended the evaluation of the herbal effectiveness in conditions where safe modern drugs are unavailable. Much interest has been focused on exploring herbal preparations, which include screening of natural bioactive compounds with the potential to cure and/or delay the progression of diabetes through different unknown mechanisms. Inspite of the presence of known anti-diabetic medicines in the pharmaceutical market, remedies from medicinal plants are used successfully to treat this disorder possibly because they are considered to be less toxic and free from side effects compared to synthetic ones.

*Gymnema sylvestre* belongs to Apocynaceae family and is a slow growing woody climber found in central and southern India (Pothuraju *et al*., 2014). The leaves of *G. sylvestre* have been used in India for over 2000 years to treat diabetes. Chewing the leaves destroys the ability to discriminate the “sweet” taste, giving it its common name “Gurmar” “sugar destroyer”.

The second phase of this study demonstrated the glucose lowering efficacy of the *G. sylvestre* in T2DM subjects with MetS. Two forms of *G. sylvestre* were used i.e. aqueous extract (GSAE) and ethanolic extract (GSEE). Subjects were selected from the above study and randomly divided into two groups.

The reduction in fasting blood glucose was seen 12.2% with GSAE and 17.5% with GSEE after three months of therapy while in post prandial blood glucose decreases were 13.9% and 15.6% in GSAE and GSEE therapy respectively (Figure 4.5). HbA1c levels were reduced by about 8.09% with GSAE, while it was reduced by 8.2% with GSEE (Figure 4.6). C-peptide levels were increased 20.8% in subjects administered with GSAE and 21.05% in GSEE administered subjects (Figure 4.7). Earlier study in STZ induced diabetic rats, oral administration of GSEE shown reduction in blood glucose levels and increase in serum insulin levels (Kang *et al*., 2012; Kumar *et al*., 2013). The aqueous extract of *G. sylvestre* improved glucose control and insulin requirement in T1DM and T2DM subjects (Khare *et al*., 1983; Baskaran *et al*., 1990; Shanmugasundaram *et al*., 1990).
Another study show that the oral administration of *G. sylvestre* capsules (OSA® 500 mg d⁻¹) for 60 days resulted in 26% reduction in fasting blood glucose and 19% in postprandial blood glucose level. It also improved glycemic control, with increased circulating levels of serum insulin levels by 34% (Al-Romaiyan *et al*., 2010). Shanmugasundaram *et al*., (1990) shown 29% reduction in fasting blood glucose and 28% HbA1c levels, and a 38% increase in insulin level after the *G. sylvestre* therapy. *G. sylvestre* was found to be responsible for the secretion of insulin from pancreatic β-cells (Baskaran *et al*., 1990; Kang *et al*., 2012) of both mouse and humans under *in vitro* conditions by Ca²⁺ influx and protein kinase activation (Al-Romaiyan *et al*., 2012). It is also responsible for enhanced glucose uptake activity (Kumar *et al*., 2016) and ameliorative effect on insulin resistance (Bhansali *et al*., 2013). Gymnemic acids present in the *G. sylvestre* inhibit the activity of α-amylase resulting in the delayed digestion of the dietary carbohydrates, and the absorption of glucose into the circulation (Ahmed *et al*., 2011). In an earlier study it has also been reported to increase insulin levels by regenerating pancreatic β cells (Baskaran *et al*., 1990; Shanmugasundaram *et al*., 1990; Aralelimath and Bhise, 2012) However, earlier study showed higher reduction in blood glucose levels which may be attributable to the duration of therapy and the type of subjects selected.

*G. sylvestre* therapy helps in the proper regulation of the blood glucose and even it shows lipid homeostasis without giving any detrimental effects on liver. *G. sylvestre* is reported to produce hypolipidemic effect by inhibition of intestinal absorption of fatty acid in rat (Wang *et al*., 1998) and increases the fecal excretion of cholesterol, neutral steroids and bile acids (Persaud and Al-Majed, 1999) and may be because of inhibition of the activity of pancreatic lipase (Manish *et al*., 2011).

The present study recorded that the *G. sylvestre* administrations produced 10.78% and 11.52% decreases in cholesterol level in GSAE and GSEE therapy, respectively; and 14.09% and 16.94% decrease in triglyceride level in GSAE and GSEE therapy respectively. HDL-C was found to increase in both groups (8.04%, & 8.53%). *G. sylvestre* therapies also reduced the LDL level; reduction in LDL may be due to
increment in insulin level, which lead to increase in the activity of lipoprotein lipase (Zuñiga et al., 2017) (Figure 4.8).

It has been reported that compounds such as phenolics (Flavonoids, Phenols, Tannins) and Triterpenoids present in the *G. sylvestre* are responsible for the radical scavenging activity (Rachh et al., 2009). There are numerous studies done exploring *in vitro* anti-oxidant activity of *G. sylvestre* (Bajpai et al., 2005; Atanassova et al., 2011; Kang et al., 2012; Ahirwal et al., 2013). *G. sylvestre* shown higher antioxidant activity than *Averrhoa bilimbi* and *Capsicum frutescens* (Mannan et al., 2014). The ability of the extracts to reduce hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and scavenger of active oxygen species (Ahirwal et al., 2013). This study shows the increase in the antioxidant molecules like GSH, SOD and Catalase, and, also, reduces TBARS levels (Figure 4.9). Earlier study also observed antioxidant activity in animal model (Kang et al., 2012).

Toxic agents may affect the kidney and impair its physiological functions. These effects are detectable and/or quantifiable by cross checking the normal functions of the kidney by its markers like urea and creatinine. This study shows significant reduction in all the parameters of kidney function. An earlier study also shown the reduction in these parameters in response to *G. sylvestre* extract (Baskaran et al., 1990). Based on this, it is evident that both the therapies are not affecting the kidney and they do not have any toxic effects.

Liver is a vital organ for regulation of the blood glucose and lipid homeostases. Thus *G. sylvestre* therapy helps in the proper regulation of the blood glucose and even it shows lipid homeostasis without giving any detrimental effects on liver. Earlier studies in animal model and human subjects also show the same (Shanmugasundaram et al., 1983; Chattopadhyay, 1998; Kumar et al., 2010; Kang et al., 2012; Shafey et al., 2013).

Favorable changes in the BMI and blood pressure in both the groups were also recorded. Body weight decrease may be due to the decrease in food intake that leads to decrease in calorie intake (Nakamura et al., 1999). *G. sylvestre* was found to reduce the
clinical symptoms of diabetes, such as excessive hunger, tiredness and polyuria. Changes were also observed in the anthropometers of subjects (Kumar et al., 2010; Zuñiga et al., 2017). The study done in animal model show the reduction of systolic blood pressure and other abnormalities related to MetS (Manish et al., 2011; Bhansali et al., 2013). Recent study in metabolic syndrome subjects also shown the same (Zuñiga et al., 2017).

However, this study did not find any significant difference between the two G. sylvestre therapies in any of the parameters monitored. It may be due to bioactive compounds of both extracts are identical (Table 2.7). Earlier study done on Cyclocarya paliurus leaves also reported no significant difference in both ethanolic and aqueous extracts (Wang et al., 2013).

On the basis of these results, it could be concluded that oral administration of G. sylvestre in either form has antihyperglycemic, antilipidemic and antioxidant potentials and appear fruitful for diabetic as well as MetS subjects. The study also revealed that therapy is not toxic to liver and kidney.

In silico docking study was performed to explore the molecular targets of G. sylvestre active constituents. Five important protein/enzymes targets involved in the insulin signalling pathway (PTP1B, IRS, PDK1, Akt/PKB and GSK3-β) were explored using PatchDock and FireDock. The docking poses are visualized by Acceryls discovery studio visualizers. Out of five target molecules, the G. sylvestre compound(s) interacted with only three targets (PTP1B, Akt/PKB and GSK3-β).

PTP1B is a key regulatory enzyme in insulin receptor activity and downstream signalling pathways. There are reports that the lack of PTP1B enzyme (inhibition of its activity) activates the insulin receptors, improves sensitivity to insulin, and stimulate, glucose uptake (Salmeen et al., 2000; Boute et al., 2003). Present study observed that the binding mode of GA, GMG with PTP1B along with the interacting amino acid residues were similar to that of proved PTP1B inhibitors (Ramírez-Espinosa et al., 2011). GA and GMG interact with amino acids ARG 24, TRY 46, ARG 254, GLN 262, and LSY 120 at the catalytic site of protein by hydrogen bond interactions with Global Energy ranging
between -36.95 & -41.02 Kcal/mol. (Table 4.13, Figure 4.12a,b). Similar study was earlier reported (Kamble et al., 2012).

Akt/PKB is a serine/threonine-specific protein kinase that plays a central role in insulin-stimulated glucose uptake in both muscle and adipose tissue (Mackenzie and Elliott, 2014). All molecules that activate a kinase must bind at an allosteric site, so that the ATP- and substrate-binding sites are available for catalysis (Cowan-Jacob et al., 2014). This study found that the bioactive compounds of *G. sylvestre* act as the allosteric site modulators because it does not bind to any ATP binding sites and binds to the known modulator sites with Global energy range between -42.5 to – 46.52 Kcal/mol (Table 4.14, Figure 4.13a,b). Earlier in silico study on *G. sylvestre* molecule also shown interactions with Akt (Rathore et al., 2016).

GSK3-β plays a vital role in two key targets of insulin action, glycogen synthase and insulin receptor substrate-1, which are suppressed by GSK-3β, and it is reported that GSK3-β activity is higher in diabetic tissues (Eldar-Finkelman et al., 1999). GSK3-β activity can be acutely modulated by allosteric regulators and phosphorylation/dephosphorylation (Macaulay and Woodgett, 2015). The inhibition of GSK3-β dependent phosphorylation activates insulin-dependent glycogen synthesis, (Eldar-Finkelman and Kaidanovich, 2002) which allows insulin to lower plasma glucose. Thus, GSK3-β inhibitor makes an efficient target for insulin resistance and T2DM (Zhang et al., 2004). Present study shows that the *G. sylvestre* molecules bind at the active site of GSK3-β and had hydrogen bond interactions on the amino acids with Global energy between -38.76 & -45.6876 Kcal/mol (Table 4.15, Figure 4.14a,b). Earlier study was done on *G. sylvestre* molecule also shown the similar inhibitory effects on GSK3-β (Kamble et al., 2012).

To confirm the in silico study results, further wet lab study was carried out in L6 skeletal muscle; it is the relevant cell line model to study the insulin signaling and GLUT 4 translocation, moreover; skeletal muscle is the primary site for glucose clearance and glucose utilization (Koivisto et al., 1991; Kim et al., 2014).

The GSEE effect on the pathway regulating insulin-mediated glucose utilization was studied. In skeletal muscle cells, binding of insulin to its receptor activates a complex
network of signalling molecules including IRS, PI3K, Akt/PKB and GSK3-β (Khan and Pessin, 2002). Acute exposure to insulin caused a robust increase in the phosphorylation of IRS-1, Akt, and GSK3-β at basal condition. *G. sylvestre* significantly stimulates the phosphorylation of IRS-1, Akt and GSK3-β upon exposure. Similar kind of study is also reported earlier in *Arnabia nobelis* (Pandeti et al., 2016).

Further, *G. sylvestre* molecule induced GLUT4 translocation was studied. GLUT4 are stored in intra cellular vesicles and upon stimulation, they translocate from cytoplasm to plasma membrane (Petersen and Shulman, 2006). GLUT4 from intracellular to the plasma membrane is one of the mechanisms by which glucose uptake regulation occur in skeletal muscle. Present study observed that, the GSEE significantly enhanced the GLUT4 translocation in the basal condition. Earlier report on methanolic extract of *G. sylvestre* in L6 myotubes shown the glucose uptake activity in dose depended manner (Kumar et al., 2016).

Insulin resistance is a hallmark of obesity and T2DM; plasma and tissue lipid levels are inversely associated with insulin sensitivity (Kelley et al., 2002). Palmitate (free fatty acid) is used to induce the insulin resistance in the *in vivo* studies (Schmitz-Peiffer et al., 1999; Sinha et al., 2004). Present study noted that GSEE induced GLUT4 translocation is not affected significantly in the presence of palmitate (insulin resistance condition) (Figure 4.18). Phosphatidylinositol 3-kinase (PI3K) plays critical role in insulin metabolism (Okada et al., 1994). Wortmannin is the potent inhibitor of PI3K (Zalkow et al., 1994). Wortmannin blocks almost all metabolic effects of insulin (Zhuo et al., 2015, 2017). The study support that activation of the PI3K is necessary to mediate insulin stimulated GLUT-4 translocation (Khan and Pessin, 2002). In the present study, GSEE was seen to induce GLUT4 translocation, which was reverted in the presence of PI3K inhibitor (Figure 4.19). It is suggested that both insulin and GSEE might activate the same signalling pathway to stimulate glucose utilization in skeletal muscle cells. GSEE acts synergistically with insulin in mediating the increase in surface GLUT4 level thus suggesting that *G. sylvestre* is a potent activator of insulin-mediated glucose utilization.
In addition to molecular mechanism of the *G. sylvestre* in augmenting GLUT4 translocation in L6 myotubes, other mechanism like anti-inflammatory activity were also explored.

Low-grade chronic inflammation which increases the systemic levels of cytokines including IL-6, TNF-α leads to the subsequent development of insulin resistance and T2DM (Spranger *et al.*, 2003; Lampropoulou *et al.*, 2014; Qu *et al.*, 2014).

Monocyte chemotactic protein-1 (MCP-1) is expressed generally in inflammatory cells. The expression level is increased in the inflammation condition (Lin *et al.*, 2014). Anti-inflammatory activity of *G. sylvestre* was reported earlier in the animal model of Carrageenan and Cotton pellet induced inflammation (Diwan *et al.*, 1995; Malik *et al.*, 2008). Recent report suggested that, the anti-inflammatory effect of *G. sylvestre* might be mediated by inhibiting the level of prostaglandin E2 (PGE2) and nitric oxide (NO) (Aleisa *et al.*, 2014). In present study, GSEE was found to significantly reduce the level of pro-inflammatory cytokines (MCP-1, IL-6 and TNF-α) and increase the level of anti-inflammatory cytokines (IL-10) in palmitate induced inflammatory condition in L6 myocytes (Figure 4.20). Thus *G. sylvestre* appear to improve insulin sensitivity also through it's anti-inflammatory function.