CHAPTER- 5

IN VITRO ANTIDIABETIC ACTIVITY OF GERMINATING FENUGREEK SPROUTS UNDER THE INFLUENCE OF CALCIUM AND NITRIC OXIDE ELICITORS

5.1 INTRODUCTION

Plants are the basic source of knowledge of modern medicine. Almost all the parts of a plant, namely leaves, flowers, fruits, bark, roots, stem and seeds are known to have various medicinal properties (Mori and Prance, 1978). The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries and for the presence of potential bioactive components (Ramma *et al.,* 2002; Senthilkumar *et al.,* 2014).

India with highest number of people suffering from diabetic disorders has been considered as the diabetic capital of the world by the 'International Journal of Diabetes in Developing Countries'. There is an alarming rise in diabetes patients in India; approximately 3.4 million deaths occur due to complication related to high blood sugar (Kumar *et al.,* 2012).

Diabetes mellitus is a serious metabolic disorder that leads to hyperglycemic condition due to decreased insulin production or inefficient insulin utilization. It is usually characterized by hyperglycemia, lipoprotein abnormalities, high basal metabolic rate, impairment in the activity of important enzymes and oxidative stress which damages the pancreatic beta cells. It is the most common endocrine disorder which disrupts glucose homeostasis causing severe diabetic associated complications in major organs like eye, blood vessels and brain (Sharma *et al.,* 2010; Jaiswal *et al.,* 2014). Multiple risk factors responsible for the disease to occur include persistent stress and depression, obesity, environmental pollutants and sedentary life style (Ghamarian *et al.,* 2012).

Some of the synthetic antidiabetic components such as metformin, acarbose, biguanides and voglibose are found to be used clinically in combination with other diet to control diabetes but moreover they exhibit adverse side effects after long term use (Asano 2009; Standl and Schnell, 2012; Patel *et al.,* 2012). To prevent or overcome the side effects

of these synthetic drugs and also to create other safer alternative drug choices, it has become essential to seek other inhibitors for further drug development. Thus, in recent years, several efforts have been made for increasing the availability of glucosidase inhibitors from natural sources (Jung *et al.,* 2006; Jin *et al.,* 2013).

Likewise, fenugreek besides having several pharmacological properties it is also reported to possess potent antidiabetic property both *in vitro* and *in vivo* system. Fenugreek is known to be a rich source of various bioactive components having different therapeutical property such as sapogenins, fenugreekine, nicotinic acid, phytic acid, and trigonelline (Puri, 1998; Randhir *et al.,* 2004). Trigonelline is a pyridine alkaloid known to be mostly found in Fabaceae members and is reported to be metabolically active as hypocholesterolemic agent along with potential hypoglycemic effect (Taguchi, 1986; Olthof *et al.,* 2011; Mathur and Kamal, 2012). Fenugreek has been successfully implemented as antidiabetic remedy for both type I and II diabetes (Zia *et al.,* 2001). Moreover, fenugreek has been reported to be enriched with wide spectrum of pharmacological and folkloric significance (Meghwal and Goswami, 2012).

The present study deals with the isolation and identification of trigonelline by 1-D and 2-D NMR from fenugreek sprouts. Further the elicitors of calcium and nitric oxide were applied for the enhancement in the alkaloid content of fenugreek sprouts along with the *in vitro* anti-diabetic property. The present study may provide an insight in the role of these signaling molecules in modulating the biosynthesis of alkaloids, a potential hypoglycemic agent; additionally the utility of 2-D NMR spectroscopy in identification of bioactive compounds.

5.2 MATERIALS AND METHODS

5.2.1 Elicitation process and germination

Mentioned in section 3.2.1

Table 5.1: Different priming agents applied

Calcium elicitors		Nitric oxide elicitors	
T0	H_2O	T0	H_2O
Tc1	CC-1mM	Tn1	SNP-10mM
Tc2	CC-2mM	Tn2	SNP-20mM
Tc3	CC-5mM	Tn3	SNP-40mM
Tc4	CC-1mM+EG500µM	Tn4	SNP-80mM
Tc5	CC-2mM+EG500µM	Tn5	SNP-120mM
Tc6	CC-5mM+EG500µM	Tn6	SNP10mM+CP125µM
Tc7	EG-2mM	Tn7	SNP20mM+CP125µM
Tc8	CC-1mM+LC500µM	Tn8	SNP40mM+CP125µM
Tc9	CC-2mM+LC500µM	Tn9	SNP80mM+CP125µM
Tc10	CC-5mM+LC500µM	Tn10	SNP120mM+CP125µM
Tc11	LC-2mM	Tn11	CP125 µM

5.2.2 Determination of antidiabetic property by *in vitro* method

5.2.2.1 Preparation of extract

The fenugreek sprouts of 3 different stages: 24h, 48h & 72h were crushed in mortar-pestle and individually processed through soxhlet extraction apparatus with methanol for 8h duration. The refluxed samples were separated from the residues by filtering through

Whatman No. 1 filter paper and the extract was concentrated to a uniform concentration of 1 g/ml using a vacuum rotary evaporator at 50°C. The obtained methanolic extracts were further used for experimental analysis for determination of antidiabetic activity and total alkaloid estimation.

5.2.2.2 Estimation of α-amylase enzyme inhibitory activity

The alpha-amylase (AA) inhibition potential of the extract was estimated by standard spectrophotometric method (Paul and Banerjee, 2013). 0.5 ml of aqueous extract was reacted with 0.5 ml of α-amylase solution and incubated at 37°C for 5 min. After incubation, 0.5 ml starch solution (1%) was added and was further incubated for 10 min. To the above reaction mixture, 1 ml of DNSA reagent was added to terminate the reaction and was heated for 10 min in a hot water bath till the colour of reaction mixture colour changed to orange-red. After change in colour, the reaction mixture was cooled and diluted up to 5 ml with distilled water. The OD value was measured at 540 nm. The α-amylase inhibitory activity was estimated by measuring the concentration of inhibitor required to inhibit 50% of the enzyme activity.

$$\% \text{ Inhibition} = (A_{540\ control} - A_{540\ sample})/(A_{540\ control}) \times 100$$

Where $A_{540\ control}$ = Absorbance of control at 540 nm and $A_{540\ sample}$ = Absorbance of sample at 540 nm.

5.2.2.3 Estimation of α-glucosidase enzyme inhibitory activity

The α-Glucosidase (AG) inhibitory property of the sample extract was assayed according to Dong *et al.,* (2012) with slight modifications. The reaction was initiated with 0.05 ml each of the samples at different concentrations in 0.2 mM phosphate buffer (pH 6.8), followed by incubation at 37°C for 15 min, after which 0.1 ml of enzyme solution was immediately added to the mixture before mixing and incubation at 37°C. Then, 3 mM *p*-nitrophenyl

glucopyranoside (pNPG) (0.25 ml) was added, after which the reaction was terminated by the addition of 4 ml of 0.1 M Na_2CO_3. Alpha-Glucosidase inhibitory activity was determined by measuring the release of pNPG at 405 nm. The control contained all reagents without the tested sample. The reactions were conducted in triplicate. The α-glucosidase inhibitory activity was calculated as follows:

$$\text{Inhibitory } \% = [1- (As- Ab)/ Ac] \times 100$$

where Ac, As, and Ab represent the absorbance levels of the control, sample, and blank, respectively. The concentration of α-glucosidase inhibitor required to inhibit 50% of α-glucosidase activity under the assay conditions is defined as the IC_{50} value.

5.2.3 Total alkaloid estimation

The extract was gently heated and methanol was evaporated on a rotary evaporator under vacuum at a temperature of 45^{o}C to dryness. The residue was dissolved in 2 N HCl and then filtered. One ml of this solution was transferred to a separation funnel and washed with 10 ml chloroform (3 times). The pH of the solution was adjusted to neutral with 0.1N Sodium hydroxide. Then 5 ml of Bromocresol Green solution and 5 ml of phosphate buffer were added to the reaction mixture. The mixture was shaken and the complex formed was extracted with 5ml chloroform by vigorous shaking. The extracts were collected in a 10-ml volumetric flask and diluted to same volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank (Shamsa *et al.,* 2008). Similarly trigonelline content was also estimated from the purified extract.

5.2.4 Isolation of Trigonelline

The extraction procedure was followed as suggested by Ahmed *et al.,* (2011) with some modifications. The fenugreek sprouts of about 100 g were homogenized in 10 M HCl solution and gently boiled for 2h in an Erlenmeyer flask. Next the mixture was filtered and zinc dust was added to the mixture and kept on magnetic stirrer for overnight. In next

morning, the mixture was filtered again and extracted with equal volume of dichloromethane (CH_2Cl_2). The CH_2Cl_2 layer containing the alkaloid was separated and later evaporated. The obtained extract after CH_2Cl_2 evaporation was re-constituted in methanol for further analysis.

5.2.5 Thin Layer Chromatography (TLC)

The extract dissolved in methanol was applied manually on activated TLC plates, along with standard trigonelline. After loading the samples the TLC plates were developed in pre-saturated chromatography solvent chamber containing solvent system composed of ethylacetate:methanol:H_2O (100:13.5:10). After running the solvent through the TLC plates, the plates were sprayed with Dragendorff's reagent to develop spot corresponding to standard trigonelline simultaneously.

After confirming the presence of trigonelline, in next TLC experiment after running the solvent through the TLC plate, the portion of plate containing pure trigonelline was cut and sprayed with Dragendorff's reagent and then the area of TLC plate containing unknown extract corresponding to band developed on the plate with standard trigonelline was scrapped and collected, dried and later dissolved in methanol for further quantitative and spectral analysis.

5.2.6 Infra-red (IR) spectral analysis

IR spectra of the crude, purified sample and standard trigonelline were recorded on FTIR spectrophotometer (model: 8300 Shimadzu) by nujol mulling for liquid sample and KBr pellets for solid sample.

5.2.7 NMR analysis

The confirmation of the structure of the extracted compound was done by NMR- spectra in 300 MHZ Bruker-Avance spectrometer using 5mm BBO probe and Methanol-d_4 was used as solvent.

5.2.8 Statistical analysis

Each experiment was performed in triplicate. The software package, MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antidiabetic activity and alkaloid content of the fenugreek sprouts. The different group means were compared by Duncan's Multiple Range Test through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $p<0.05$ was considered significant in all cases. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC_{50} values of antidiabetic activity and their standard error of estimates.

5.3 RESULTS AND DISCUSSION

The key enzymes in the digestive system, α-amylase and α-glucosidase are actively involved in the release of glucose in the blood via breakdown of starch. Hence, the inhibition of these enzymes would minimize the breakdown of starch, which may lead to a reduction in the post-prandial hyperglycemic level (Tarling *et al.,* 2008). Therefore, determination of the capability of an extract to inhibit the activity of these enzymes α-amylase and α-glucosidase is essential for investigating the antidiabetic potentiality of the extract. Since fenugreek has been well known for its antidiabetic activity, the present study aimed at enhancing the antidiabetic activity of fenugreek sprouts through elicited sprouting. The inhibition capacity of the sprouts was expressed in terms of IC_{50} values. The results revealed that the fenugreek sprouts exhibited potential antidiabetic property, and interestingly, it was also observed that the exogenous supply of Ca^{2+} and nitric oxide resulted in the enhancement of this activity in fenugreek sprouts. Both the α-amylase and α-glucosidase inhibition capacity of fenugreek sprouts was enhanced by CC (Table 5.2) and SNP (Table 5.3).

In many plant species the alkaloids are reported to get accumulated in seeds in relatively higher amount as a chemical defense. During germination these alkaloids are metabolized and their nitrogen is further used for seedling's metabolism (Wink and Witte, 1985). The antidiabetic property of most legumes including fenugreek is usually attributed to their trigonelline content, an alkaloid with scientifically well characterized hypoglycemic effect (Randhir *et al*., 2004; Yoshinari *et al.,* 2013). Trigonelline is reported to act by regulating cell regeneration, insulin secretion, enzymes associated with glucose metabolism. It is further known to mitigate oxidative stress during the diabetic complications (Yoshinari *et al.,* 2009; Amaro *et al.,* 2014). Considering such potential role of alkaloids in controlling the diabetic disorders, the alkaloid content of the fenugreek seedlings subjected to various priming agents were determined. Interestingly, it was found that the sprouts pre-treated with CC and SNP exhibited significantly higher alkaloid content with respect to control sprouts Figure 5.1. Similar trend in the effect of different priming agent was observed in the trigonelline content of the fenugreek sprouts (Figure 5.2). The potential hypoglycaemic property of trigonelline was further supported by our results which clearly depicted the significant influence of trigonelline content on the AG (Figure 5.3) and AA (Figure 5.4) inhibitory capacity of fenugreek sprouts pre-treated with different priming agents. On the other hand, the antagonists, EG, LC and CP showed inhibitory effect on the alkaloid accumulation in the sprouts. In agreement to the earlier cited statement, it was noted that the sprouts with higher trigonelline content exhibited stronger α-glucosidase and α-amylase inhibition activity thus suggesting the probable role of alkaloids especially trigonelline as hypoglycemic compounds in the management of diabetic disorders.

Furthermore, various authors have proved that the enhancement in the hypoglycemic activity of fenugreek is considerably associated with increase in the level of bioactive components such as trigonelline and 4-hyroxy leucine (Ahmed *et al.,* 2011; El-Soud *et al.,* 2007; Narender *et al.,* 2006).

Table 5.2: *In vitro* α-amylase and α-glucosidase inhibition activity of fenugreek sprouts under the influence of different nitric oxide elicitors.

Treatment	24h		48h		72h	
	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)
T0	1010±47.76e	1186.75±38.72de	715.20±31.24c	840.36±42.36d	1122.81±29.98d	1319.30±24.21c
Tn1	533.33±22.16b	981.33±11.05c	420.01±9.22ba	772.8±8.72c	765.14±21.94b	1407.86±18.44d
Tn2	377.86±12.78a	581.90±12.62a	350.91±8.56a	540.41±7.16a	630.38±17.04a	970.78±8.54a
Tn3	635.81±8.15c	915.57±9.29b	451±11.05b	649.44±7.95b	839.75±18.69c	1209.25±12.05b
Tn4	822.96±12.33d	1431.95±12.67f	836.32±12.06d	1455.21±18.52g	1215.47±27.24e	2114.92±22.48i
Tn5	1313.16±11.22g	1497.0±26.38g	1464.12±19.14i	1669.09±18.32h	1962.27±39.97i	2236.99±19.86j
Tn6	1341.30±12.56gh	1166.93±9.08d	1354.88±14.17h	1178.75±10.22f	2375.56±23.15j	2066.73±21.62hi
Tn7	1160.0±9.42df	1032.40±14.12bc	1197.09±12.32f	945.71±8.25e	1992.78±13.73i	1574.29±18.24e
Tn8	1071.33±11.24f	1229.90±8.54e	1146.82±13.16e	860.11±8.66d	1406.67±15.67f	1448.86±16.92d
Tn9	1376.40±13.05h	1575.97±14.16h	1294.28±12.11g	1481.95±18.45g	1625.24±17.14g	1860.90±24.68f
Tn10	1704.09±19.19j	1917.0±17.32j	1820.76±22.04k	2048.36±24.35j	1819.86±12.05h	2047.33±18.78h
Tn11	1559.38±14.22i	1748.06±21.41i	1608.13±14.28j	1802.71±22.08i	1768.12±11.36h	1982.06±21.86g

Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) in columns are significantly ($p<0.05$) different from each other by Duncan's multiple range test

Table 5.3: *In vitro* α-amylase and α-glucosidase inhibition activity of fenugreek sprouts under the influence of different calcium elicitors

Treatment	24h		48h		72h	
	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)	AG (mg/ml)	AA (mg/ml)
T0	1010±47.76b	1186.75±38.72c	715.20±31.24c	840.36±42.36d	1122.81±29.98d	1319.30±24.21c
Tc1	336.52±28.05a	619.20±15.02b	330.0±22.37a	607.20±12.08b	717.50±21.94b	1320.20±19.12c
Tc2	339.42±22.32a	522.71±10.33a	301.12±13.77a	463.71±20.48a	599.80±17.04a	923.69±14.34a
Tc3	348.94±16.69a	502.48±12.05a	464.68±23.13b	669.15±23.95c	795.38±18.69c	1145.35±12.72b
Tc4	1055.23±22.42b	1202.97±32.15c	773.20±16.08d	1345.36±27.99i	1176.25±27.24d	1576.17±18.79e
Tc5	1593.33±12.54d	1386.20±36.44e	1289.22±18.33g	1121.43±11.86f	1869.88±39.97h	1626.79±16.76ef
Tc6	1634.5±15.69d	1291.97±9.56d	1610.71±25.36h	1272.46±13.40h	1906.36±23.15h	1506.02±23.17d
Tc7	2276.66±29.58e	1707.50±24.45f	1732.11±17.87i	1299.08±13.25hi	2284.50±13.73i	1713.37±13.90g
Tc8	1047.41±10.56b	1199.29±13.22c	832.85±12.42e	953.63±10.55e	1326.92±15.67e	1519.32±17.09d
Tc9	1141.54±12.36c	1284.23±12.42d	1075.21±19.11f	1209.62±19.23g	1598.89±17.14f	1798.75±20.33h
Tc10	1148.09±9.44c	1287.02±8.56d	1258.18±12.45g	1410.42±17.19j	1738.04±12.05g	1885.77±18.90i
Tc11	2545±34.22f	1679.70±22.72f	1294.58±14.62g	1513.34±12.14k	1700.80±11.36g	1632.76±18.24df

Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) in columns are significantly ($p<0.05$) different from each other by Duncan's multiple range test

Table 5.4: Two way ANOVA analysis of antidiabetic and trigonelline content of differently primed fenugreek sprouts at various days of germination stage

Source of Variation	df	*F crit*	AG		AA		TC	
			F	***P-value****	***F***	***P-value****	***F***	***P-value****
Treatment	23	1.604555	91163.68	1.1E-287	70049.33	1.8E-279	1314.238	2.6E-155
Days of germination	2	3.058928	131427.4	1.5E-235	178824.7	3.5E-245	1838.919	3E-103
Interaction	46	1.453191	4813.891	5.2E-209	7558.876	4.1E-223	69.94316	1.9E-78
Within	144							
Total	215							

*Significant at P<0.01

Further, two-way ANOVA analysis with replication was performed to determine the impact of different priming agents and various germination stages on antidiabetic and trigonelline content of fenugreek sprouts. As a result, a significant impact of both the variance i.e. priming agents and germination stages was found on the AA and AG inhibitory activity along with the trigonelline content (Table 5.4). Interactions between both were also significant at $p<0.05$ level.

The crude extract and the purified extract of fenugreek sprout were subjected to IR spectral analysis. The finger print region of both the crude and purified extract matched exactly with the that of standard trigonelline (Figure 5.6), this indicates all the samples are of same nature, and further confirms the predominance of trigonelline in both the extract (crude and purified). In the frequency range 3000 to 3500 cm^{-1} broad strong absorption indicates presence of -OH groups, both intra and intermolecular H-bonded. At 1715 cm^{-1} the absorption corresponds the presence of carboxylic acid group.

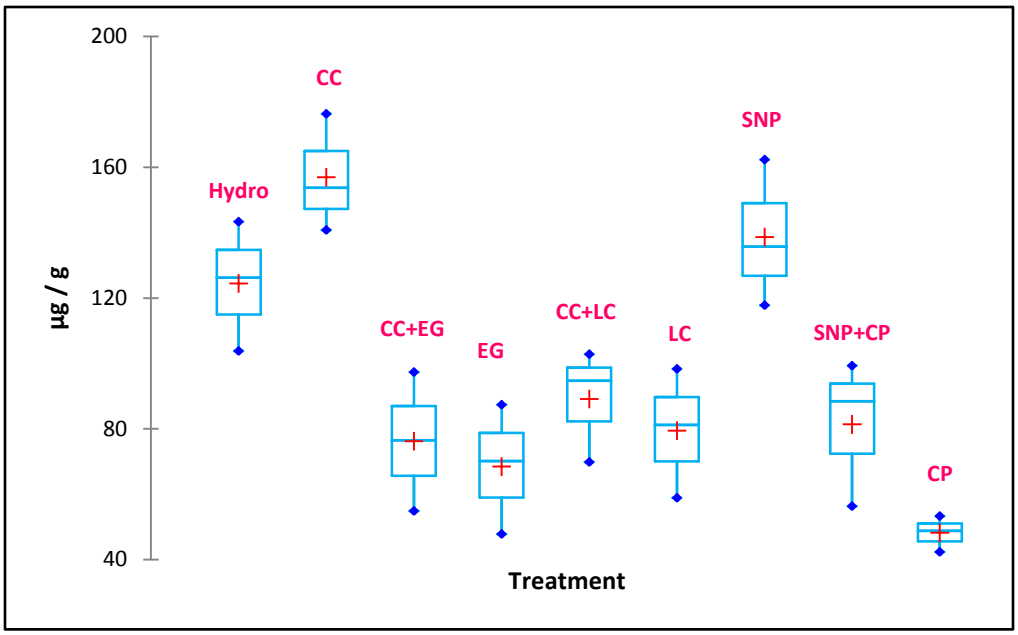

Figure 5.1: Effect of different priming agents on total alkaloid content of fenugreek sprouts

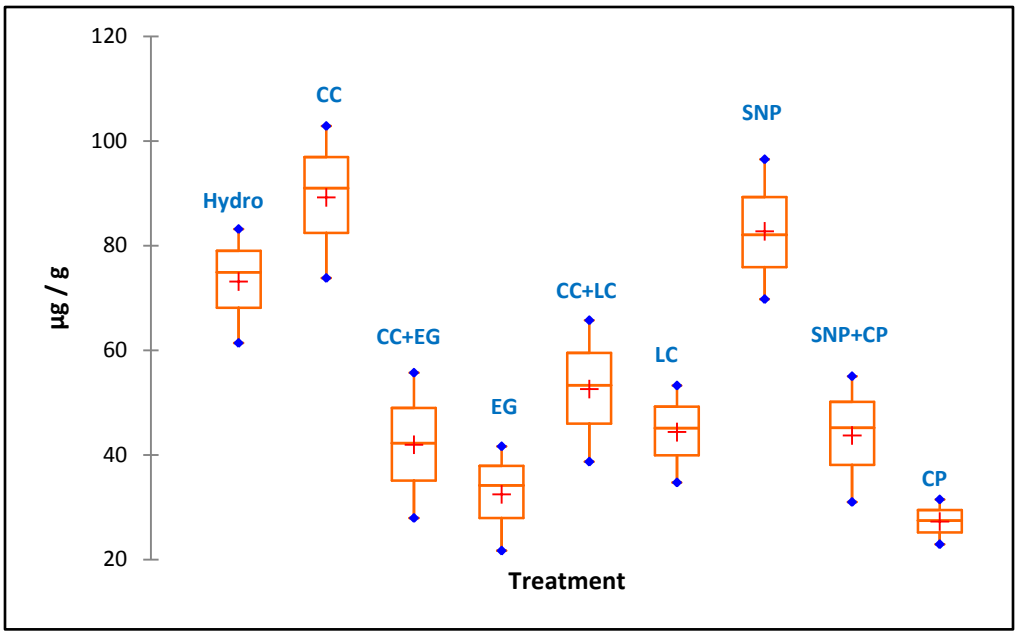

Figure 5.2: Effect of different priming agents on trigonelline content of fenugreek sprouts

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

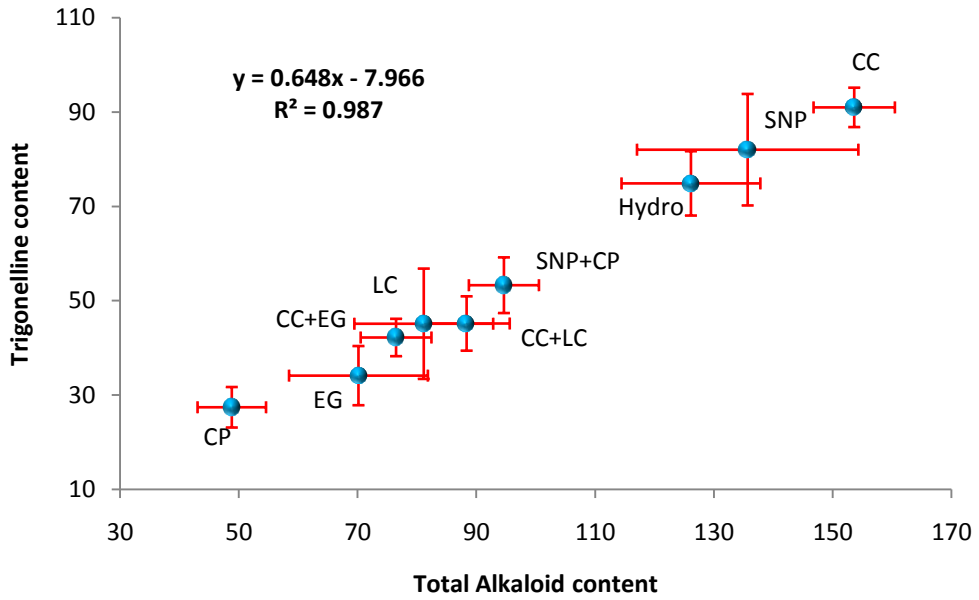

Figure 5.3: Effect of different priming agents on the total alkaloid and trigonelline contents of fenugreek sprouts

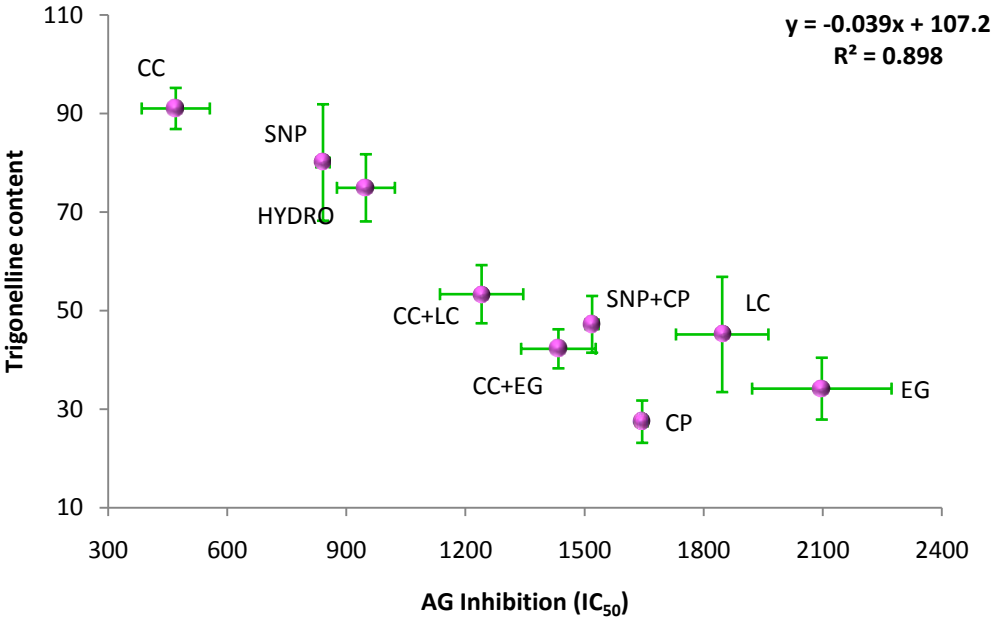

Figure 5.4: Alteration in the α-glucosidase inhibition activity of fenugreek sprouts with respect to the trigonelline contents of fenugreek sprouts resulted due to different priming agents

Abbr. used: Hydro: hydro primed; CC: calcium chloride; EG: EGTA; LC: lanthanum chloride; SNP: sodium nitroprusside; CP: c-PTIO

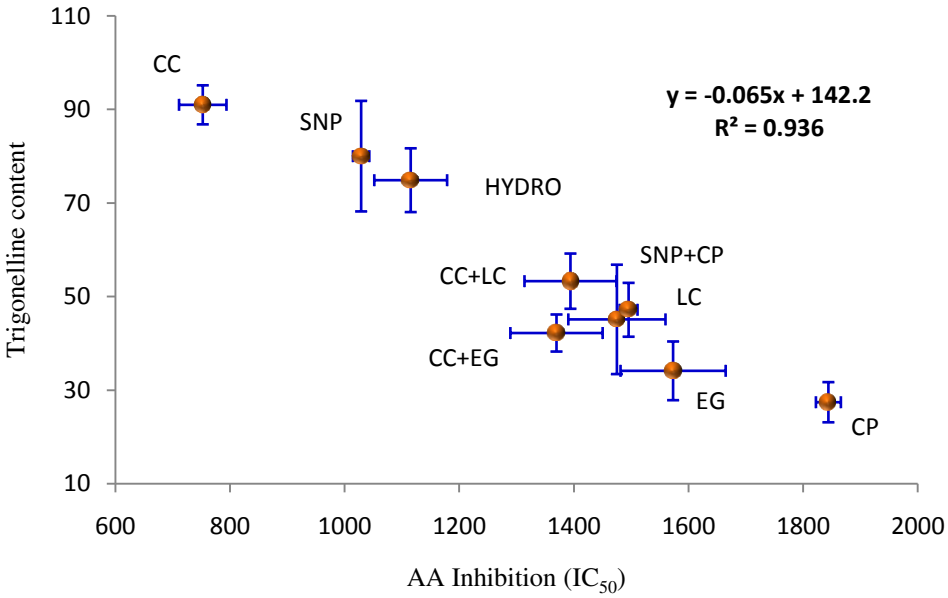

Figure 5.5: Alteration in the α-glucosidase inhibition activity of fenugreek sprouts with respect to the trigonelline contents of fenugreek sprouts resulted due to different priming agents

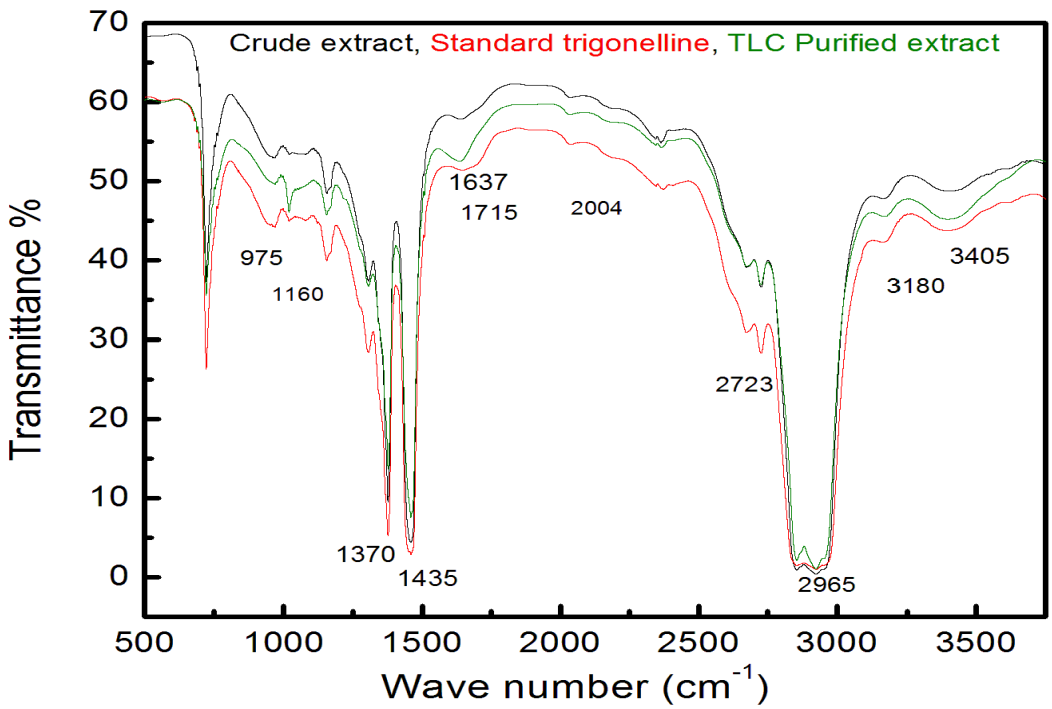

Figure 5.6: IR spectra of crude extract and TLC purified extract compared with standard trigonelline

The presence of some sharp medium absorption peaks at around 2725 cm^{-1} indicates presence of C=C-H aromatic bonds supported by the presence of aromatic ring unsaturation vibrational bands around 1600cm^{-1}, the weak absorption for the ring bending of benzene derivatives in the range of 900 cm^{-1} was also observed. The aliphatic C-H stretching modes at around 2900$cm^{-1}$are masked with that of the nujol. After confirming the presence of trigonelline in purified extract by IR analysis the extract was further analysed by NMR spectroscopy.

The implementation of NMR spectroscopy for metabolite fingerprinting has been considered highly reliable by the researchers. One dimensional NMR (1D-NMR) spectra has been used successfully for analyzing compounds in a crude extract, further two dimensional NMR (2D-NMR) spectra have been reported to be more efficient in identification of bioactive compounds in plant extracts (Kim *et al.,* 2010; Farag *et al.,* 2015). Likewise, the purified extract was subjected to 2D-NMR for the identification of compound. The ^{1}H NMR spectra of purified fenugreek extract obtained by NMR spectroscopy is depicted in (Figure 5.7 & 5.8). The proton (^{1}H) NMR spectra derived from the purified extract of fenugreek was characterized by N^{+}-CH_3 (3H) peak at 4.5 ppm (δ); aromatic protons are observed at 8.1 ppm (δ) (1H), at 9 ppm (δ) (2H) and at 9.3 ppm (δ) (1H). In proton correlated spectroscopy (COSY) (Figure 5.9) and total correlation spectroscopy (TOCSY) spectra the correlation of the protons are confirmed and in the heteronuclear correlation (HETCOR) (Figure 5.10) spectra correlation of the spectra with that of carbon shifts are ascertained. This spectral analysis unequivocally suggests that the purified extract contains 1-Methylpyridinium-3-carboxylate (trigonelline). Overall, it was observed that among all treatments, CC and SNP priming exhibited enhancement in the alkaloid and antidiabetic potential of fenugreek, among which CC was found to exhibit the best result.

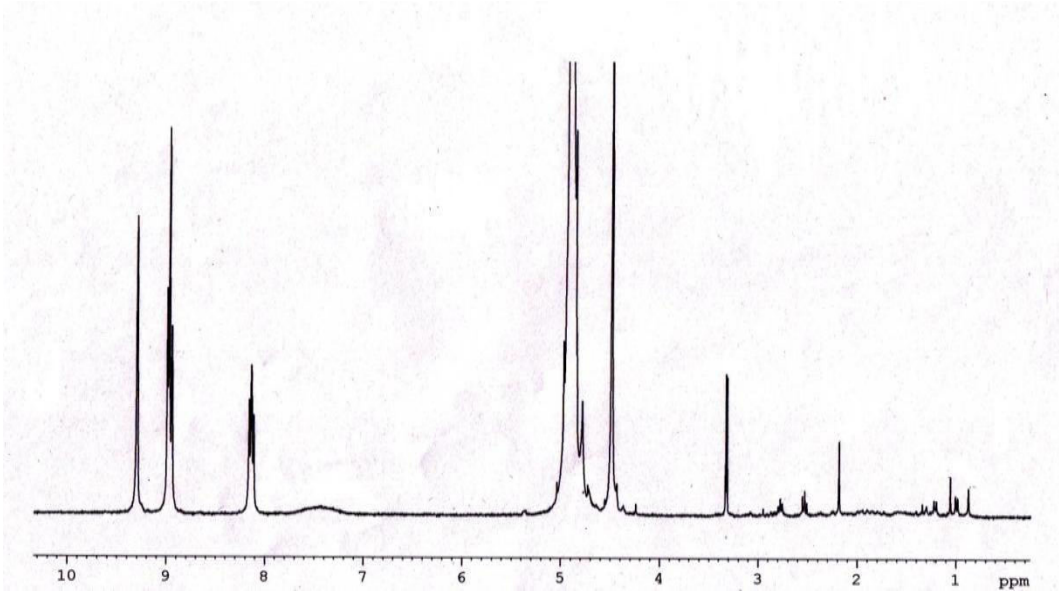

Figure 5.7: ^{1}H NMR spectra of TLC purified extract of fenugreek sprout

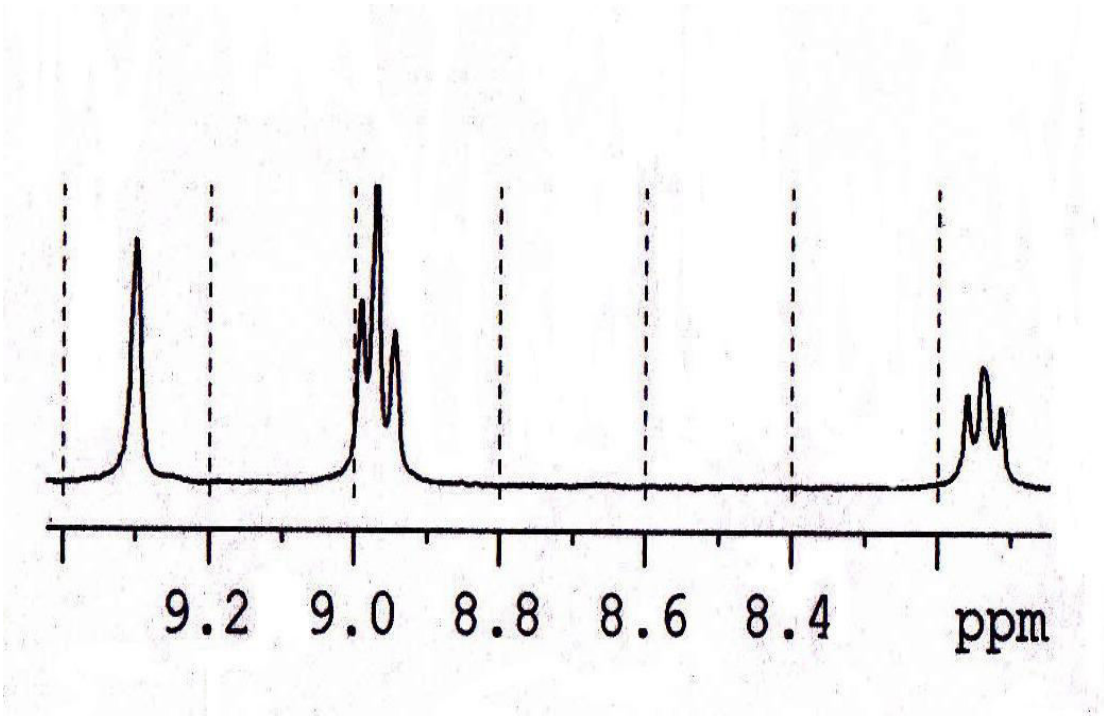

Figure 5.8: Expanded regions of ^{1}H NMR spectra of TLC purified extract of fenugreek sprout

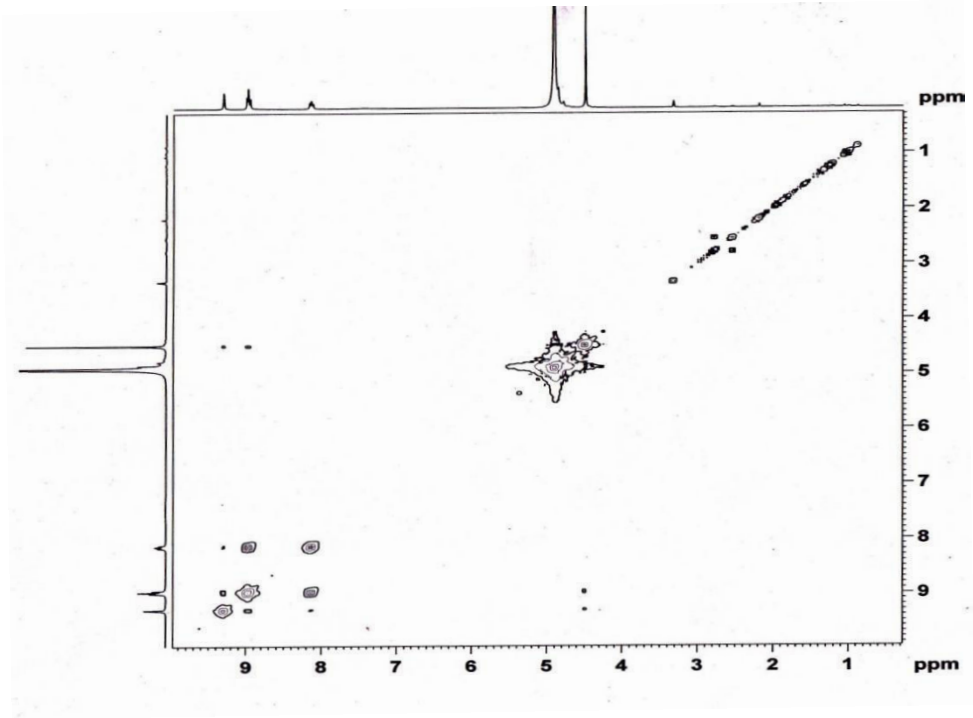

Figure 5.9: COSY spectra of TLC purified extract of fenugreek sprout

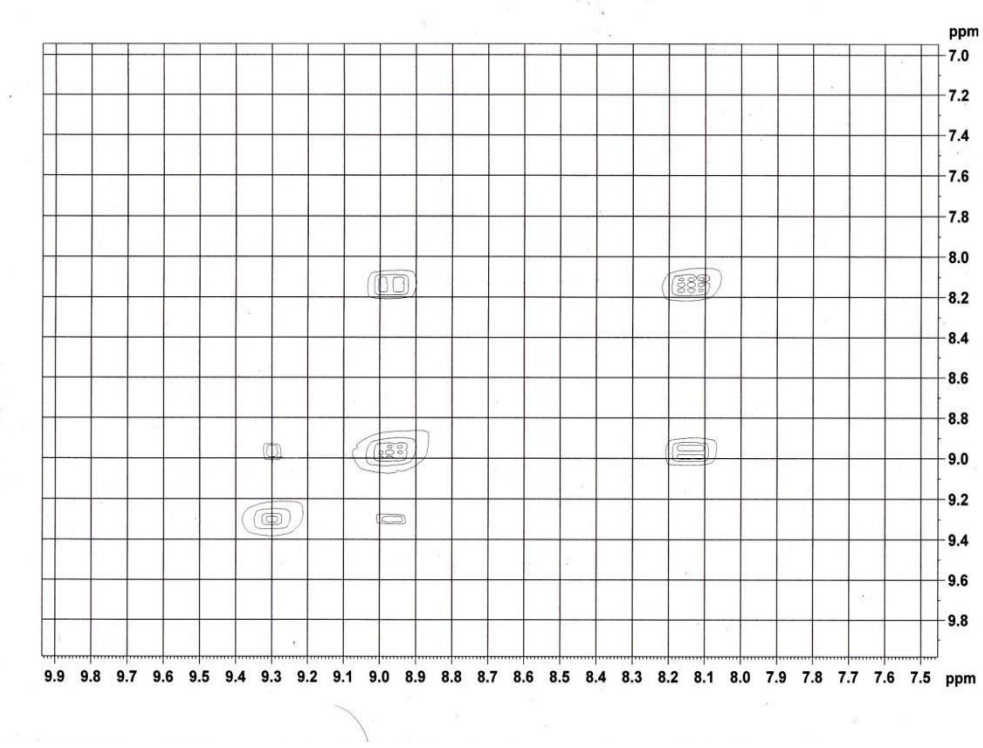

Figure 5.10: HETCOR spectra of TLC purified extract of fenugreek sprout

Such enhancement in the antidiabetic activity and the level of trigonelline in fenugreek has reported by various authors through biotic and abiotic elicitors (Ahmed, 2011; Qaderi, 2016). The therapeutic property of trigonelline is highly attributed to its hypoglycaemic effect, thus the enhancement in the biosynthesis of alkaloids further boosts the antidiabetic potential of plant. The observation of best antidiabetic activity of sprouts during 48h stage indicates that the components including trigonelline responsible for the therapeutic potential were elicited appropriately at this stage. The impact of nitric oxide scavenger (CP), calcium chelator (EG) and calcium channel blocker was also assessed on the antidiabetic as well as alkaloid content of the fenugreek sprouts. The effect of these elicitors was found to be deteriorative on both the aspects of the sprouts, i.e. therapeutic (antidiabetic) as well phytochemical (alkaloid) content.

It can be suggested that the increase in the alkaloid content was found to be responsible for the enhancement in antidiabetic property of fenugreek sprouts. The negative effects of the antagonists of calcium and nitric oxide indicate that the flux of these signalling molecules within the cellular system is essential during germination phases to regulate the mechanisms responsible for the biosynthesis of antidiabetic compounds. The presence of trigonelline as a major alkaloid component was further confirmed by IR and NMR analysis. Thus, this knowledge can be used to design the priming based sprouting techniques which might have potential application in improving the nutraceutical quality of legume sprouts.