In-Silico and In-Vitro Evaluation of the Anti-Diabetic Activity of Gmelinol

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Abstract

Purpose

The present study was investigated the anti-diabetic activity of Gmelinol using the in-silico molecular docking and in-vitro study.

Methods

In-silico study was performed using TriposSybyl-X 2.0 (TriposInc, St Louis, MO, USA) software and the evaluation was conducted based on the total score and visualization. In-vitro study was performed using the α-amylase inhibitory assay and α-glucosidase inhibitory assay and measured the percentage inhibition of the enzyme. The glucose uptake assay was also performed using L6 myoblast cell and measured the glucose utilization.

Results

Molecular docking studies demonstrated that Gmelinol was able to bind the active sites of proteins and the total score of Gmelinol with α-amylase and α-glucosidase were found 6.33 and 5.04 respectively. Gmelinol showed significant α-amylase and α-glucosidase inhibitory activities (IC$_{50}$ = 45.92 µg/mL on α-amylase and IC$_{50}$ = 49.25 µg/mL on α-glucosidase) compare with Acarbose (IC$_{50}$ = 70.63 µg/mL α-amylase and IC$_{50}$ = 64.82 µg/mL on α-glucosidase). Gmelinol showed significant glucose utilization activities in the L6 myoblast cell.

Conclusion

Gmelinol has shown anti-diabetic activity in terms of reducing the hyperglycaemia through the increased glucose utilization and inhibition of enzyme involved in carbohydrate metabolism namely α-amylase and α-glucosidase.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder, which is characterized by hyperglycemia (an elevated level of blood glucose), carbohydrate, protein and fat metabolic disturbance. It causes by failure of insulin secretion or action or both. (1) Type-2 diabetes is more important than type-1 diabetes because it is considered a preventable disease. Type-2 diabetes is brought about by an imbalance between glucose ingestion and insulin discharge. Controlling blood sugar levels is the basic way for preventing type-2 diabetes.(2)
World Health Organization (WHO) estimates that in 2019, diabetes was the 9th leading cause of death with an estimated 1.5 million deaths directly caused by diabetes occurred before the age of 70 years. More than 400 million people live with diabetes. This number will probably double by 2030. Although diabetes is more prevalent in developed countries, it is likely that the developing world will bear the brunt of epidemic in the future. (3)

Prevention of diabetes through healthy lifestyles (e.g., healthy diet, physical activities, weight reduction, etc.) is better than a cure. To date, diabetes has no cure, and the common oral hypoglycaemic synthetic drugs currently used in its management are accompanied by absolute side effects. (4) Researchers are now in search of alternative and complementary drugs to give continuing solutions to this metabolic disease, with limited side effects. (5) Many herbal remedies are effective anti-hyperglycemic agents and they are also a very important component of the health care. (6) In one of their offers, the WHO supported the assessment of medicinal plants based on their viability, low cost and possession of very few adverse effects. (7) However, not enough studies have been done to comprehensively evaluate the dosage, safety, and anti-hyperglycemic potentials of most of the medicinal plants. Therefore, there is a need to scientifically evaluate their toxicities and antidiabetic potentials through in vivo and/or in vitro studies before exposure to animal and human.

In present study, we selected lignan namely, Gmelinol which is present in the some plants part like heartwood of Gmelina arborea. (8)

The total lignan from the plant has been proven to be a safer antidiabetic agent and might help to prevent diabetic complications. The consumption of lignin containing seeds produced moderate reduction in glucose levels and hyperlipidemia, together with improvement in the impaired organs’ function. The daily administration of polyphenols and lignans compounds could impact therapeutic potential in diabetes management. (9, 10) Based on the literature, different extract of various part of plant of G. arborea possess various pharmacological activities including antidiuretic, antipyretic, antianalgesic, antioxidant, antidiabetic, antihelminthic, antibacterial, antifungal, cardioprotective, antiulcer, anticancer, antihyperlipidemic and immunomodulatory activity etc.. (11) However its active lignan Gmelinol has not been evaluated for anti-diabetic activity till date. Thus, very first aim will be to evaluate Gmelinol in-silico and in-vitro anti-diabetic activity.

The molecular docking is expected the structure of the ligand-receptor complex using computational approaches. Docking can be performed in two interdependent steps: first is by sampling conformations of the ligand in the active site of the protein and second is by rating of prepared conformations using a scoring function. (12)

Alpha amylase is a one of the most important enzymes in the digestive process found in the saliva and pancreatic juice. Alpha amylase activates the process of carbohydrate digestion by hydrolysis of polysaccharides (starch) to disaccharides (maltose), which leads to prevent high postprandial blood glucose. Alpha glucosidase is also one of the essential enzymes of the digestion and secreted from the intestinal chorionic epithelium is responsible for the degradation of complex carbohydrates. Its inhibition
delays the process of digestion, absorption of carbohydrates by blocking of glycosidase. Hence, inhibitors of α-amylase and α-glucosidase are beneficial in the control of hyperglycaemia as they delay carbohydrate digestion, which consequently reduce the postprandial plasma glucose level, which may probably suppress diabetes progression.(13, 14)

The liver and skeletal muscle are major tissues responsible for the maintenance of the body’s glucose homeostasis.(15) Glucose is the main energy source for cells, there has been extensive research on its actions as a cellular metabolite and on the mechanisms controlling its accumulation in tissue. Glucose uptake transport systems are abundant in animal cells and are responsible for transporting glucose across cell surface membranes. Evaluation of glucose uptake is crucial in the study of numerous diseases and metabolic disorders such as myocardial ischemia, diabetes mellitus, and cancer.(16)

This present research was planned for pilot based to determine the anti-diabetic activity of Gmelinol by in-silico docking model and in-vitro α-amylase inhibitory assay, α-glucosidase inhibitory assay and glucose uptake assay. The binding affinity of the Gmelinol with selected protein was measured from the docking study. The inhibition of enzyme was obtained from the in vitro α-amylase inhibitory assay and α-glucosidase inhibitory assay and the glucose consumption was measured from the glucose uptake assays in L6 myoblast cell. The purpose of these studies was to minimize avoidable discomfort to experimental animals.

Methodology

Material and Equipment

In-silico Docking Study

Ligand Preparation: Gmelinol was downloaded from Pubchem in sdf format including 3D coordinates. It was converted to a .pdb file format using the OpenBable2.3.2 application. The file was opened in TriposSybyl-X 2.0 (TriposInc, St Louis, MO, USA). Then after added hydrogen and Gasteiger–Hückel charges were assigned to atoms. To optimize the structure, energy minimization was performed with the standard tripos force field and maximum iterations used was 5000 and energy change kept 0.5 kcal/ (mol*A).

Protein Preparation: The three-dimensional crystal structures of protein α-amylase (PDB: 1C8Q) was obtained from Research collaborator for structure bioinformatics (RCSB) in protein data bank (pdb) format. All the proteins were pre-processed by deleting inhibitor and water molecules. Then after added hydrogen AMBER7FF99 charges were assigned to atom. Minimization of protein was done by using Powell method with gradient termination. Maximum iterations used was 5000 and energy change kept 0.5kcal/ (mol*A).

Molecular Docking Analysis: All molecules were docked into the active site of enzymes using SurflexDock module of TriposSybyl X 2.0 software package.(17) Ligand binding site called protomol was
constructed by removing the attached ligand in crystal structure of protein. Docking was performed using default Surflex-Dock parameters. Binding affinity of each molecule were calculated by total score value. H-bonding interactions of molecule were taken in to consideration for its binding to the protein.

**In-vitro Study:**

**α-amylase Inhibition Assay** (18)

The study was carried out according to described by Quangin Fei et al., et al. Five different concentrations ranging from 20, 40, 60, 80 and 100 µg/mL in DMSO solutions were prepared for the Gmelinol and Acarbose (standard) and mixed with 0.25 mL of α-amylase solution (0.02 mol/L, pH 6.8) and incubated at 37°C for 5 min. The reaction was started by the addition of 0.5 mL 1% (w/v) starch substrate solution to the incubation medium. Reaction was ended by adding 0.5 mL of DNS reagent (1% dinitrosalicylic acid, 0.05% Na₂SO₃ and 1% NaOH solution) to the reaction mixture boiled at 100°C for 5 min. After cooling the solution at room temperature, absorbance was taken at 540 nm by UV-spectroscopy. Acarbose was used as standard drug (positive control). Reaction mixture without the sample served as negative control and reaction mixture without the substrate served as blank. The result of triplicate determinations of α-amylase inhibitory activity was performed and expressed as percentage inhibition by the below mentioned equation:

\[
\%\text{inhibition activity of } \alpha - \text{amylase} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100
\]

From the standard calibration curve (absorbance against concentration), IC₅₀ was calculated from the liner equation of the plotted graph.

**α-glucosidase Inhibition Assay** (19)

The study was carried out according to described by Ju-Sung et al. Five different concentrations ranging from the 20, 40, 60, 80 and 100 µg/mL in DMSO solution were prepared for the Gmelinol and Acarbose (standard) and incubated with α-glucosidase solution (50 µl, 0.5 Units/mL) and 0.2M potassium phosphate buffer (1500 µl, pH 6.8) at 37°C in a water bath for 15 min. Then, 250 µl of 3mM p-nitrophenyl glucopyranoside (PNPG) was added as substrate. The reaction was incubated again for 10 min and then stopped by the addition of 750 µl of 0.1M Na₂CO₃. The absorption of 4-nitrophenol, a product after the reaction was taken at 405 nm by UV-spectroscopy. Acarbose was used as standard drug (positive control). Reaction mixture without the sample served as negative control and reaction mixture without the substrate served as blank. The result of triplicate determinations of α-amylase inhibitory activity was performed and expressed as percentage inhibition by the below mentioned equation:

\[
\%\text{inhibition activity of } \alpha - \text{glucosidase} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100
\]
From the standard calibration curve (absorbance against concentration), IC$_{50}$ was calculated from the linear equation of the plotted graph.

**Glucose uptake assay**

L6 is a myoblast cell isolated from the skeletal muscle from a rat and it was obtained from National Repository of Animal Cell Culture, National Centre for Cell Sciences (NCCS), Pune, India. The glucose utilization in L6 myoblasts cells was determined according to the methods described by Van de Venter et al. (20)

**Cell Cultivation and Seeding**

The L6 cells were cultivated in DMEM growth medium supplemented with 10% foetal calf. The cells were grown in incubator at 37°C and 5% CO$_2$. Confluent cells (70–80%) in Corning flasks were detached using a trypsin solution followed by removing the trypsin. The cells were re-suspended in DMEM growth medium and counted using a hemacytometer. Cell densities were adjusted to $1 \times 10^5$ cells/ml. The suspension of cells (1000 µl) was seeded into a pre-labelled 24 well culture plate. Three set for replica for each concentration of compound used were made. The tubes were incubated at 37°C and 5% CO$_2$ for 24 hours.

**Assay Procedure**

The cells were allowed to adhere until 90% confluence was reached. Two cell-free wells were also included to serve as blanks for glucose utilization assay. After 90% confluence, the culture medium was removed and replaced with DMEM containing 2% FBS and cultured for additional five days. Fourty-eight hours (48 hrs) prior to the glucose utilization assay, the culture medium was replaced and five different concentrations ranging from 3.125, 6.25, 12.5, 25 and 50 µg/mL in DMSO solutions were prepared for the Gmelinol (test) and added to separate labelled wells. Three different concentrations ranging from 12.5, 25 and 50 µg/mL in DSMO solutions were prepared for the Metformin and Insulin and added to separate labelled wells. After 48 hrs of exposure, glucose solution was added in all wells to set total concentration 100 mg/dl, shake carefully, and put in incubator for 12 hrs. Every hour 5 µl media was collected for glucose testing (Autospan® Glucose kit, GOD POD end point assay from Arkray, India). The amount of glucose utilized was calculated as the difference between the glucose standard 100 mg/dl and cell-containing wells. The percentage of glucose uptake was calculated.

**Statistical Analysis**

All the presented data were expressed as Mean ± SEM for three determinations. The presented data were analysed for statistical significant using the GraphPad Prism 8.02.

**Results**

**Molecular Docking**
The molecular docking was performed between the Gmelinol and Acarbose with the protein targets such as α-amylase and α-glucosidase. The binding of Gmelinol and Acarbose with the α-amylase and α-glucosidase during the docking were given in Fig. 1 and Fig. 2. During the docking, the docking score of Acarbose and Gmelinol for binding with α-amylase were 5.10 and 6.33 respectively and binding score with α-glucosidase were 8.04 and 5.04 respectively. Docking result shows that the Gmelinol and Acarbose binding affinities with target proteins and its interacting residues with docked ligand were presented in Table 1.

### Table 1
Docking Results with Interacting Residues

<table>
<thead>
<tr>
<th>Targeted Protein</th>
<th>Ligand</th>
<th>Total Docking Score</th>
<th>Interacting residue with docking ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-amylase</td>
<td>Acarbose</td>
<td>5.10</td>
<td>LYS227, TYR2, ILE230, GLY249</td>
</tr>
<tr>
<td></td>
<td>(Standard)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gmelinol</td>
<td>6.33</td>
<td>TYR2, LYS208</td>
</tr>
<tr>
<td>α-glucosidase</td>
<td>Acarbose</td>
<td>8.04</td>
<td>ASN470, GLU471, PRO475, GLU530, ASP531, CYS533, ASN535</td>
</tr>
<tr>
<td></td>
<td>(Standard)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gmelinol</td>
<td>5.04</td>
<td>TRP618, ARG600, TRP481, ASP282, ALA284</td>
</tr>
</tbody>
</table>

### α-amylase Inhibition Assay

Gmelinol and Acarbose were showed good % inhibition of α-amylase enzyme with increasing order of concentration. The inhibitory effects of Gmelinol and Acarbose on α-amylase are presented in Table 2. The maximum concentration of Gmelinol was reported 75.72% (0.58) inhibition (SEM) when compared to Acarbose was reported 61.81% (0.22) inhibition (SEM). The half maximumal inhibitory concentration (IC$_{50}$) of Gmelinol (70.63) and Acarbose (45.92) was calculated from the liner equation obtained from the graphs and shown in Table 2. Even from the statistical analysis that the Gmelinol showed significantly (p < 0.05) higher inhibitory activity of α-amylase than the standard Acarbose. The graphical comparison of % inhibitory activity of Acarbose and Gmelinol on α-amylase are presented in Fig. 3.
### Table 2

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>% Inhibition of α-amylase</th>
<th>Acarbose</th>
<th>Gmelinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>26.93 ± 0.58</td>
<td>36.42 ± 1.01</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>38.63 ± 0.58</td>
<td>46.58 ± 0.58</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>45.47 ± 0.58</td>
<td>58.50 ± 1.17</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>54.53 ± 0.22</td>
<td>67.99 ± 1.17</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>61.81 ± 0.22</td>
<td>75.72 ± 0.58</td>
<td></td>
</tr>
<tr>
<td><strong>IC\textsubscript{50} (µg/mL)</strong></td>
<td></td>
<td><strong>70.63</strong></td>
<td><strong>45.92</strong></td>
</tr>
</tbody>
</table>

Values expressed as MEAN ± SEM, where n = 3

### α-glucosidase Inhibition Assay

Gmelinol and Acarbose were showed good % inhibition of α-glucosidase enzyme with increasing order of concentration. The inhibitory effects of Gmelinol and Acarbose on α-glucosidase are presented in Table 3. The maximum concentration of Gmelinol was reported 83.15% (0.36) inhibition (SEM) when compared to Acarbose was reported 65.35% (0.43) inhibition (SEM). The half maximumal inhibitory concentration (IC\textsubscript{50}) of Gmelinol (64.82) and Acarbose (49.25) was calculated from the liner equation obtained from the graphs and shown in Table 3. Even from the statistical analysis that the Gmelinol showed significantly (p < 0.05) higher inhibitory activity of α-glucosidase than the standard Acarbose. The graphical comparison of % inhibitory activity of Acarbose and Gmelinol on α-glucosidase are presented in Fig. 4.

### Table 3

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>% Inhibition of α-glucosidase</th>
<th>Acarbose</th>
<th>Gmelinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>23.54 ± 0.43</td>
<td>26.64 ± 0.67</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>33.57 ± 0.43</td>
<td>44.56 ± 0.84</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>51.25 ± 0.55</td>
<td>61.41 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>62.84 ± 0.63</td>
<td>72.28 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>65.35 ± 0.43</td>
<td>83.15 ± 0.36</td>
<td></td>
</tr>
<tr>
<td><strong>IC\textsubscript{50} (µg/mL)</strong></td>
<td></td>
<td><strong>64.82</strong></td>
<td><strong>49.25</strong></td>
</tr>
</tbody>
</table>

Values expressed as MEAN ± SEM, where n = 3
Glucose uptake assay

The results obtained for glucose uptake assay using the L6 myoblast are presented in Fig. 5. The result also showed concentration-dependent increases in glucose uptake in the Gmelinol, Metformin and Insulin treated cells. According to the results, the concentration (12.5 µg/mL) of Gmelinol was shown a significant (p < 0.05) increase in glucose uptake in L6 myoblast when compared to the untreated control (normal), Metformin and Insulin. The Gmelinol compared to the untreated treated controls, a significant (p < 0.05) dose-dependent increase in glucose uptake across all the concentrations and time manner (Fig. 5) but somewhat lesser glucose uptake than Metformin and Insulin. The maximum concentration (50 µg/mL) of Gmelinol (53.81%) was shown a significant (p < 0.05) increase in glucose uptake in L6 myoblast at all the concentration tested in a concentration-dependent manner when compared to the untreated control (normal) (4.54%) but somewhat lesser than Metformin (75.47%) and Insulin (81.49%).

Discussion

DM is a leading metabolic disorder in worldwide and India. DM is a situation which is caused by failure of insulin secretion or action or both, the body does not produce enough insulin or properly respond to insulin. Insulin is a hormone which is produced in β cells of the pancreas and it stimulates the body cells to absorb glucose from the blood. DM is one of the most common endocrine and metabolic disorder which cause various microvascular complications such as retinopathy, neuropathy and nephropathy, and macrovascular complications such as heart attack, stroke, and peripheral vascular diseases.(21)

Molecular docking was performed to understand the mechanism by which Gmelinol would inhibit the selected proteins (α-amylase and α-glucosidase enzymes). Total Score value of docked compound implies binding capacity of ligand (Gmelinol). The total docking score of Gmelinol (6.33) was higher with α-amylase and lower with α-glucosidase (5.04) when compared with Acarbose (5.10 and 8.04 respectively), but it also has good binding affinity (Fig. 1 and Fig. 2).

In general, in vitro antidiabetic assays help to assess the effects of compounds on the inhibition of the two key enzymes involved in carbohydrate metabolism, namely, α-amylase and α-glucosidase, which has been described by several publication.(22, 23)

Gmelinol was reported best inhibitory activity against α-amylase than standard drug Acarbose in a concentration dependent manner (Table 2). The calculated IC₅₀ value of Gmelinol from α-amylase assay was 45.92 µg/mL which was comparable with the IC₅₀ value of the standard drug Acarbose (70.63 µg/mL). Gmelinol was reported best inhibitory activity against α-glucosidase than standard drug Acarbose in a concentration dependent manner (Table 3). The calculated IC₅₀ value of Gmelinol from α-amylase assay was 49.25 µg/mL which was comparable with the IC₅₀ value of the standard drug Acarbose (64.82 µg/mL). In the present study, there was a dose-dependent, α-amylase and α-glucosidase inhibition were observed in Gmelinol. That the concentration was increased, inhibition of activity of α-
amylase and α-glucosidase were also significantly (p < 0.05) increased. By comparing with standard (Acarbose), Gmelinol produced good inhibition.

In the present study, we examined glucose uptake action of gmelnol in L6 myoblast cell; the results revealed that standard drug metformin and insulin significantly (p < 0.05) enhanced glucose uptake activity which was higher than Gmelinol. Gmelinol did not exhibit higher glucose uptake action like standard drug metformin and insulin. However, results confirmed that Gmelinol enhanced glucose uptake activity when compared to untreated control group and also Gmelinol produced dose-dependent glucose uptake action. (Fig. 5) The Gmelinol effectively prevents hyperglycemia and thereby it controls lipid metabolism.

Metformin lowers glucose, sensitizes insulin by reducing gluconeogenesis and opposing glucagon-mediated signalling in the liver and in a lesser extent by increasing glucose uptake in skeletal muscle. It exerts its hypoglycemic effect through activation of the AMP activated protein kinase (AMPK) in the liver. (24) Most abundant tissue in the whole body is skeletal muscle. Hence, proper function of skeletal tissue is important to maintain normal blood glucose level.(25, 26)

Insulin increases the glucose uptake in the skeletal muscle by increasing functional glucose transport molecules in the plasma membrane. Common pathological condition in non-insulin dependent diabetes mellitus is, the defect in insulin stimulated skeletal muscle glucose uptake.(27)

This data have given clear evidence that the selected Gmelinol possibly acts to suppress glucose release and improve the glucose uptake in the skeletal muscle. It could be a potential antidiabetic drug, which could improve hyperglycemia by enhancing glucose uptake.

**Conclusions**

The anti-diabetic activity of Gmelinol was evaluated by in-silico and in-vitro methods. Gmelinol reported a significant relationship in binding affinity towards α-amylase and α-glucosidase in an in-silico molecular docking study. Inhibitory activity against the α-amylase and α-glucosidase were confirmed by in-vitro α-amylase and α-glucosidase assay. Hence it was concluded that Gmelinol possesses significant anti-diabetic activity on laboratory scale. Further, studies need to be conducted to evaluate the anti-diabetic activity through in-vivo study on animal.

**Abbreviations**

°C: Celsius

CO₂: Carbon Dioxide

DM: Diabetes mellitus

DMEM: Dulbecco's Modified Eagle Medium
DMSO: Dimethyl sulfoxide

GOD: Glucose Oxidase

Hr: Hour

IC₅₀: Half-maximal inhibitory concentration

M: Mol

Min: Minutes

mL: Milliliter

Na₂SO₃: Sodium Sulphite

NaOH: Sodium Hydroxide

Nm: Nanometer

PDB: Protein Data Bank

PNPG: p-nitrophenyl glucopyranoside

POD: Peroxidase

RCSB: Research Collaborator for Structure Bioinformatics

SEM: Standard Error of the Mean

UV: Ultraviolet

WHO: World Health Organization

μg: Microgram

Declarations

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Conflict of Interest:
None
Acknowledgement:

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References


Figures
Figure 1

Molecular docking Interacting Residue with Ligand; a) interaction residue with Acarbose-α-amylase and b) interaction residue with Gmelinol-α-amylase

Figure 2
Molecular docking Interacting Residue with Ligand; c) interaction residue with Acarbose-α-glucosidase and d) interaction residue with Gmelinol-α-glucosidase

Figure 3

α-amylase inhibition assay

% of inhibition

Concentration (µg/mL)

Figure 3

α-amylase activity of Acarbose and Gmelinol
Figure 4

α-glucosidase Inhibition Assay of Acarbose and Gmelinol

Glucose Utilization in L6

- Normal
- Metformin 12.5
- Metformin 25
- Metformin 50
- Insulin 12.5
- Insulin 25
- Insulin 50
- Gmelinol 12.5
- Gmelinol 25
- Gmelinol 50
Figure 5

Glucose Utilization of Metformin, Insulin and Gmelinol in L6