Phytochemical screening and antidiabetic activity of Mangifera indica (Seed kernels) in streptozotocin-induced diabetic rats

Dharmendra Arya, Kiran Bhagour, Mahima Sharma and RS Gupta

Abstract
The study was designed to explore the phytochemical analysis of methanolic extract of Mangifera indica (MiMtE) and to evaluate its antidiabetic effect on streptozotocin-induced diabetic rats. Gas chromatography-mass spectrometric analysis was performed to identify the presence of different phytocompounds in MiMtE. For the assessment of antidiabetic activity, MiMtE was orally administrated to STZ (60 mg/kg b.wt) induced diabetic rats at the doses of 50 and 100 mg/kg b.wt for 21 days. The result showed MiMtE promoted a significant (p < 0.05) antidiabetic effects in diabetic rats through reducing the levels of blood glucose, glycosylated hemoglobin and also restored body weight, liver glycogen content and serum insulin level in diabetic rats in a dose-dependent manner. A significant (p < 0.05) improvement in serum lipid profile, hepatic and nephrotic markers after treatment with MiMtE, also signified its protective nature against long-term diabetic complications. Phytochemical screening of MiMtE indicates the presence of various phytocompounds, which might contribute to the antidiabetic activity. Therefore, it could be concluded that the MiMtE might be used safely as an adjunct therapy in the management of diabetes and its associated complications. Therefore, it could be used as a safer complimentary drug in the management of diabetes and associated complications.

Keywords: Mangifera indica; antidiabetic; phytocompounds; hepatic

1. Introduction
Diabetes mellitus (DM) is a metabolic syndrome characterized by hyperglycemia, hyperinsulinemia and hyperlipidemia resulting from defects in insulin secretion, insulin action or both (ADA, 2010). The incidence of DM has greatly increased, and the numbers of patients have risen to more than 422 million until now, and expected to reach this figure to 592 million in 2035. Currently, the global prevalence has been accounted for 8.5% among adults, which is rising more rapidly in middle and low-income countries (IDF, 2017) [2]. DM is caused due to impairment occurs in carbohydrate, lipid and protein metabolism resulting in either low insulin level in the blood or the insensitivity of target organs to insulin (Maiti et al., 2004) [3]. Long-term DM has been also associated with several types of complications such as neuropathy, nephropathy, retinopathy and coronary artery disease (Lyra et al., 2006) [4]. Although, several therapies are in use for treatment of diabetes include oral synthetic hypoglycemic agents and insulin therapy, but these treatments are certain limitations due to high cost and side effects viz. insulin resistance, anorexia nervosa, brain atrophy and fatty liver (Piedrola et al., 2001; Yaryura-Tobias et al., 2001) [5, 6]. Therefore, there is a need to explore new, safer and cost-effective alternatives.

Since ancient times, medicinal plants and their products have been practiced in our traditional medical system for the treatment of diabetes Recent scientific studies have also been explored the hidden wealth of medicinal plants (Valiathan, 1998) [7]. Mangifera indica, commonly known as mango, is a flowering plant species belongs to Anacardiaceae family, generally found all over the world. Several pharmacological properties including Immunomodulatory, hypolipidemic, analgesic and anti-inflammatory have been reported for M. indica (Makare et al., 2001; Anila and Vijayalakshmi, 2002; Garrido et al., 2001) [8, 9, 10]. Therefore, the present study was designed to perform phytochemical screening of methanolic seed kernel extract of M. indica (MiMtE) and evaluate its antidiabetic activity in STZ-induced diabetic rats.

2. Materials and Methods
2.1 Chemicals
Streptozotocin and glibenclamide were procured from Himedia Laboratories, Mumbai, India. All chemicals and reagents used were of analytical grade and procured from Sigma Chemicals Co., USA and HiMedia Laboratories, Mumbai, India.
2.2 Plant materials
The fresh seed kernels of *M. indica* were collected from the local market of Jaipur, Rajasthan and authenticated by the Department of Botany, University of Rajasthan, Jaipur, India. A voucher specimen (RUBL 211353) has been deposited at the Department of Botany, University of Rajasthan, Jaipur, India for future reference.

2.3 Preparation of plant extracts
The seed kernels of *M. indica* were shade dried and powdered in a mechanical grinder. The powdered material (2 kg) was extracted in 7 L methanol (MeOH) using a Soxhlet apparatus at an ambient temperature for 72 hr. Later, the extract was filtrated under vacuum, concentrated in a rotary evaporator and then lyophilized to yield 80 g brownish crude extract. The obtained extract was stored in the deep freezer for further use.

2.4 Gas chromatography-mass spectrometry (GC-MS) analysis of MiMtE
GC-MS analysis of MiMtE was performed by using Thermo GC-Trace Ultra-version 5.0 gas chromatography interfaced to Thermo MS DSQ II mass spectrometer instrument employing the following conditions: DB5-MS capillary standard non polar column (30 x 0.25 mm x 0.25 µm) and helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. The oven temperature was kept at 70°C and was programmed to reach 260°C at a rate of 6°C/min. Mass range was 50 to 650 (m/z). The total running time was completed within 43 min. The chromatogram obtained from gas chromatography was analyzed by mass spectrometry to get the mass of all fractions. The identification of phytochemical components was achieved through retention time and mass spectrometry by comparing the mass spectra of unknown peaks with those available in Wiley 9 GC-MS library.

2.5 Animals
Colony-bred, adult, male albino rats of ‘Wistar strain’ (190±10 g) were fed with conventional diets (commercial pelleted diet procured from Aashirwad Industries, Chandigarh, India) and water ad libitum and they were maintained under standard conditions of humidity (55±5%), temperature (25±3°C), and light (12-h light: 12-h dark cycle).

2.6 Acute toxicity studies
The acute toxicity study of MiMtE was performed as per guidelines of the OECD-423 received from the Committee for the Purpose of Control and Supervision of Experiments on Animals ( CPCSEA). The rats were divided into three groups and orally administered with MiMtE at increasing dose levels of 500, 1000 and 2000 mg/kg b.wt, respectively (Gosh, 1984). All animals were observed for gross behavioral, neurological, autonomic and toxic effects at short intervals of time for 5 hrs after administration and then for next 24 hrs. Food consumption and body weights were recorded daily for 7 days. On the 7th day, the animals were sacrificed and all the organs were removed for gross pathological examination (Turner, 1965).{[12].

2.7 Induction of hyperglycemia
Hyperglycemic condition was induced by a single intraperitoneal injection of STZ (60 mg/kg b.wt), freshly prepared in 0.1 M sodium citrate buffer and the animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. After 72 hrs of STZ administration, fasting blood glucose level was measured by Glucometer (one touch, Johnson & Johnson) and the rats with moderate diabetes, having hyperglycemia (blood glucose range of above 250 mg/dl) were considered as diabetic rats and employed in the antidiabetic study.

2.8 Experimental design
A total of thirty rats randomly divided into five groups containing six animals each. Group I served as Normal control rats received only distilled water; Group II served as Diabetic control rats received STZ (60 mg/kg bwt, i.p.); Group III and group IV served as treated groups received MiMtE at the dose of 50 and 100 mg/kg b.wt, respectively. Group V served as a positive control received glibenclamide (a reference standard drug, 10 mg/kg b.wt). All treatments were given orally after 72 hrs of STZ induction except normal control and the body weight of animals was recorded initially and at the end of the last dose whereas blood glucose levels were measured by Glucometer (one touch, Johnson & Johnson) at 1, 7, 14 and 21 day of the study. The percentage value of glycated hemoglobin (HbA1c) was evaluated on 21 day in blood using the method of Bannon 1982.[13].

2.9 Serum and tissue collection
At the end of treatments, the blood was collected from the heart via cardiac puncture and allowed to clot at room temperature, further centrifuged at 4°C at 3000 rpm for serum separation. The animals were sacrificed using mild anesthesia and liver was dissected and preserved in the deep freezer at -20ºC for the further biochemical analysis.

2.10 Serum analysis
The concentration of serum hepatic marker enzymes (ALT, AST and ALP), nephritic markers (serum creatinine, serum urea) and lipid profile (total cholesterol (TC), triglycerides (TG), LDL-C and HDL-C) were determined spectrophotometrically using commercially available kits (Accurex Biomedical Pvt. Ltd. Mumbai, India). The concentrations of serum insulin were assessed according to the standard protocol using radioimmunoassay (RIA).

2.11 Tissue biochemical analysis
Glycogen content was determined in the liver by the anthrone reagent method as adopted by Carrol et al., 1956. In brief, 500 mg of fresh liver tissue was homogenized in 1.5 ml of 5% potassium hydroxide and boiled for 30 min. Later, 5 mL of 95% ethanol was added to precipitate glycogen. After precipitation, test tubes were centrifuged and the supernatant decanted. Finally, the pellet was dissolved in anthrone reagent and change in the green color was measured at 620 NM.

2.12 Ethical aspects
The ethical committee, Center for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur (India), approved the study. The Indian National Sciences Academy, New Delhi (INSA, 2000), guidelines were followed for the maintenance of animals during the experiment.

2.13 Statistical analysis
Statistical analysis was performed using SPSS, version 21.0 (SPSS, Chicago, IL). All the results were expressed as mean ± SEM for six rats in each group, and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparison test. The values of p < 0.05 were considered as statistically significant.
3. Results
3.1 GC-MS analysis methanolic extract of Mangifera indica (seed kernels)

The chemical components present in the MiMIE were identified by GC-MS analysis (Fig. 1). The active compounds with their retention time (RT), molecular formula, and molecular weight (MW) are presented in Table 1.

**Table 1: Compounds identified in the methanolic extract of Mangifera indica in GC-MS.**

<table>
<thead>
<tr>
<th>Retention time</th>
<th>Compound Name</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
<th>Peak Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.86</td>
<td>Propanoic acid, 2-(aminooxy)-</td>
<td>C3H4NO</td>
<td>105.093</td>
<td>3.37</td>
</tr>
<tr>
<td>20.18</td>
<td>2H-Pyran, 2-(4 chlorobutoxy) tetrahydro-</td>
<td>C8H12ClO</td>
<td>192.683</td>
<td>2.74</td>
</tr>
<tr>
<td>21.82</td>
<td>Trimethyl [4(1,1,3,3,4,4,4,4tetramethylbutyl) phenoxyl] silane</td>
<td>C8H14O3Si</td>
<td>278</td>
<td>3.32</td>
</tr>
<tr>
<td>22.64</td>
<td>1H-Benzimidazole,2,2'-[thiobis(methylene)]bis-</td>
<td>C10H8N2S</td>
<td>294.376</td>
<td>2.17</td>
</tr>
<tr>
<td>23.05</td>
<td>N-Methoxy-N-methylacetamide</td>
<td>C9H12NO2</td>
<td>103.121</td>
<td>3.54</td>
</tr>
<tr>
<td>23.39</td>
<td>(2,2Dimethylhydradino) fluoroethylphosphine sulfide</td>
<td>C8H12F2N2PS</td>
<td>170.184</td>
<td>3.47</td>
</tr>
<tr>
<td>23.63</td>
<td>Bis(trimethylsilyl)1,4chloro-1,2cyclohexene1,2dicarboxylate</td>
<td>C14H16ClO2S2</td>
<td>348</td>
<td>3.15</td>
</tr>
<tr>
<td>23.71</td>
<td>phenacyle 2-methoxybenzoate</td>
<td>C13H16O2</td>
<td>270.284</td>
<td>2.83</td>
</tr>
<tr>
<td>23.76</td>
<td>3,6-Bis(trimethylsilyl)-1,4-cyclohexadiene</td>
<td>C14H20Si2</td>
<td>224.494</td>
<td>2.43</td>
</tr>
<tr>
<td>23.88</td>
<td>2-(1-hydroxyhexyloxy)butanoic acid</td>
<td>C12H14O3</td>
<td>186.251</td>
<td>3.81</td>
</tr>
<tr>
<td>23.94</td>
<td>3-(4-methoxyphenyl)-7-methylchromen-4-one</td>
<td>C15H14O3</td>
<td>266.296</td>
<td>1.38</td>
</tr>
</tbody>
</table>

**Fig 1: Chromatogram of methanolic extract of Mangifera indica (Seed kernels) by GC-MS**

3.2 Acute Toxicity

In acute toxicity studies, oral administration of MiMIE at the dose of 500, 1000 and 2000 mg/kg b.wt did not induce any changes in behavior and no mortality was observed during the study. There was no significant difference in the body weight and food consumption when compared to the vehicle-treated group. On the 7th day, macroscopic pathology observations revealed no visible lesions in any animals after sacrifice. Therefore, MiMIE can be safely used as a dose up to 2000 mg/kg b.wt for therapeutic use.

3.3 Effect of MiMIE on body weight, liver glycogen content and glycosylated hemoglobin

The Antidiabetic activity of MiMIE was observed through determination of body weight, liver glycogen content and glycosylated hemoglobin in normal and experimental rats. Table 2 shows that there was a significant (p<0.05) decrease in body weight (30%), liver glycogen content (70.07%) and an elevation in glycosylated hemoglobin (6.55%) in STZ-induced diabetic rats as compared to normal control rats. Oral administration of MiMIE significantly (p<0.05) increased the body weight, liver glycogen content and decreased the glycosylated hemoglobin in diabetic rats as compared to diabetic control rats.

**Table 2: Effect of oral administration of MiMIE and glibenclamide on body weight, glycosylated hemoglobin and liver glycogen content in normal and streptozotocin-induced diabetic rats (Duration-21 days).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Glycosylated hemoglobin (%)</th>
<th>Liver glycogen (mg/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Group-I Normal control</td>
<td>199±6.54</td>
<td>202±6.76</td>
<td>5.7±1.11</td>
</tr>
<tr>
<td>Group-II Diabetic control</td>
<td>186±5.94</td>
<td>140±4.32</td>
<td>12.12±1.07</td>
</tr>
<tr>
<td>Group-III Diabetic + MiMIE (50 mg/kg bwt/day)</td>
<td>195±6.00</td>
<td>167±5.47</td>
<td>9.52±2.03</td>
</tr>
<tr>
<td>Group-IV Diabetic + MiMIE (100 mg/kg bwt/day)</td>
<td>183±6.16</td>
<td>175±6.18</td>
<td>7.09±1.84</td>
</tr>
<tr>
<td>Group-V Diabetic + Gliben. (10 mg/kg bwt/day)</td>
<td>210±5.55</td>
<td>183±3.12</td>
<td>7.98±1.92</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM (n=6).

* = (p < 0.05); ** = (p<0.01) statistically significant difference when compared with Normal control.

* = (p < 0.05); ** = (p < 0.01) statistically significant difference when compared with Diabetic control.
3.4 Effect of MiMtE on blood glucose level

Fig. 2 shows the mean value of blood glucose level in diabetic and normal rats. There was a dramatically increased (p<0.05) in the level of blood glucose after STZ induction as compared to normal controls. Following MiMtE administration at the dose of 50 and 100 mg/kg b.wt to diabetic rats caused a significant reduction in the level of fasting blood glucose (from 329 to 192 and 176 mg/dL, respectively) when compared to diabetic control rats (Fig. 2). In a similar way, glibenclamide lowered the level of blood glucose up to 164 mg/dl, signified that MiMtE has a potent hypoglycemic activity.

![Fig 2: Effect of MiMtE on blood glucose level in STZ-induced diabetic rats. Data represented as mean ± SEM (n=6). *= (p < 0.05); **= (p<0.01) statistically significant difference when compared with Normal control. a = (p < 0.05); b = (p < 0.01) statistically significant difference when compared with Diabetic control.](image)

3.5 Effect of MiMtE on serum insulin level

As depicted in Fig. 3, STZ administration caused a massive reduction in serum insulin level (63.69%) in rats when compared with normal control rats. Oral administration of MiMtE and glibenclamide brought the serum insulin level back to the normal range (43.48 and 78.82% at the doses of 50 and 100 mg/kg b.wt, respectively).

![Fig 3: Effect of MiMtE on serum insulin level in STZ-induced diabetic rats. Data represented as mean ± SEM (n=6). *= (p < 0.05); **= (p<0.01) statistically significant difference when compared with Normal control. a = (p < 0.05); b = (p < 0.01) statistically significant difference when compared with Diabetic control.](image)

3.6 Effect of MiMtE on serum lipid profile

In case of serum lipid profile, STZ administration caused a significant (p<0.05) elevation in the level of total cholesterol, triglycerides and LDL-C while HDL-C level was declined in diabetic rats as compared to normal control. Administration of MiMtE brought back the levels of serum lipids to near normal values, signified the hypolipidemic activity of MiMtE (Fig. 4).

![Fig 4: Effect of MiMtE on serum lipid profile in STZ-induced diabetic rats. Data represented as mean ± SEM (n=6). *= (p < 0.05); **= (p<0.01) statistically significant difference when compared with Normal control. a = (p < 0.05); b = (p < 0.01) statistically significant difference when compared with Diabetic control.](image)
administration to normoglycemic rats caused a significant elevation (p<0.05) in the level of AST, ALT, ALP, serum urea and creatinine. However, administration of MiMtE to diabetic rats reduced the level of hepatic and nephrotic marker in a significant manner (p<0.05) (Table 3).

Table 3: Effect oral administration of MiMtE and glibenclamide on AST, ALT, ALP, Serum urea and Serum creatinine in normal and streptozotocin-induced diabetic rats (Duration- 21 days).

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/dl)</th>
<th>ALT (U/dl)</th>
<th>ALP (U/dl)</th>
<th>Serum urea (mg/dl)</th>
<th>Serum creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I Normal control</td>
<td>43.69±3.01</td>
<td>28.57±5.44</td>
<td>57.01±3.36</td>
<td>23.99±1.67</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>Group-II Diabetic control</td>
<td>76.11±1.55</td>
<td>49.11±2.69</td>
<td>79.22±7.14</td>
<td>72.58±2.18</td>
<td>1.15±0.05</td>
</tr>
<tr>
<td>Group-III Diabetic + MiMtE (50 mg/kg bwt/day)</td>
<td>62.09±3.32</td>
<td>39.10±5.05</td>
<td>72.17±3.21</td>
<td>47.62±1.90</td>
<td>0.84±0.01</td>
</tr>
<tr>
<td>Group-IV Diabetic + MiMtE (100 mg/kg bwt/day)</td>
<td>56.17±2.86</td>
<td>31.11±3.71</td>
<td>65.69±4.05</td>
<td>33.81±2.29</td>
<td>0.69±0.017</td>
</tr>
<tr>
<td>Group-V Diabetic + Gliben. (5 mg/kg bwt/day)</td>
<td>59.11±2.08</td>
<td>34.68±6.00</td>
<td>61.76±3.10</td>
<td>33.85±2.55</td>
<td>0.60±0.013</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM (n=6).
* = (p < 0.05); ** = (p<0.01) statistically significant difference when compared with Normal control. 
* = (p < 0.05); b = (p < 0.01) statistically significant difference when compared with Diabetic control.

4. Discussion

Currently available treatments for the management of diabetes have a limited efficacy and associated with certain side effects. Therefore, there is a need to find a safer alternative antidiabetic drug. In the present investigations, the phytochemical analysis and Antidiabetic activity of MiMtE was screened in STZ-induced diabetic rats. STZ-induced diabetic rat model was used in this study, as the STZ causes a massive destruction of beta cells of the pancreas, reduces insulin availability to cells, resulting in hyperglycemia (Rakieten, 1963) [15]. A reduction in blood glucose level after MiMtE treatment indicates that the drug has insulin secretion and beta cell regeneration property. In diabetic rats, reduction in body weight changes observed might be the result of degradation of structural proteins due to deficiency of carbohydrate for the energy metabolism (Pepato et al., 1996) [16]. A significant increase in body weight of diabetic rats treated with MiMtE showed the blood glucose stabilization effect which in turn prevents the loss of body weight.

A marked increase in HbA1c was observed in STZ-induced diabetic rats and it is in accordance with hyperglycemia. High level of blood glucose increases the affinity of glucose molecules to bind to hemoglobin in RBC's lead to an elevated level of HbA1c (Sampson et al., 2002) [17]. MiMtE treatment showed a significant reduction in HbA1c via interfering with glucose metabolism.

STZ caused a reduction in the serum insulin level and it might due to the destruction of the beta cells of the endocrine pancreas. The STZ administration selectively destroys the pancreatic cells and induce hyperglycemia (Gilman, 1990; Kurup and Bhonde, 2000) [19]. Similar observations were found earlier in the STZ treated rats, the levels of serum insulin significantly reduced (Gupta and Gupta, 2011) [20]. The elevation in serum insulin level in MiMtE treated diabetic rats indicates that the drug has insulin secretion and beta cell regeneration property.

STZ administration produced a significant rise in hepatic marker enzymes such as AST, ALT and ALP levels in diabetic rats that may be due to exposure of these enzymes from the liver cytosol into the bloodstream and it may be induced due to liver dysfunction (Navarro et al., 1993; Ghosh and Suryawansi, 2001) [21, 22]. Administration of MiMtE revealed its protective nature on liver tissue by reducing the
elevated levels of AST, ALT and ALP. Therefore, MimiE could be a better hepatoprotective agent in diabetes-associated complications.

A marked increase in the concentration of serum urea and creatinine was observed in STZ-induced diabetic rats. Previous investigations have been reported a significant increase in the rate of kidney cell damage (nephropathy) in diabetes (Ahmed and Osman, 2006) [23]. Finally, this nephropathy reduces the physiological function and changes in the structure of kidney in diabetes (Rosolowsky et al., 2008) [24]. Hyperglycemia increases the generation of free radicals by glucose auto-oxidation and the increment of free radicals may lead to kidney cell damage (Sharma et al., 2006) [25]. These changes lead to impairment in the physiological function of the kidney as well as an increase in serum urea and creatinine levels (Mariee et al., 2009) [26].

The long-term diabetes caused an increase in TG, TC and LDL-C and decrease in HDL-C levels (Al-Shamaony et al., 1994) [27]. The hyperlipidemic condition occurs in diabetes is might be due to insulin deficiency that inactivates the lipoprotein lipase promoting liver conversion of free fatty acids into phospholipids and cholesterol, which get discharged into the blood resulting in elevated serum lipid level (Pari and Latha, 2002; Bopanna et al., 1997) [28, 29].

Previous studies have been also reported an elevation in TC, TG and LDL-c levels and reduction in HDL-C levels in diabetic models (Howard et al., 2000) [30]. MimiE treatment significantly restored TC, TG, LDL-C and HDL-C to their normal values in diabetic rats and it may be due to its lipid-lowering activity, signified its hypolipidemic potential in diabetes.

In diabetes, the excessive blood glucose concentration is generally accompanied with a reduced level of hepatic glycogen content, suggesting that there is a deficit in the accumulation or retention of glycogen in the liver. STZ causes selective damage to pancreatic β-cells, declined insulin level leads to diminished glycogen synthesis (Whitton and Hems, 1975; Rossetti and Giaccari, 1990) [32, 32]. MimiE administration restored the hepatic glycogen content and it might be a result of its insulin secretory activity.

5. Conclusion
In conclusion, the present study provides an evidence of the anti-diabetic activity of MimiE in STZ-induced diabetic rats. It is also inferred that the MimiE possesses various phytochemicals that may be responsible for their hypoglycemic property. Also, the MimiE treatment showed an improvement in the parameters of hepatic, nephrotic and lipid profile. Thus, it can be useful in the treatment of hepatic, nephrotic dysfunction and dyslipidemia, associated with diabetes. Therefore, it could be concluded that the methanolic seed kernels extract of Mangifera indica might be used safely as an adjunct therapy in the management of diabetes and its associated complications. Further comprehensive pharmacological investigations are needed to elucidate the exact mechanism of action of the antidiabetic effect of methanolic seed kernels extracts of Mangifera indica.

6. Conflict of interest statement
The authors declare that there are no conflicts of interest.

7. Acknowledgment
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