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Antidiabetic Activity of Green Gold-Silver Nanocomposite with *Trigonella foenum graecum* L. Seeds Extract on Streptozotocin-Induced Diabetic Rats

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ABSTRACT

The present study investigated the green synthesis of gold and silver (Au/Ag) nanocomposite using aqueous *Trigonella foenum-graecum* (fenugreek) seed extract and to evaluate the anti-diabetic activity of the prepared nanopcomposite. Antidiabetic potential of orally administered bulk aqueous extract of *Trigonella* (TBE) and Au/Ag nanocomposite with *Trigonella* seed extract (TN) on streptozotocin (70 mg/kg) induced diabetic rats was assessed for a period of 7 weeks. Metformin was used as a standard drug. The results of Zetasizer and Transmission and Scanning Electron Microscopy images showed the presence of spherical to irregularly shaped green gold and silver (Au/Ag) nanopcomposite with an average size of 73.18 nm with polydispersity. A typical optical absorption peak at approximately 2.30 keV and 3.0 keV corresponded to the surface plasmon resonance property of gold and silver nanoparticles respectively. The green gold and silver (Au/Ag) nanocomposite showed a profound antihyperglycemic effect which was significantly better than all other treated groups. The results on serum ALT, creatinine levels and blood urea also showed a comparable ameliorative effect of both the bulk *Trigonella* seed extract (TBN) and the green nanocomposite (TN), being more efficacious than metformin. Thus, the green synthesis and application of the Au/Ag nanoparticles offer a novel approach in nanomedicine for diabetes management.

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Green synthesis, *Trigonella foenum graecum*, Diabetes, Gold/Silver, Nanocomposite.

INTRODUCTION

Diabetes mellitus – Type 2 (DM-2) is a major chronic metabolic disease afflicting a large proportion of the population worldwide. Diabetes is an endocrine disorder characterized by hyperglycemia and dyslipidemia. It is also recognized for associated complications such as diabetic nephropathy, neuropathy, and retinopathy (Xue *et al*., 2007) and hepatic damage (Harris, 2005). These complications have been attributed to the morbidity and mortality in DM. The WHO recognizes DM as a growing global epidemic which could be a major cause of disease and disability in the next quarter of the century. Worldwide around 230 million people have been affected by diabetes and the number are expected to reach around 366 million by 2030 (Rahiman and Tantry, 2012).The management of DM-2 thus is a challenge both for the patients and the medical fraternity.

The conventional therapy includes a lifestyle management, nutritional intervention and pharmacological management. An alternative approach to the management strategy of the disease has been the use of medicinal herbs. Ethanopharmacological surveys indicate that more than 1200 plants are used in traditional medicine for their allied hypoglycemic activity (Kesari *et al*., 2007). Fenugreek, *Trigonella foenum-graecum* L. (Leguminosae), is a medicinal plant of Mediterranean origin, used by ancient Egyptians and cultivated worldwide. Its seeds are used as condiment for seasoning in food preparations; is assumed to possess nutritive and restorative properties (Petit *et al*., 1993) and has been used in folk medicine for centuries for a wide range of diseases including diabetes (Renuka *et al*., 2009). Previous studies have reported the hypoglycemic and hypocholesterolemic effects of *Trigonella foenum-graecum* seeds on type 1 and type 2 diabetes mellitus patients and experimental diabetic animals (Xue *et al*., 2007; Renuka *et al*., 2009; Laila and Murtaza, 2015).

The development of engineered nanoparticles, is a fast growing industry with great potential and applications in many biomedical fields, including nanomedicine. Nanomaterials have unique properties and applications that can be used judiciously in drug delivery and imaging, which improves the diagnostics and therapy for many human diseases. Use of metal nanoparticles is a new era in diabetes management. Both gold and silver nanoparticles have been evaluated for their antidiabetic potential and

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have shown promising results (Rahiman and Tantry, 2012; Alkaladi *et al*., 2014). The present study was designed to evaluate a novel therapeutic approach towards diabetes using a combination of gold and silver nanoparticles with a natural ingredient, *Trigonella* seed extract.

MATERIALS AND METHODS

Chemicals

All chemicals and drugs were obtained commercially and were of analytical grade. Streptozotocin was used to induce diabetes (Sigma, U.S.A.). Metformin was the synthetic drug used (Merck Serono Middle East). Silver nitrate from Techno Pharmchem (India), Chloroauric acid from Loba Chemie (India).

Plant material and aqueous extract

Trigonella foenum-graecum (fenugreek) seeds were purchased from the local market, and cleaned. The seeds were identified by the Department of Botany at King Saud University, Riyadh.

Dried fenugreek seeds (4g) were washed well and soaked overnight in 200 mL of boiled distilled water. The extract was centrifuged for 7 min at 8000 rpm at room temperature and filtered again when used for preparation of the nanoparticles.

Synthesis of silver and gold nanoparticles

Synthesis of green silver nanoparticles: 50 ml of distilled water was used to dissolve 0.001M silver nitrate under vigorous stirring at 75°C for 9 min which gave a colorless solution. To this 6 ml of *T. foenum-graecum* extract was added which changed the colour of the solution to brown, which confirmed the reduction of Ag ions and the formation of green silver nanoparticles. This was followed by the synthesis of green gold nanoparticles. Chloroauric acid (0.0009M) was dissolved in 50 ml distilled water under vigorous stirring at 75°C for 9 min and a light yellow color colloidal solution was formed. To this colloidal solution, 6 ml of *T. foenum-graecum* extract was added and the color changed to red, confirming the reduction of Au^{3+} ions and the formation of green gold nanoparticles. These color changes in reaction mixtures were noted by visual observation. To finally synthesize the green gold/silver (Au/Ag) nanocomposite, the colloidal solutions which had been prepared were mixed together to obtain the final solution which contained silver and gold nanoparticles.

Characterization of green gold-silver nanocomposite

The samples were dried at room temperature and then analyzed for sample composition of the synthesized nanoparticles. Elemental analysis on single particles was carried out. The green nanoparticles (Au/Ag) were characterized using Zetasizer, Nano series, HT Laser, ZEN3600 (Molvern Instrument, UK). The characterization included the measurement of the average size of green Au /Ag nanocomposite. Transmission electron microscopy (TEM) (JEM-1011, JEOL, Japan) was employed to characterize the size, shape and morphology of biosynthesized silver/gold nanocomposite. Scanning electron microscopy (SEM) was employed to characterize the shape and morphologies of formed bio synthesized nanoparticles using JEOL-FE SEM (Japan) and Energy Dispersive Spectrometer (EDS) analysis was performed for the confirmation of elemental silver and gold.

Experimental animals

Male Wistar rats weighing 200-245 g were used for the study. The animals were fed with standard laboratory chow and had free access to water under well ventilated conditions of 12 h day and 12 h dark cycles. The animals were acclimatized to laboratory conditions prior to the experiment. All animal procedures were performed in accordance with the standards set forth in the Guidelines for the Care and Use of Experimental Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) (http://icmr.nic.in/bioethics/final_cpcsea.pdf). The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University.

Induction of diabetes

The rats were made to fast 12 h before the induction of diabetes. Thereafter, they were injected with streptozotocin (70 mg/kg, i.p.). Five days after injection, the rats with fasting blood glucose levels higher than 200 mg/dl were considered diabetic and used for the experiment. Feeding was stopped 12 h before blood sampling.

Experimental design

The experimental period was eight weeks. The first 7 days were for the induction of diabetes in rats and the following seven weeks were the investigational period with crude aqueous extract of *Trigonella* (fenugreek) seeds and Au/Ag nanocomposite with the fenugreek seed extract which were administered orally and separately. There were five groups of five rats each: Group 1, Normal saline treated rats (Normal control-NC); Group 2, Normal saline treated diabetic rats (Diabetic Control-DC); Group 3, Metformin (600 mg/kg body weight) treated diabetic rats (MT); Group 4, *Trigonella foenum-graecum* bulk seed extract (200 mg/kg body weight) treated diabetic rats (TBE); Group 5, Au/

Ag nanoparticles with *Trigonella foenum-graecum* seed extract (the rats were injected with green nanocomposite at a dosage of 2 mg/kg.b.wt/day) (TBN).

At the end of the experimental period, rats were anaesthetized using carbon dioxide. Venous retro orbital blood samples (Yadav *et al.*, 2002) were collected in the fasting state using a glass capillary and collected in polystyrene tubes without the anticoagulant. Serum was separated by centrifugation at 3000 rpm for 10 min after which it was tested for parameters such as, blood glucose, blood urea, serum creatinine, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Samples were stored at -20°C until assayed.

Biochemical analysis

Commercial Reflotron kits (Roche, Switzerland) were used for the estimation of blood glucose, urea and serum creatinine. Commercial ELISA kits from Cusabio (China) were used to determine the serum ALT and AST levels. The optical density was determined using a microplate reader set to 450 nm. The AST and ALT activities were expressed as units per gram of wet tissue.

Statistical analysis

Results were presented as the mean standard deviation (SD). A one-way analysis variance was performed using SPSS-17. A post-hoc Tukey's test was used for group comparisons. The values were considered significantly different at the p value was lower than 0.05.

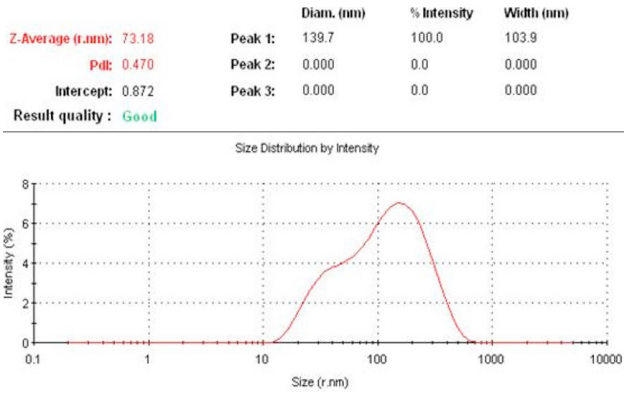

Fig. 1. Zetasizer measurement of the average size of green silver/gold noncomposite.

RESULTS

Nanoparticle size determination using Zetasizer

Figure 1 shows the average size of the formed nanoparticle which was 73.18 nm with polydispersity and large difference in size. This is observed clearly from the appearance of a board peak with intensity 100 % and width 103.9 nm, which gives very little stability of nanoparticles over a long time.

TEM analysis of green gold-silver nanocomposite

The shape and morphology of the green nanoparticles formed was analyzed by TEM. The observations confirm that the morphology of nanocomposite is highly variable with a variety of size and shapes. The images showed the shape being spherical and irregular (Fig. 2A, B, C, D), suggesting that the biomolecules such as protein in *T. foenum-graecum* extract acted as capping agents, supporting the formation of spherical-shaped nanoparticles and were adhered to their surfaces. The TEM images of green silver Au / Ag nanoparticles corresponded with the data obtained from the dynamic light scattering (DLS) and revealed the polydispersive distribution and variable size of the nanoparticles.

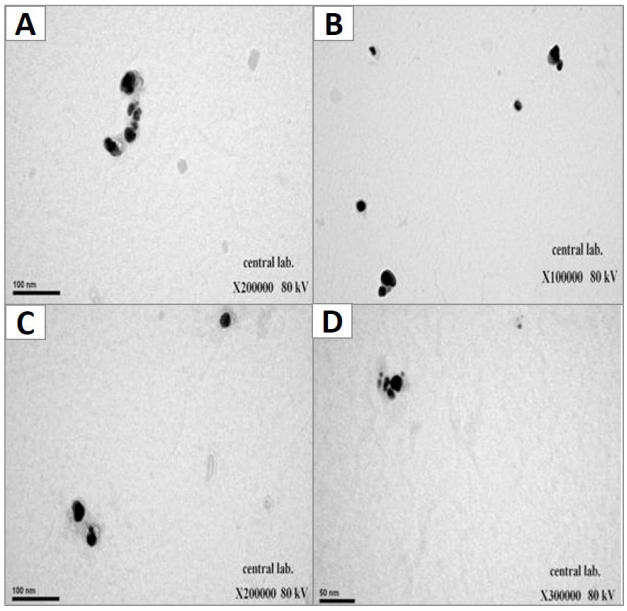

Fig. 2. TEM images of the green silver/gold nanocomposite.

SEM and energy-dispersive spectrometer (EDS) analysis of green gold-silver nanocomposite.

The images from scanning electron microscopy (SEM) showing the structures and morphologies of the formed nanocomposite are shown in Figure 3A. It was observed that the green Au/Ag nanocomposite had different sizes and shapes such as spherical and irregular with the *T. foenum-graecum* extract being used as reducing and capping agents. The green synthesized silver / gold nanoparticles were investigated further using energy-dispersive spectrometer (EDS), which is known to provide

information on the chemical analysis of the elements. The spectrum analysis (Fig. 3B) revealed signals in the silver and gold conforming the formation of silver and gold nanoparticles without contaminants.

Noble metal silver nanoparticles and gold nanoparticles showed a typical optical absorption peak at approximately 3 keV, and 2.30 keV, respectively. The percentage of silver and gold in the bio nanoparticle suspension are tabulated below (Table I).

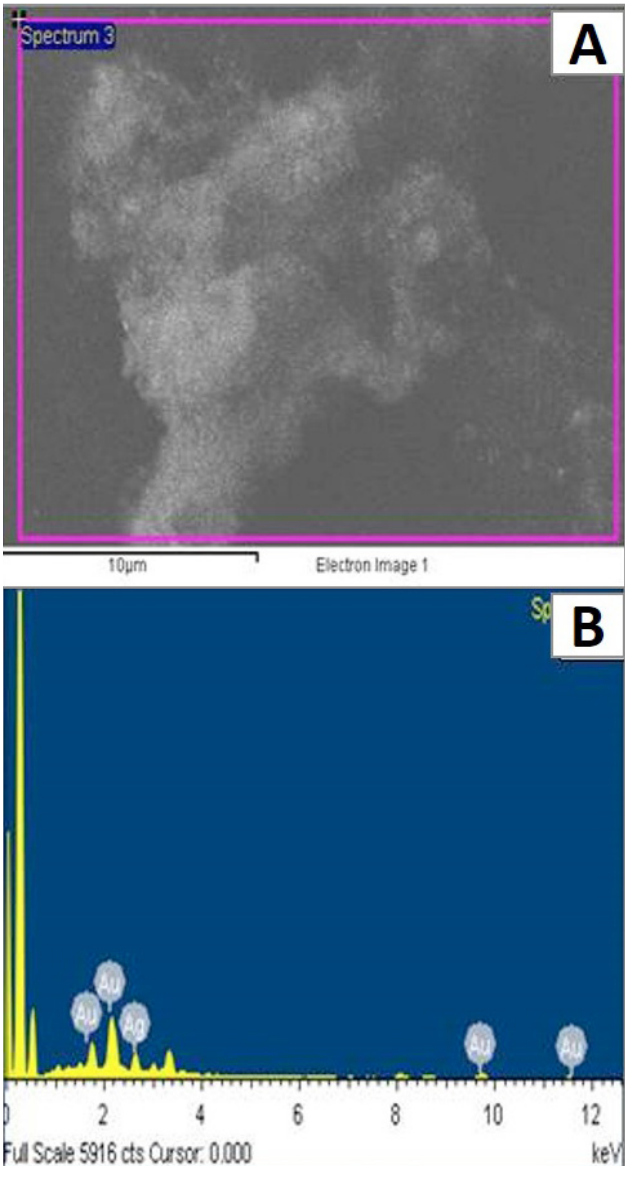

Fig. 3. A, Images from scanning electron microscopy (SEM) showing the structures and morphologies of the formed nanocomposite; B, Spectrum analysis showing the presence of the silver and gold in green Au/Ag nanocomposite.

Table I.- Percentage of silver and gold in the bio nanoparticle suspension.

Element	Weight%	Atomic%
Ag L	18.59	29.42
Au M	81.41	70.58
Total	100.00	-

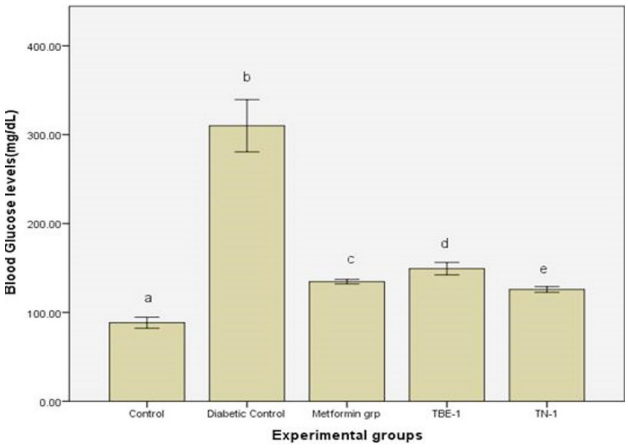

Fig. 4. Effect of Au/Ag nanocomposite with aqueous *Trigonella* seed extract (TN) and bulk *Trigonella* seed extract (TBE) treatment on blood glucose levels (mg/dL) in diabetic rats. Values are expressed as mean ± standard error (n=5). Different letters indicates significant differences between groups ($p \geq 0.05$).

Antihyperglycemic effect

There was no statistical difference observed in the basal blood glucose levels of rats from all experimental groups (94-96 mg/dl). Five days after streptozotocin (STZ), administration, blood glucose values were significantly 3-folds higher ($p<0.05$) in all the diabetic groups in comparison to the negative control group. Further, no statistical difference was observed among the diabetic groups before the treatment. A significant ($p<0.05$) decrease in the blood glucose level was observed in all the diabetic groups with the treatments, metformin, *Trigonella* bulk extract (TBE) and Au/Ag *Trigonella* nanopcomposite (TN) in comparison to DC (310.00±23.65 mg/dL). The least blood glucose level was recorded in the rats from the group with TN treatment (125.80±2.58 mg/dL) being significantly ($p<0.05$) lower than the group with TBE treatment (149.20±5.63 mg/dL) (Fig. 4).

Liver function tests

The most common liver function tests include determination of levels of the serum aminotransferases, such as alanine aminotransferase (ALT) and aspartate

aminotransferase (AST). A measure the serum levels of these intracellular hepatic enzymes serve as a marker of hepatic damage.

The serum AST levels were significantly ($p<0.05$) elevated in all the diabetic groups (DC, Metformin, BE and TN treated) in comparison to the negative control (271.80±21.82 U/L). However, the treatment with metformin and TBE did show a significant decrease in the AST levels in comparison to the DC. The AST levels recorded for the group treated with TN were comparable to the DC as there was no significant difference observed between the two groups (Fig. 5A).

The induction of diabetes resulted in a significant ($p<0.05$) increase in the serum levels of ALT in the serum of rats. In comparison to the negative control (68.60±6.42 U/L) the levels in the DC showed a four-fold increase (457.60±36.72 U/L). The treatments, metformin, TBE and TN significantly ($p<0.05$) reduced the serum ALT levels being more effective in the groups treated with *Trigonella*, TBE and TN in comparison to the metformin. The reducing effect on the serum AST levels was more pronounced in the group treated with TBE. However, the groups treated with *Trigonella*, TBE and TN, had a profound effect on reducing the serum ALT levels (Fig. 5B).

Kidney function

Blood urea nitrogen (BUN) and creatinine are the simplest indicators to monitor diabetes induced kidney dysfunction. There was a significant elevation in the urea and serum creatinine levels of the diabetic rats when compared with those from the negative control group.

Administration of metformin and *Trigonella* extract, TBE and TN decreased the blood urea levels in the diabetic rats. However, the effect was significant ($p<0.05$) and profound in the groups treated with *Trigonella* extract (both TBE and TN) in comparison to the metformin.

The different treatments (metformin, TBE, TN) reduced the serum creatinine levels, however it was statistically significant ($p<0.05$) in the groups treated with *Trigonella* extract (both TBE and TN) (Fig. 5C, D).

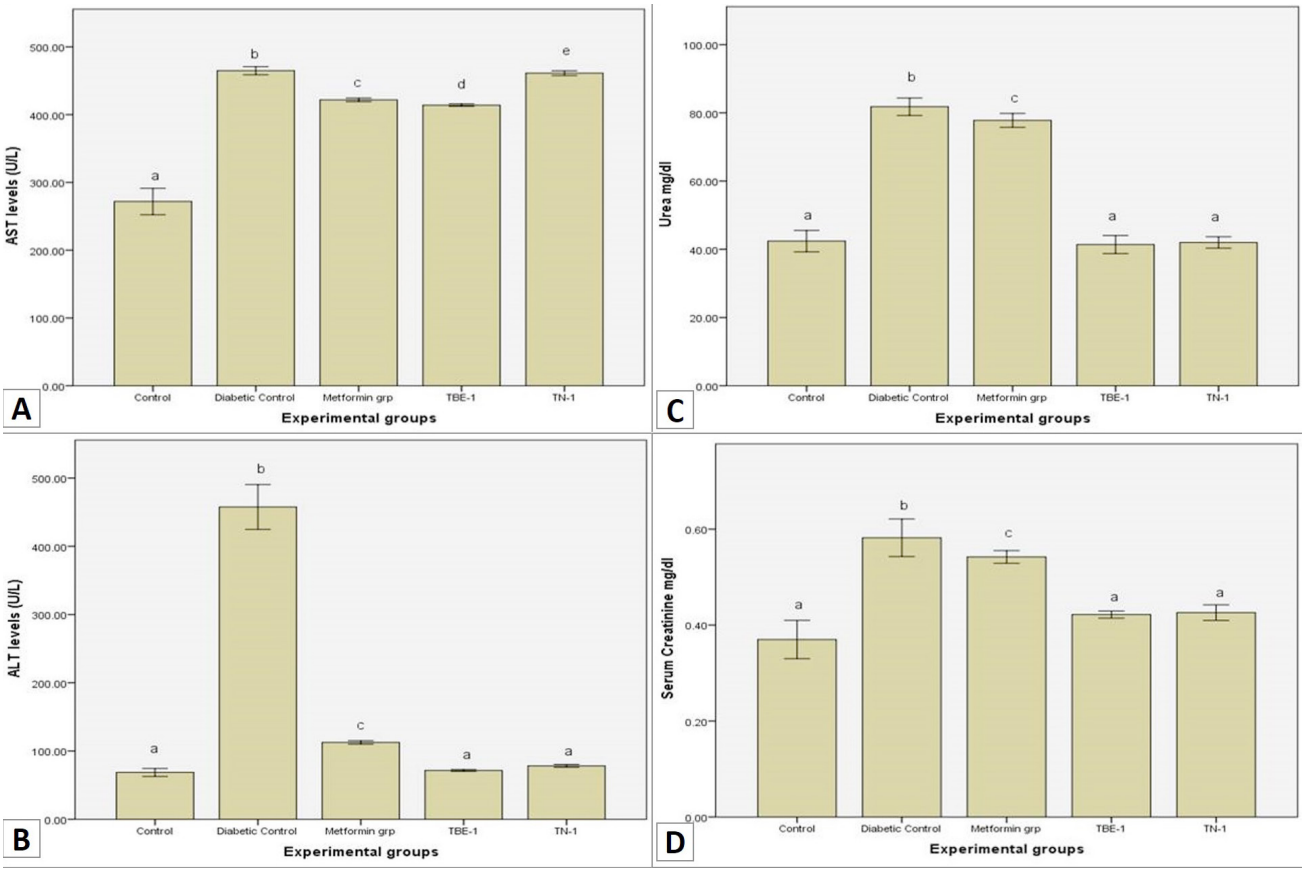

Fig. 5. Effect of Au/Ag nanocomposite with aqueous *Trigonella* seed extract (TN) and bulk *Trigonella* seed extract (TBE) treatment on serum AST levels (U/L) (A), ALT levels (U/L) (B), urea levels (mg/dL) (C) and creatinine levels (mg/dL) (D) in diabetic rats. Values are expressed as mean ± standard error (n=5). Different letters indicates significant differences between groups ($p\geq0.05$).

DISCUSSION

The present study evaluated the possible therapeutic effect of *Trigonella* gold and silver nanocomposite on streptozotocin-induced diabetic rats in comparison to a synthetic anti-diabetic drug, metformin. The metal nanoparticles such as, zinc, silver, iron and gold, have been reported to have wide applications in the field of biomedical science. Oxidative stress plays an instrumental role in etiology of diabetes and its associated complications (Hirst *et al*., 2009; Giugliano *et al*., 1996; Feldman *et al*., 1997; Mishra *et al*., 2008). The production of high level of reactive oxygen species (ROS) is incriminated in the pathogenesis of the disease. The metal nanoparticles used currently in diabetic treatment act as free radical scavengers (Warheit, 2004) thus alleviating the oxidative damage caused. The gold nanoparticles (AuNPs), are known for their tremendous applications in the field of therapeutics and diagonosis. The ability of gold nanoparticles in inhibiting the lipid peroxidation thereby preventing the ROS generation restores the antioxidant imbalance (Yakimovich *et al*., 2008). Barath Mani Kanth *et al*. (2010) reported the anti-oxidative and antihyperglycemic activities of gold nanoparticles. Further nanoparticles of another noble metal, silver have also been reported to have antidiabetic activity (Alkaladi *et al*., 2014) and Ag^{+} loaded zirconium phosphate nanoparticle plays a crucial role in diabetic wound healing (Ruggiero *et al*., 1997). A green synthesis of Au /Ag nanocomposite with the *Trigonella* seed extract in this study is an attempt to amalgamate the properties of the three constituents to enhance the therapeutic effect on diabetes and its associated risks.

The characterization of the green Au/Ag nanoparticles showed an average size of 73.18 nm with polydispersity. The TEM and SEM images in the present study showed the shape of the nanoparticles as spherical and irregular, suggesting that the biomolecules such as proteins in the *Trigonella* seed extract acted as a capping agent, supporting the formation of spherical-shaped nanoparticles and were adhered to their surface (Ahmad *et al*., 2010). The TEM images of green Au / Ag nanoparticles were in line with the data obtained from the DLS and revealed the polydispersity distribution and difference in the size of nanoparticles. The characterization of the nanoparticles showed that, Au and Ag nanoparticles exhibited a typical optical absorption peak at approximately 2.30 keV and 3.0 keV which is attributed to the surface plasmon resonance of gold and silver nanoparticles, respectively (Arunachalam *et al*., 2013; Velmurugan *et al*., 2013). The phytochemical analysis of the dried seed extract of fenugreek has been reported to show the presence of proteins, vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin and plant sterol (Bukhari *et al*., 2008). The flavonoids present in the seed extract are powerful reducing agents which may be responsible for the reduction of chloroauric acid used in the preparation of the nanoparticles while the carboxylate group present in proteins can act as surfactant to attach on the surface of Au/Ag NPs and this further stabilizes NPs through electrostatic stabilization. A previous study on the green synthesis of Au nanoparticles with *Trigonella (*fenugreek) seed extract reported that owing to its phytoconstituents, the seed extract has the ability to perform dual functions of reduction and stabilization of Au NPs (Aromal and Philip, 2012). It has been suggested that, polyphenols (-OH group), amides and amines present in the fenugreek extract caps the silver nanoparticles surface, thereby restricting the aggregation of silver nanoparticles and stabilizes them (Suganya and Devasena, 2014).

The treatment with Au/Ag nanoparticles with the fenugreek extract also exhibited a marked antihyperglycemic effect in the diabetic rats which was significantly more effective than the bulk treatment of the seed extract (TN). The synergistic anti-diabetic effect of all three constituents of the nanopartclies, Au, Ag and phytocompounds in the fenugreek seed extract clearly explains the results.

The serum AST and ALT levels increases as a result of metabolic changes in the liver, such as administration of toxin, cirrhosis of the liver, hepatitis and diabetes (Chalasani *et al*., 2004). Similarly, in the present study, the induction of diabetes in the rats, elevated the serum levels of transaminases, such as ALT and AST. It may be due to leaking out of enzymes from the tissues and migrating into the circulation by the adverse effect of STZ (Stanely *et al*., 1999). AST and ALT were used as markers to assess the extent of liver damage in streptozotocin induced diabetic rats (Hwang *et al*., 2005). The different treatments (MT, TBE, TN) given did alter the levels of the transaminases as a significant decrease was observed in comparison to the diabetic control. The effect on serum AST level was more profound in the groups treated with MT and TBE, however both the *Trigonella* treated groups (TBE, TN) were more effective in reducing the serum ALT levels in comparison to the MT group.

Diabetic nephropathy takes many years to develop. However, biomarkers of early tubular dysfunctioning are studied to observe the progression of the kidney dysfunction in diabetes. Blood urea and serum creatinine levels are established biomarkers used to assess diabetes related renal dysfunctioning. A significant elevation in blood urea and serum creatinine were observed in diabetic rats (DC), when compared to control rats. These results were similar to a study that reported a significant increase

in serum creatinine in albino diabetic rats administered Alloxan for 14 days (Shanmugasundaram *et al*., 2011) increase in the blood urea and serum creatinine level, indicating an abnormal glomerular filtration. An increase in the blood urea levels is attributed to a high rate of protein breakdown during diabetes and the failure of excretion due to an abnormal renal filtration (Palanivel *et al*., 2001). All the treatments (MT, TBE, TN) given during the experimental period did reduce both the urea and creatinine levels. The effect was more significant in the groups receiving the *Trigonella* seed extract (TBE, TN) in comparison to the metformin treated group.

CONCLUSION

Stable Au/Ag nanoparticles with aqueous extract of *Trigonella* seed extract were synthesized which were nearly spherical and stable. The phytoconstituents of the seed extract acted as reducing agents and stabilized the structure. It is found that the possible reducing agent are flavonoids and the capping material responsible for stabilization are the proteins present in the seed extract. The method of synthesis employed in the present study is simple, economic, nontoxic and efficient. The as-prepared Au/Ag nanocomposite showed a potent antihyperglycemic activity. A broad assessment of the other end points evaluated, showed that the nanocomposite used was effective in alleviating the diabetes related hepatic damage and renal dysfunctioning. Thus, the present synthesis approach could be extended as a potential nano therapeutic strategy in the management of type II diabetes and other metabolic disorders. The synthesis of Au/Ag nanocomposite with other phytocompounds could also be a promising approach in the field of nanomedicine.

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Statement of conflict of interest

The author has declared no conflict of interest.

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