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RESEARCH ARTICLE

Hypoglycemic Properties of Sphaeranthus indicus and *Nigella sativa* in Alloxan Induced Diabetes Mellitus in Rats; A New Therapeutic Horizon

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ABSTRACT

The current therapies used to cure diabetes mellitus *viz.* based on some oral hypoglycemic preparations or insulin are not sufficient and accompany a lot of side effects. Complementary and alternative remedies are being considered in managing diabetes. Therefore, the current study was planned to investigate the anti-diabetic efficacy of *Sphaeranthus indicus* and *Nigella sativa* (alone and in combination) in comparison to rational anti-diabetic drug metformin. For this purpose, 54 albino rats were selected and randomly divided into six equal groups after inducing diabetes. Extracts of *Sphaeranthus indicus* and *Nigella sativa* were given alone and in combination to animals of three groups, respectively, whereas metformin was administered as standard therapy to animals of the respective group. One group served as positive control while the other one as a negative control. Evaluation criteria were based on serum glucose and insulin levels, oxidative stress biomarkers and gene expression. Provision of respective treatments led to a statistically significant decrease in serum glucose levels and vice versa in serum insulin levels; this change was statistically non-significant among all the treated groups at the end of the study period. A similar trend was observed in the case of oxidative stress biomarkers *viz.* total oxidative stress and total antioxidant capacity, where the former was decreased and later was increased significantly after provision of treatments. Similarly, the provision of *Sphaeranthus indicus* and *Nigella sativa* alone or in combination upregulated the insulin signaling pathway genes while downregulated the genes for MAPK downstream JNK pathway.

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INTRODUCTION

Presently all over the world, diabetes mellitus has become the most common endocrine disorder. In the next 25 years, diabetes is anticipated to be the world's primary life-threatening condition compared to cancer and cardiovascular diseases. In Pakistan, during 2011, the reported prevalence of diabetes was 11% which is expected to be 15% by 2030 (Hussain and Ali, 2016). Currently, Pakistan seats at 7th position in the list of diabetes prevalent countries (Qidwai and Ashfaq, 2010). At present, routine therapy for diabetes generally comprises of insulin and a number of oral hypoglycemic drugs like sulfonylurea, metformin, troglitazone and glucosidase inhibitors etc. Such synthetic agents are valuable but are reported to be associated with numerous untoward effects like digestive problems, liver problems, abdominal pain, weight loss and lactic acidosis (Asche *et al*., 2008). Even with the insidious use of hypoglycemic drugs, diabetes continues to be a topmost health problem globally.

Coupled with the existing therapeutic regime, alternative remedies are becoming more popular in managing diabetes. Medicinal plants remain to be imperative therapeutic support for relieving various diseases of both humans and animals (Zaman *et al*., 2017; Ashraf *et al*., 2021). A robust traditional medicine system like Ayurvedic, Unani and Chinese has been practiced over many years. Minimum side effects, less cost and easy availability make the herbal formulations the monarch of all the existing therapies (Zaman *et al*., 2017). Several plants have been used for the treatment of diabetes, and there are various mechanisms by which bioactive compounds of plants may exert their anti-diabetic effects like regeneration of beta cells of the pancreas or decreasing insulin resistance (Dwivedi and Daspaul, 2013).

JNK pathway activation leads to destruction of insulin secreting beta-cells through interleukins and interferons. Many studies have revealed that expression of insulin genes have been reduced by oxidative stress after activation of JNK-pathway (Kaneto *et al*., 2002; Jaeschke *et al*., 2005). Moreover, it was further suggested that activation of JNK pathway leads to decreased PDX-1 activity which in turn results into insulin gene's down regulation (Kaneto *et al*., 2002).

Both *Sphaeranthus indicus* (Gul-e-mundi) and *Nigella sativa* (black seed) are widely used in folk medicine. Even though the efficacy of constituents of individual plant is well established (Prabhu *et al*., 2008), the use of mixture or polyherbs in folklore is considered better theoretically in achieving good outcomes as compared to the individual plant. Hypoglycemic activity of *Sphaeranthus indicus* and *Nigella sativa* individually has been reported. However, the effect of the combination of both plants in diabetes has not been investigated yet. Considering the concept of synergism in use of polyherbal medicine, the present study was designed to investigate the anti-diabetic efficacy of *Sphaeranthus indicus* and *Nigella sativa* alone and in combination in comparison to rational anti-diabetic drug metformin.

MATERIALS AND METHODS

Extract preparation of selected plants: Alcoholic extract of *Sphaeranthus indicus* flowers and *Nigella sativa* seed was prepared as per Anusha *et al*. (2011) after identifying the plant materials from plant taxonomist Department of Botany, Faculty of Basic Sciences, UAF.

Study protocol: Fifty-four healthy albino rats of either sex were selected for the study and equally divided into six groups. Diabetes was induced in all the animals except animals of group negative control group (NG). Induction of diabetes was carried out by administering the Alloxan monohydrate (150mg/kg) intra-peritonealy. Inclusion criteria for the diabetic rats in the study were blood glucose levels more than 300mg/dl (Aruna *et al*. 1999). In Positive control (PG), only the routine diet was offered to diabetic rats. Animals of group Met received hypoglycemic drug Metformin orally (400mg/kg) once a day. Alcoholic extract of *Sphaeranthus indicus* and *Nigella sativa* (300mg/kg/day) was given to rats of group SI and NS, respectively. At the same time, a combination of extracts of both plant materials was given to group SI+NS. Blood samples were collected at day 0 (after diabetes induction), at week four and week eight post-initiation of treatments for the different biochemical tests. At the end of the trial, pancreatic tissues were harvested from animals of each group and stored in liquid nitrogen for gene expression analysis.

Evaluation parameters: Serum glucose, serum insulin and oxidative stress biomarkers were checked to evaluate the efficacy of treatment. Quantitative real-time PCR (qRt-PCR) was also carried out to determine the expression level of different genes (Ins-1, Ins-2, PDX-1, Calm-2, Grk-2, Pias-2, MAPK-8, involved in insulin signaling pathways in pancreatic cells.

Determination of Serum Glucose and Insulin: Levels of glucose in the serum was checked through Flutiest ® GLU- Analyticon diagnostic kit while insulin level was determined by using Abcam's ab200011- Insulin Human Simple step ELISA® kit.

Estimation of Oxidative Stress Biomarkers: Total oxidative stress (TOS) and total antioxidant capacity (TAC) were measured by using the method described by (Erel, 2005).

Gene Expression analysis: RNA was isolated from pancreatic tissue samples by using TRIZOL (ThermoFisherScientific, Massachusetts, USA) reagent as described by Liu and Patel (1995). Synthesis of cDNA was carried out through RevertAid cDNA synthesis kit according to the instructions given by the manufacturer. qRt-PCR data was analyzed by using $2^{*}(-\Delta\Delta ct)$ method. The primers used for this purpose are mentioned in Table 1.

Table-1: Primers used for assessment of gene expression

Genes	Primers
Ins-1 F	AGGCTCTGTACCTGGTGTGTG
Ins-1 R	CGGGTCCTCCACTTCACGAC
Ins-2 F	GGAGCGTGGATTCTTCTACACA
Ins-2 R	AGTGCCAAGGTCTGAAGGTCAC
PDX-1 F	TCCCGAATGGAACCGAGACT
PDX-1 R	TTCATCCAGGGAAAGGGAG
Calm-2 F	AAGTGTGGAGTTGTGAGCGT
Calm-2 R	GAGTACCGGACAGAACACCC
Grk-2 F	GGAGTCCTACAGAAGGGCG
Grk-2 R	CCCGCAACAACTTGAAGAGC
Pias-2 F	CCAGTAGAGCCTGACTTGGC
Pias-2 R	TGACGGTGAATGAGGTGCAA
MAPK-8 F	CTCAGCATCCGGTCTCTTCG
MAPK-8 R	CTGCTGTCTGTACCGAGGC
Traf-4 F	CGACTACAAGTTCCTGGAGAAGC
Traf-4 R	AGGTGTCGCAGAAGCGGTG
Traf-6 F	GGCGCCTAGTAAGACAGGAC
Traf-6 R	ACATGCATGCTCTGCGTTTC

Statistical analysis: Data thus generated were analyzed using ANOVA and Duncan's Multiple Range test was used to compare differences in means.

RESULTS

Serum Glucose (mg/dL): Induction of diabetes by alloxan led to a statistically significant ($P<0.05$) increase in serum glucose values of all the animals at week 0 of the study when compared to the negative control group. Provision of metformin as treatment of diabetes resulted in a statistically significant ($P<0.05$) decrease in serum glucose values at week 4 of the study. A similar trend was observed in the group at week 8, which was the last sampling time point of the study. Administration of allocated treatments to animals of group SI, and combination group led to a similar pattern of serum glucose values (Fig.1. A). The values indicate very clearly that all the treatments effectively declined the elevated serum glucose values in diabetic rats, but the combination of extract of both plants reverted the serum glucose values close to the negative control group.

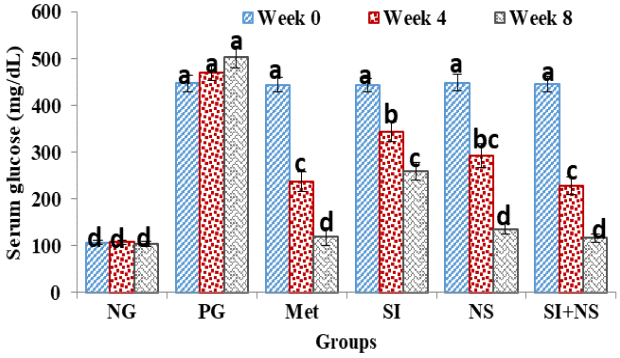

Fig. 1: A. Effect of *S. indicus* and *N. sativa* alone and in combinationin comparison with rational treatment on Serum Glucose (mg/dL; Mean±SE).

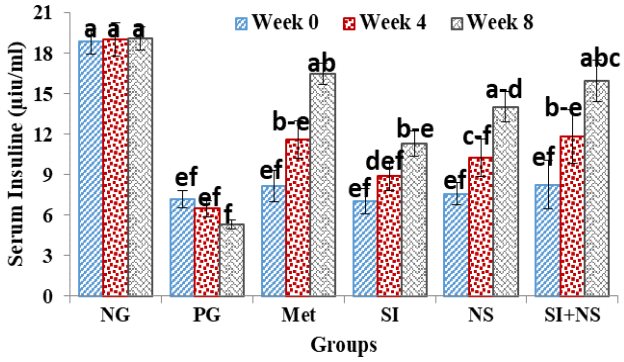

Fig. 1: B. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on Serum Insulin (µiu/ml; Mean±SE)

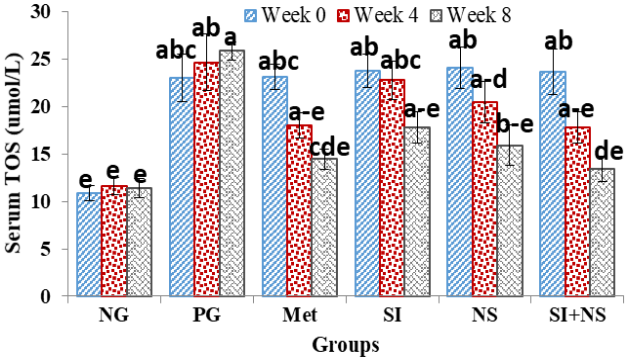

Fig. 1: C. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on Serum Total Oxidative Stress (µmol/L; Mean±SE).

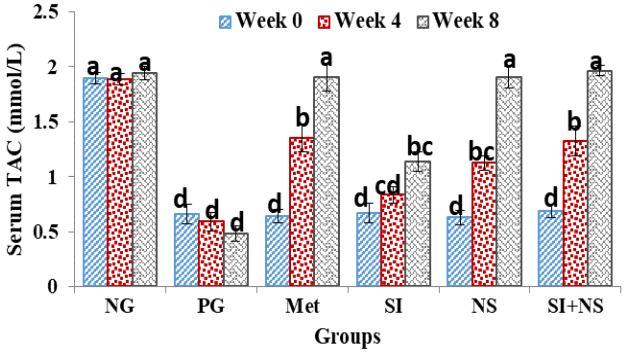

Fig. 1: D. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on Serum Total Antioxidant Capacity (mmol/L; Mean±SE).

Serum Insulin (µiu/ml): In terms of serum insulin level, a statistically significant (P<0.05) decrease was observed after induction of diabetes in the animals of groups positive control, or treated with metformin, *S. indicus*, *N. sativa* or a combination of *S. indicus* and *N. sativa* at day 0 sampling time point compared to the negative control group (Fig.1. B). A statistically non-significant decrease in serum insulin was further observed in animals of the negative control group at subsequent sampling time points (P>0.05) from previous ones (Fig.1. B). Treatment of diabetic rats with metformin resulted in an increase in serum insulin of the animals throughout the study period being statistically significant (P<0.05) from the previous sampling time point (Fig. 2). A similar trend in terms of serum insulin values was observed in other treatment groups. It is evident from these values that the Met group achieved serum insulin values close to NG followed by the combination group.

Serum Total Oxidative Stress (TOS;µmol/L): Dynamics of serum TOS values in alloxan-induced diabetic rats and treated with allocated treatments are shown in Fig. 3. Induction of diabetes led to a significant (P<0.05) rise in serum TOS values of all the rats at week 0 of the trial as compared to the negative control group (Fig. 1C). This rise continued its trend in non-treated diabetic rats of the positive control group throughout the study period being statistically non-significant (P>0.05) at each sampling time point. Provision of allocated treatments *viz.* metformin, *S. indicus*, *N. sativa* and combination of later two to the respective groups resulted in a decline in serum TOS values of the diabetic rats. There was a statistically significant (P<0.05) difference between serum TOS values of the combination group at week eight compared to the values of week 0 of the respective group.

Serum Total Antioxidant Capacity (TAC; mmol/L): Regarding serum TAC values in experimental groups, results showed that induction of diabetes by alloxan resulted in a significant (P<0.05) decrease in serum TAC values of all the rats at week 0 of the trial as compared to negative control group where diabetes was not induced (Fig. 1D). This decrease continued its trend in non-treated diabetic rats of the positive control group throughout the study period being statistically non-significant (P>0.05) at each sampling time point. Provision of allocated treatments to the respective groups resulted in an increase in serum TAC values of the diabetic rats. There was a statistically significant (P<0.05) difference between serum TAC values of all the groups at week 8 compared to the values of week 0 of the respective groups. The group receiving a combination of (SI+NS) achieved serum TAC values close to NG, followed by the NS group at week 8.

Gene expression analysis

Insulin signaling pathway: In the release of insulin from β pancreatic cells, the insulin signaling pathway is considered to be the cornerstone. The level of expression of INS-1, INS-2 and Pdx-1 genes were assessed in the current study (Fig. 2A to 2C). The findings of the study reveal that in animals which were subjected to induction of diabetes, there was a significant (P<0.05) downregulation in the above-mentioned genes as compared

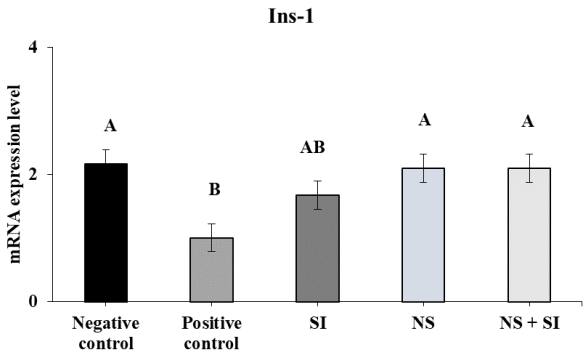

Fig. 2: A. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Ins-1 gene.

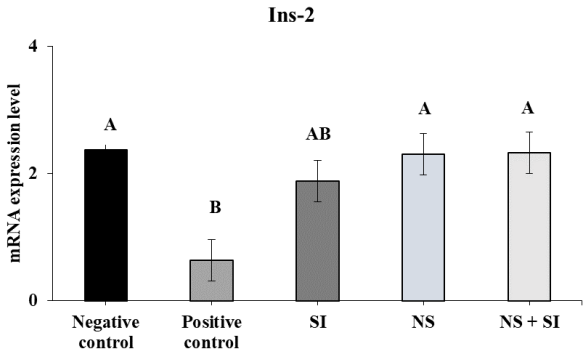

Fig. 2: B. Effect of *S. indicus*and *N. sativa*alone and in combination to diabetic rats in comparison with rational treatment on expression of Ins-2 gene.

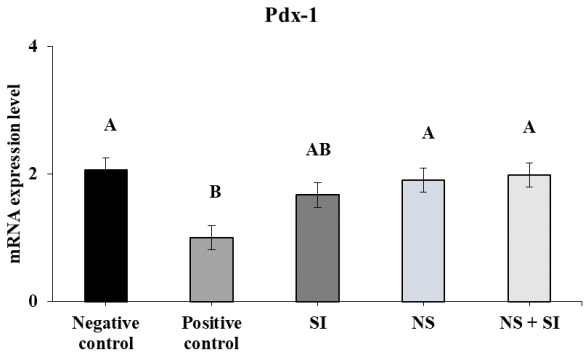

Fig. 2: C. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Pdx-1gene.

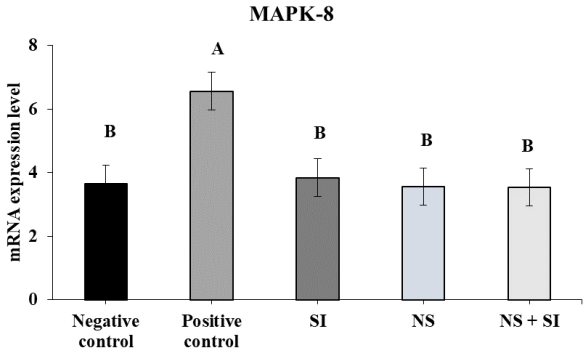

Fig. 3: A. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of MAPK-8 gene.

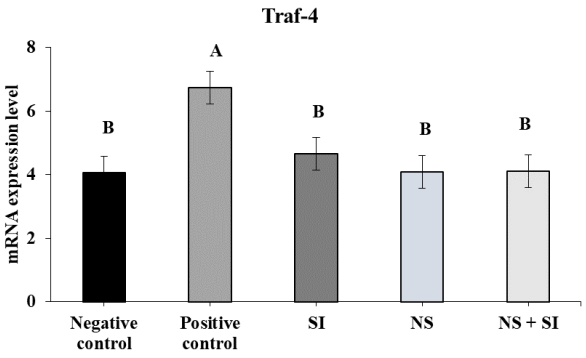

Fig. 3: B. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Traf-4 gene.

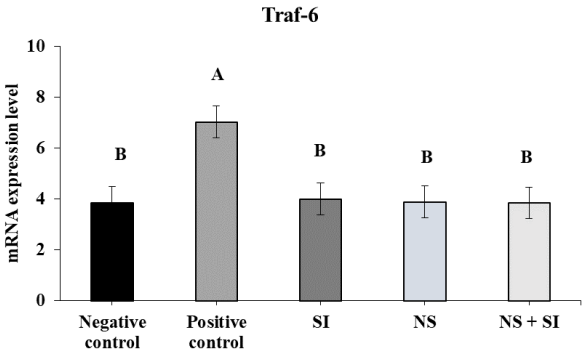

Fig. 3. C. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Traf-6 gene.

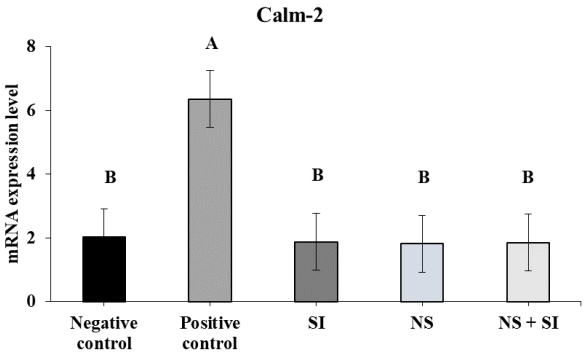

Fig. 4. A. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Calm-2 gene.

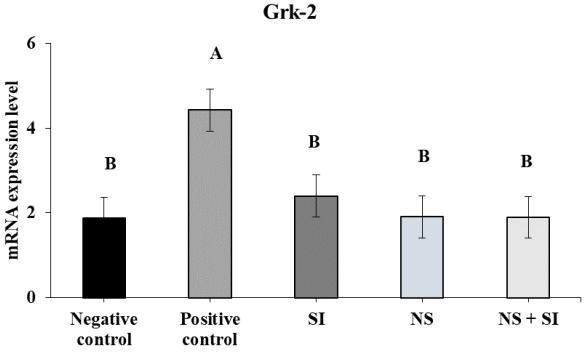

Fig. 4. B. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Grk-2 gene.

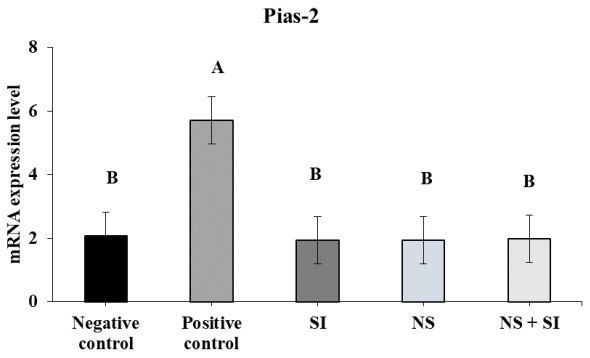

Fig. 4. C. Effect of *S. indicus* and *N. sativa* alone and in combination to diabetic rats in comparison with rational treatment on expression of Pias-2 gene.

to the negative control group. Provision of allocated treatments to diabetic groups resulted in a significantly (P<0.05) higher gene expression of these genes compared to the positive control group.

MAPK downstream JNK pathway: Kinases pathways are leading phenomena for the establishment of cellular stress, and among these, MAPK pathway is the largest one. To assess MAPK downstream JNK pathway, MAPK-8, Traf-4 and Traf-6 gene expression level was determined (Fig. 3A to 3C). The results indicated that induction of diabetes in animals of the positive control group led to significant (P<0.05) up-regulation of these genes compared to the negative control group. Treatment of diabetic rats with allocated treatment protocols significantly (P<0.05) down-regulated these genes when compared to positive control group.

Calcium signaling pathway: Assessment of expression level of Calm-2, Grk-2 and Pias-2 genes was carried out for determination of Calcium signaling pathway (Fig.4A to 4C). The findings of the study revealed a significantly (P<0.05) higher expression level of the aforementioned genes in diabetic rats of positive control compared to negative control. Provision of treatment protocols *ut supra* led to a significantly (P<0.05) lower expression level of genes under reference.

DISCUSSION

The common treatment approach for diabetes treatment mainly relies on decreasing and regulating blood sugar level within the normal range through β-cells stimulation, increasing insulin receptor sensitivity or improving utilization of glucose. Presently routine therapy for diabetes generally comprises insulin and several oral hypoglycemic agents such as troglitazone, metformin, sulfonylureas, glucosidase inhibitors, etc. Such synthetic drugs are valuable but are reported to be responsible for some untoward effects like digestive problems, liver problems, abdominal pain, weight loss and lactic acidosis (Asche *et al*., 2008). Owing to the glitches associated with the existing therapeutic protocol for diabetes, alternative remedies are becoming more popular. Herbal plants are very commonly consumed by healthy individuals as well as by patients for treatment or prophylaxis. Minimum side effects, less cost and easy availability make the herbal formulations the monarch of all the existing therapies (Zaman *et al*., 2017). Therefore, in the current study, flowers of *Sphaeranthus indicus* and seeds of *Nigella sativa* were evaluated for their hypoglycemic properties by determining their role in decreasing the oxidative stress and upregulating the insulin signaling pathways.

The current study revealed that treatment of diabetic rats with alcoholic extract of selected plant materials resulted in a significant decrease in serum glucose level while an increase in the level of serum insulin compared to non-treated diabetic rats where a significant increase in serum glucose and decrease in serum insulin was observed. A similar trend was reported by Bensiameur-Touati *et al*. (2017) who reported the beneficial effect of *Nigella sativa* on serum biochemical parameters of diabetic rats. Likewise, Parbhu *et al*. (2008) also indicated the decrease in hyperglycemic changes by giving *S. indicus* in STZ-nicotinamide induced diabetes in rats. Antihyperglycemic activity of these medicinal plants material could be associated with the presence of flavonoids in them which may be responsible for the rise in insulin secretion from the intact pancreatic cells by exerting a positive impact on beta cells regeneration and thus improving the glucose transportation in the tissues (Cheng *et al*., 2013; Oza and Kulkarni, 2018).

It has been established that diabetes may result in severe oxidative stress leading to damage to various organs of the body. The findings of the present study also indicated the oxidative stress as induction of diabetes resulted in a significant increase in oxidative stress biomarkers. A significant increase in total oxidative stress (TOS) values and decrease in total oxidant capacity (TAC) was found in diabetic rats, which were reverted towards normal after treatment with the *N. sativa* and *S. indicus* comparable to rational treatment with metformin. Previous research studies also supported the findings of the current study in terms of the decrease in oxidative stress by the other medicinal plants depicting their antioxidant potential (Assi *et al*., 2016; Mao *et al*., 2018). Antioxidant activity of *N. sativa* described by Bensiameur-Touati *et al*. (2017) also prop up the findings of the present study.

Quantitative real-time PCR(qRT-PCR) based gene expression analysis was carried to investigate the mRNA expression level of various genes involved in cellular stress. These include MAPK downstream JNK pathway, calcium signaling pathway and insulin signaling pathways. Apoptosis of beta-pancreatic cells is attributed to cellular stress cascade, whereas neogenesis, regeneration, proliferation and secretion of insulin from beta pancreatic cells is considered dependent on insulin signaling pathway. It is now very well established that ROS overproduction due to alloxan leads to apoptosis of beta cells (Chang and Karin, 2001; Bhattacharya *et al*., 2011). A number of signal transduction pathways, including MAPK downstream JNK pathways, are activated in various cells, including beta-pancreatic cells due to the production of ROS (Chang and Karin, 2001). To assess MAPK downstream JNK pathway, MAPK-8, Traf-4 and Traf-6 gene expression level was determined. The results indicate that induction of diabetes in animals of the positive control group led to significant (P<0.05)

up-regulation of these genes compared to the negative control group. Treatment of diabetic rats with allocated treatment protocols significantly ($P<0.05$) downregulated these genes when compared to the positive control group. A similar pattern was observed in terms of calcium and insulin signaling pathways. A number of studies in the recent past have reported that downregulation of the JNK pathway protects the beta-pancreatic cells from oxidative stress damage (Etuk, 2010; Yang *et al*., 2014). JNK pathway activation leads to destruction of insulin secreting beta-cells through interleukins and interferons. Many studies have revealed that expression of insulin genes have been reduced by oxidative stress after activation of JNK-pathway (Kaneto *et al*., 2002; Jaeschke *et al*., 2005). Moreover, it was further suggested that activation of JNK pathway leads to decreased PDX-1 activity which in turn results into insulin gene's down regulation (Kaneto *et al*., 2002).

Conclusions: Keeping in view the comparable results of *S. indicus* and *N. sativa* alone and in combination in amelioration of alloxan-induced diabetes with rational treatment, one can fancy a chance to make herbal anti-diabetic product of above-mentioned combination. This product can be an economic one with few side effects but equal efficacy to routine therapies. Hence, this study has opened a new window for the pharmaceutical industry to look forward to a new solution to this huge threat to the human population with fewer side effects. External validation of the findings by reciprocating the results on a large population and different spp. is suggested prior to human trial.

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