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EVALUATION OF METHANOLIC NIGELLA SATIVA L SEED EXTRACT ON BLOOD GLUCOSE LEVELS IN NORMAL AND ALLOXAN-INDUCED DIABETIC MICE

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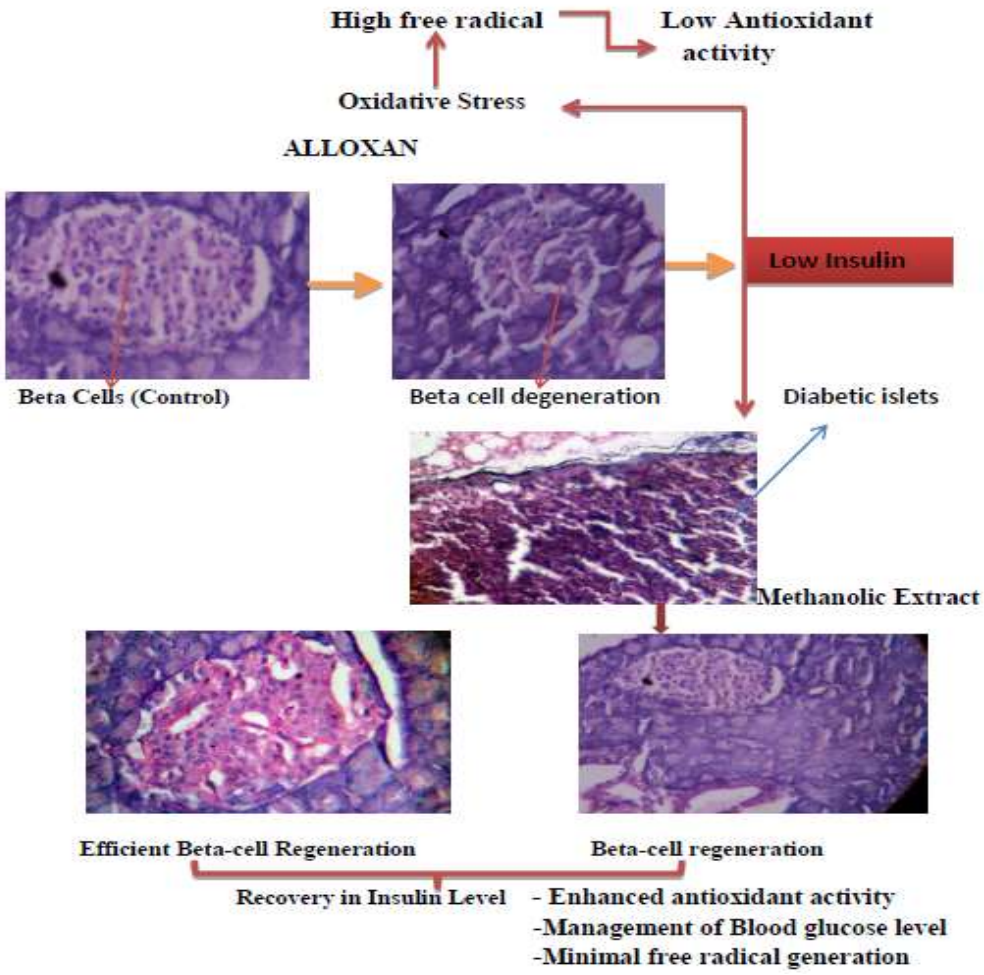
INTRODUCTION

Nigella sativa, a member of Ranunculacae family is an annual, herbaceous, capsulated, dicotyledon medicinal plant cultivated for its seeds and is categorized as an edible medicinal part. *Nigella sativa* has inflated capsule composed of 3-7 united follicles, each containing numerous black trigonal seeds **(Prain (2008), Mozzafari (2000).**The seeds are frequently used for centuries and have kept its popularity for historical, cultural and religious reasons to treat various human ailments **(Salem, 2005).** The notable potential for cultivation, propagation and production of *Nigella sativa* has been reported in Asia, Africa and Europe (**Sharma *et al.*, 2009, Iqbal *et al.*, 2010; Rabbani *et al.*, 2011)**



The *Nigella sativa* (Black seeds) are very rich and diverse in chemical composition containing sterols, triterpenes, tannins, flavanoids, cardiac glycosides, alkaloids, saponins, volatile oils, volatile bases, glucosinolates and anthraquinones **(A1-Yahya, 1986),** important minerals like calcium, phosphorus and iron were found to be in appreciable amounts, while zinc, calcium, magnesium, manganese and copper in meager quantities (**Ali and Blunden, 2003**) and amino acids, carbohydrates, fixed and volatile oils **(Khan, 1999).** The yield of black seed fixed oil ranges from 22.0 to 40.35% **(Cheikh-Rouhou *et al*., 2007).**

The field of diabetes care and research is rapidly growing and changing with advancement in treatments that can improve the health and well-being of people with diabetes (**ElSayed NA *et al*., 2023)**. Diabetes is one of the main threats to human health in the 21 century and is believed to be one of the most dangerous and life threatening diseases in the world today, involving the pancreas, **(Verbrugge *et al*., 1989).** From the beginning of the 20th century, the subject had developed mainly on botanical side being concerned with history, identification, collection, preparation and storage of botanical drugs. Currently, plant based drugs are

researched, dispensed, formulated and manufactured in modern framework (**Kinghorn, 2002).** More than half a billion people are living with diabetes worldwide, affects people regardless of country, age group, or sex, and that number is projected to more than double to 1.3 billion people in the next 30 years. **(Ong, Kanyin Liane, et al. (2023); IDF, 2012).**

Despite the intensive use of current anti-diabetic agents, many (more than 50%) type 2 diabetic patients still exhibit poor glycemic control and some (18%) develop serious complications within six years of diagnosis Clearly, there is a need for new anti-diabetic agents. Therefore, much effort has been devoted to the search and development of optimal therapeutic regimens for the management of diabetes **(Acharya and Shrivastava, 2008).**

This has open many research opportunities to biotechnologists and pharmacognosists ranging from characterizing biologically active principles, designing suitable biotechnological approaches and analytical methods for quality control and standardization, activity based screening and drug development. Its increasing prevalence and high cost of treatment in the world is a cause of concern **(Kanter *et al*., 2003)** and is posing a continuous threat to human health since decades.

MATERIALS AND METHODS

Target Animal

The experiment was conducted on healthy experimental animals.

Strain	-	Swiss albino mice
Age	-	8- 10 weeks
Sex	-	Either sex
Body weight	-	25 ± 35 gm

The experimental animals were obtained from the animal house Pinnacle Biomedical Research Institute (PBRI) Bhopal India (CPCSEA Reg.No.1283/c/09/CPCSEA, Protocol Approval No. PBRI/10/IAEC/PN185) before starting the study.

Alloxan: Alloxan monohydrate dissolved in 0.9 sodium chloride solution (normal saline) was used for this study. Alloxan used to induce diabetes in experimental animals damages insulin secreting pancreatic beta cells **(Omamoto *et al.*, 1981)**. Molecular formula C4H2N2O4, Molar mass 142.07 g/mol., Melting point 256 °C (decomposition) Solubility Freely soluble in water.

Nigella sativa Linn Seeds: Acquisition of *Nigella sativa* seeds (Black seeds, locally known as Kalonji) were purchased from a herbal store in Bhopal, Madhya Pradesh India.
The seeds were identified and authenticated. Voucher specimens of the black
Seeds were kept at Pinnacle Biomedical Research Institute Bhopal, India.

Sample preparation: The *Nigella sativa* L. seeds were examined to determine physical condition and extent of contamination. The seeds were made dust free followed by washing with distilled water. The seed samples were then air dried in an oven at 300 C overnight to stabilize the seed samples. However care was taken to avoid drying at high temperature and longer duration which may result in thermal decomposition. The *N. sativa* L. seeds were grinded to fine powder (0.5 to 1.0-rnm) particle size to ensure homogeneity. After grinding and homogenization, the *Nigella sativa* L. seed samples were thoroughly dried, sealed, and placed under refrigerated conditions until completion of the experimental work.

Macroscopic evaluation

The black seed sample was taken for various macroscopic organoleptic evaluations like colour, odour, size, shape, taste and appearance. The results of organoleptic evaluation of black seeds and its powder are shown in (Table 2 & 3) respectively.

Preparation of *Nigella sativa* L. seed extracts

The *Nigella sativa* L. seed powder was subjected to Soxhlet extraction by using methanol (HiMedia laboratories Mumbai India, NLT 99.0% Assay GC) in 1:5 w/v ratio at 45-50̊C for 48 till it showed dark reddish brown colour of the crude. After extraction was performed, the extract was filtered and the solvent was evaporated in rotary evaporator at 40˚C and stored in refrigerator for further analysis.

Plant Collection and Preparation of extracts

The plant material of dried seeds of *Nigella sativa* used for investigation was collected. The seeds were dried in shade and made to dry powder by grinding process using sophisticated instruments. The dried powder was then passed through the 40 mesh sieve. A weighed quantity of the powder was subjected to continuous hot extraction in soxhlet apparatus. The extract was evaporated under reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample.

Phytochemical screening of *Nigella sativa* L. seed crude extracts

The preliminary phytochemical screening for principle bioactive compounds in methanolic extract of *Nigella sativa* black seeds was performed by standard methods **(Harbone, 2008; Jeffrey, 2007)**

In-vitro anti-oxidant activity of *Nigella sativa* L. seed

The *invitro* antioxidant activity of *Nigella sativa* Linn seed methanolic extract was carried out by 2, 2-Diphenyl-1-picrylhydrazyl radical DPPH free Radical Scavenging Activity **(Mansouri *et al*., 2005)**

Acute toxicity study

The acute toxicity of the extract of *Nigella sativa* seeds was carried out on experimental animals under the Organization for Economic Cooperation and Development **(OECD)** guidelines. Different doses were selected for further study. The *Nigella sativa* Linn seed methanolic extract was prepared in DMSO at the volume 1ml/100g b.w. of experimental

animals. Animals were fasted prior to dosing (only water was withheld overnight). On next day, the fasted body weight of each animal was determined and the dose was calculated according to the body weight. *Nigella sativa* L. seed methanolic Extract was administered orally to different groups (each of three animals) in increasing dose levels of 5, 50, 300, and 2000 mg/kg body weight.

Antidiabetic Activity & Dose study

Preparation of extract solution

The Methanol extract of *Nigella sativa* L*inn* black seeds was dissolved in normal saline (Parenteral, Drugs, Ltd. India**)** for oral administration. The solution of methanol extract was prepared at a dose of 200 and 400 mg/kg body weight.

Standard drug: Glibenclamide was dissolved in normal saline and was administered by oral route at a dose of 10 mg/kg bodyweight.

Induction of diabetes by using alloxan

After an overnight fasting, the mice were injected freshly prepared freshly prepared 2 % solution of alloxan monohydrate in 0.9 % sodium chloride solution (normal saline). The dose injected was 150 mg/kg body weight.

Procedure

The animals were divided into five groups consisting of six animals. Each group consisted of six animals. The animals were then subjected to the following treatments for 14 days.

Group I: Served as Normal control and received Distilled water for 14 days. Group II: Served as Diabetic control and received Alloxan (150 mg/ kg b.w. i.p) on first day + distilled water for 14 days. Group III: Alloxan (150 mg/kg. b.w. i.p.) on first day+ Glibenclamide 10mg/kg/day. Group IV: Alloxan (150 mg/kg. i.p.) on first day + Methanol extract (200 mg/kg/day. Group V: Alloxan (150 mg/kg. i.p) on first day + Methanol extract (400 mg/kg/day.

Antidiabetic evaluation of treatment groups

After one hour of alloxan administration, animals were given feed *ad libitum* and 1ml of (100 mg/kg b.w) glucose i.p. and 5 % glucose solution orally for 12 hours to combat ensuring severe hypoglycemia. After one week of alloxan injection mice were tested for evidence of diabetes by estimating their blood glucose level with the help of glucometer (Accu-check,

Roche Diagnostic, Germany) using strip method**.** The animals with blood glucose level above 200 mg/dl were considered as diabetic mice and were used for the experiment. The test samples were administered accordingly in a single dose by gavage using a stomach tube.

Blood sampling and Blood glucose level estimation

Blood samples were withdrawn from retro-orbital sinus under light ether (Merck, Mumbai, India) anesthesia, using heparinized capillaries and were measured immediately for glucose level determination on 0th, 7th and 14th day of experiment with the help of glucometer using strip method. **(Priyanka *et. al*., 2010; Prasad *et al*., 2009)**

Statistical analysis

All the data was expressed as MEAN ± SD. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni multiple comparison tests. Values were considered statistically significant at $p<0.001$.

Histopathological studies

Tissue preparation for histology

After sacrificing the experimental mice by cervical dislocation, pancreas tissues were collected, washed in normal saline and fixed by using fixative (picric acid, formaldehyde 40% and glacial acetic acid) for 24 hours and dehydrated with alcohol. The tissues were cleaned and embedded by using xylene and paraffin (melting point 58-600 C). Tissues were stained by double staining. To differentiate the nucleus and cytoplasm, the basic dye haematoxylin and the acid dye eosin were used (Kehar and Wahi 1967; Pearse 1968). Thin Sections were observed under microscope (Olympus 'CH20I' Trinocular) at 40X and 100X. Photographs of slides were taken using Sony digital camera attached to microscope.

Table 1: Organoleptic evaluation of *Nigella sativa* L. Black seeds.

Test Sample	Analysis Parameters					
	Colour	Appearance	Odour	Taste	Size	Shape
Black seed (*Nigella sativa* L.)	Black	Solid seeds	Aromatic	Bitter	Length 2.5-3.5mm width 1.5 to 2mm	Trigona, ovoid

Table 2: Organoleptic evaluation of *Nigella sativa* L. Black seed powder.

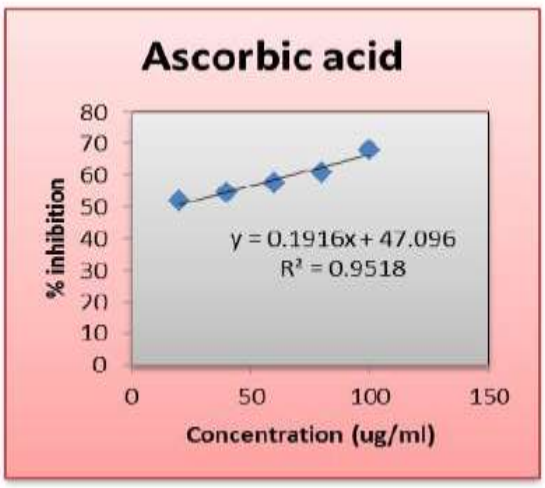
Test sample	Analysis parameters				
	Colour	Odour	Taste	Flavour	Touch
Black seed powder	Greyish-Black	Spicy	Oregano like	Pungent	Oil to touch

Table 3: percentage yield, phytochemical analysis, Physical evaluation of *N. sativa* L. seed methanolic extract.

Sample	Extract	% Yield	Tests	Results	Parameters	Observations
N. sativa L	Methanolic Extract	34.25 ±0.49	Test for alkaloids	Positive	Colour	Reddish brown
			Test for terpenoids	Positive	Odour	Sweet
			Test for flavonoids	Positive	Consistency	Sticky
			Test for saponins	Positive	Taste	Fruity
			Test for Protein and Amino Acids	Positive	Solubility	DMSO Soluble
			Test for carbohydrates	Positive		

Table 2: % Inhibition data of DPPH of free radical scavenging assay by Ascorbic Acid, methanol extract and IC_{50} value.

Conc. (µg/ml)	% Inhibition Of ascorbic acid	% Inhibition of Methanol	IC_{50} Value Ascorbic acid	IC_{50} Value Methanol extract
20	51.9461	45.6853	15.23 ug/ml	34.51 µg/ml
40	54.6407	51.269		
60	57.485	57.868		
80	60.9281	63.1134		
100	67.9641	67.6819		



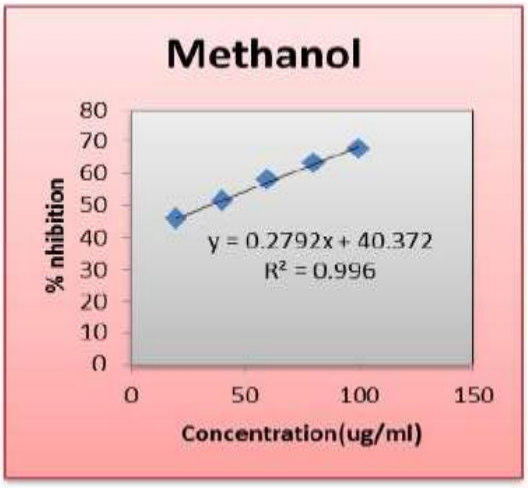


Fig. 1: Standard curve of A. acid by DPPH assay Fig.2: % DPPH inhibition curve of Methanolic extract.

Table 3: Assessment of acute oral toxicity study according to OECD 423 guidelines.

S. No.	Dose	No. of Animals	Mortality			
			24 hour	72 hour	7th day	14th day
1.	5 mg/kg	Three	0/3	0/3	0/3	0/3
2.	5 mg/kg	Three	0/3	0/3	0/3	0/3
3.	50 mg/kg	Three	0/3	0/3	0/3	0/3
4.	50 mg/kg	Three	0/3	0/3	0/3	0/3
5.	300 mg/kg	Three	0/3	0/3	0/3	0/3
6.	300 mg/kg	Three	0/3	0/3	0/3	0/3
7.	2000mg/kg	Three	0/3	0/3	0/3	0/3
8.	2000 mg/kg	Three	0/3	0/3	0/3	0/3

Table 4: Showing effect of *N. sativa* L. seed methanolic extract on blood glucose levels (mg/dl) in Normal and Alloxan-induced diabetic mice.

Group n= 6	Treatment	Dose	Blood glucose level (mg/dl)		
			0th DAY	7th DAY	14th DAY
Group I	Normal control	D/W	92.6±2.160	93.8±1.471	94.6±1.366
Group II	Diabetic control	150 mg/kg	283.8±1.561	291.6±2.447	297.1±1.474
Group III	Glibenclamide	10 mg/kg	289.6±2.266	208.3±2.157	142.7±3.407
Group 1V	MeOH Extract	200 mg/kg	289.5±4.375	255.2±3.180	214.7±4.273
Group V	MeOH Extract	400 mg/kg	288.6±2.825	226.1±3.576	177.8±4.745

Values are given as MEAN ± SD for groups of six animals *p<0.001 when compared to all groups with diabetic control group.

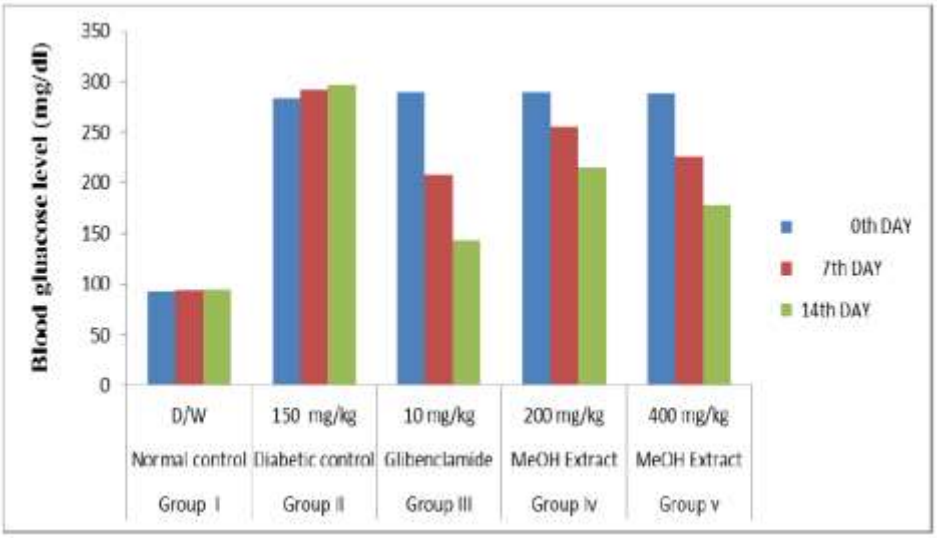


Fig. 3: showing variation in blood glucose levels (mg/dl) in various study groups on 0th, 7th and 14th Day.

DISCUSSION

The Organoleptic studies are the major and reliable criterion of identification of plant drugs. Macroscopical studies revealed that Seeds were flattened, oblong, angular, rugose tubercular, small, funnel shaped, 0.2 cm long and 0.1 cm wide. It had black colour, slightly aromatic odor and bitter taste. The black seed powder depicted its spicy odour, oregano like taste, pungent flavor. Almost similar observations were also reported by **Aqel and Shaheen, 1996.**

In recent years photochemistry has again become a field of active interest. Preliminary qualitative phytochemical analysis of *Nigella sativa* methanolic extract showed the presence of alkaloids, flavonoids, saponins, amino acids, and terpenoids. The results are in justification with the studies carried out by **(Brutis and Bucar 2000).**

Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. The herbal drugs with anti-diabetic activity are yet to be commercially formulated as modern medicines**.** Anti-diabetic drugs like Sulphonylurea (by increasing the insulin production from beta cells of Langerhans in pancreas) and biguanides (by working like insulin) play a critical role in treatment of diabetes, but due to failures in achieving ideal results, increasing side effects and high cost, there has always been a need and desire for a natural, more effective and economically feasible alternative with fewer side effects. Botanical remedy is one important way for this purpose because some of herbs have immense therapeutic effects without obvious side effects. Various synthetic anti-diabetic drugs with

their mechanism, site of action and side effects have been described **(G.B. Kavishankar *et al*., 2011).**

Antioxidants have a wide range of biological and pharmacological activities and are considered to be of great benefit in nutrition and health, as oxidative stress is an important factor in cell damage and it has been implicated in the development of diabetes, certain cancers and neurodegenerative diseases **(Newman *et al*., 2000)**. As antioxidants play an important role in inhibiting and scavenging radicals, thereby providing protection to humans against infection and degenerative diseases **(Ahmed 2005).** Flavonoids block free radical formation at several steps: by scavenging superoxide anions (in both enzymatic and non-enzymatic systems), by quenching intermediate peroxyl and alkoxyl radicals, and by chelating iron ions, which catalyze many Fenton reactions leading to free radical formation **(Musonda and Chipman 1998).**

The extract was investigated for its antioxidant activity using 2, 2-diphenyl-1-picryal-hydrazyl (DPPH). The antioxidant activity of the samples was expressed as IC_{50} values, the concentration (in µg/ml) of the standard or drug that inhibits the formation of DPPH radicals by 50%. The lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH radical scavengers. IC_{50} values were estimated from the percentage inhibition versus concentration plot, using a non-linear regression algorithm. Methanol crude extract of *Nigella sativa* Linn black seed showed strongest ability to act as DPPH radical scavengers by showing lower IC_{50} values of 34.51µg/ml for methanol extract.

For selection of dose acute oral toxicity was performed as per OECD 423 guidelines. This method is a stepwise procedure with the use of three animals per step. Depending on mortality and moribund status of the experimental animals, on average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test sample. In the present study the testing animals were given oral doses of 5mg/kg, 50mg/kg, 300mg/kg and 2000mg/kg b.w. 2000mg/kg b.w was considered as NOAEL (Not Observed Adverse Effect Limit), hence $1/10^{th}$ and $1/5^{th}$ of 2000mg/kg were calculated as 200mg/kg and 400mg/kg b.w and thus selected for evaluation of antidiabetic activity. In comparison with our results much higher LD50, i.e., 2.4 g/kg (2400mg/kg) has been reported in literature for *Nigella sativa* thymoquinone given orally **(Badary *et al*., 1998).** This difference might be due to the differences in the vehicle and the method used for the estimation of LD50. Our results present NsME as a relatively safe in the experimental animals, when given orally. These results are in

agreement with various former studies that revealed a high degree of safety in the acute administration of black seed extracts (**El-Daly 1998**) and essential oil (**Khanna *et al.* 1993**).

Alloxan, a urea derivation and beta cytotoxic cause's massive destruction of β-cells of the islets of langerhans resulting in reduced synthesis and release of insulin. This leads to hyperglycemia and diabetes **(Szuldelski, 2001).** Oral administrations of the *Nigella sativa* methanolic extract (NsME) with (200 and 400 mg/kg body weight) for 14 days in such mice caused gradual partial regeneration/proliferation of pancreatic beta-cells and decrease in the elevated blood glucose. Here we report that administration of alloxan resulted in the degeneration of β-cells in islets all over the pancreas. NsME given daily (200 mg/kg by gastric gavage) reduced blood glucose from **289.5** mg/dl before treatment to **255.2833** and **214.7833** mg/dl after the 7^{th} day and 14^{th} day respectively. NsME given daily (400 mg/kg by gastric gavage) reduced blood glucose from **288.6** mg/dl before treatment to **226.1833** mg/dl and **177.85** mg/dl after the 7^{th} day and 14^{th} day respectively.

From the experimental investigations and histopathological studies it can be said that *Nigella sativa* L. helps regeneration of beta-cells and stimulates insulin secretion from them. It can be thought that in the experimental diabetic mice NsME prevents lipid peroxidation and increases anti-oxidant defence system activity. The medicinal properties of plant could be based on the antioxidant properties of the phytochemicals in them **(Adesokan *et al*., 2008).**

CONCLUSION

The study dealt with antidiabetic potential of *Nigella sativa* so as to set the basis for utility of this plant in the treatment of diabetes. It clearly concludes that *N.sativa* L. seed extract, extracted by using the traditional Soxhlet approach are rich in bioactive compounds, containing alkaloids, saponins, flavonoids, and terpenoids. Additionally the methanolic extract was potent in terms of antioxidant potential. This could serve as a biotechnological tool for promoting it as a potential as well as natural antioxidant and antidiabetic agent. Hence synthetic antioxidants and antidiabetic drugs could be replaced by major bioactive compound from *N. sativa* Linn black seed.

The experiments have found use of *Nigella sativa* Linn seed extract safe in appropriate doses.The *Nigella sativa* being antihyperglycemic may increase the body's ability to produce insulin or to use insulin. The observations lead to a conclusion that herbal preparations of *Nigella sativa* may be effective, economical, and safe treatment for diabetics. The study

encourages the use of *Nigella sativa* for further pre-clinical and clinical trials for diabetes and other diseases may also be studied in safe doses in human patients through well designed clinical trials. However a word of caution will still be required. Longer duration studies of *Nigella sativa* L. and its isolated compounds on chronic models are necessary to elucidate the exact mechanism of action so as to develop it as a potent antidiabetic drug.

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