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ANTIDIABETIC POTENTIAL OF *HELIANTHUS ANNUUS* L. SEED EXTRACT: EVIDENCE FROM GLUCOSE UPTAKE, PKC MODULATION, OXIDATIVE STRESS, AND IN VIVO STUDIES

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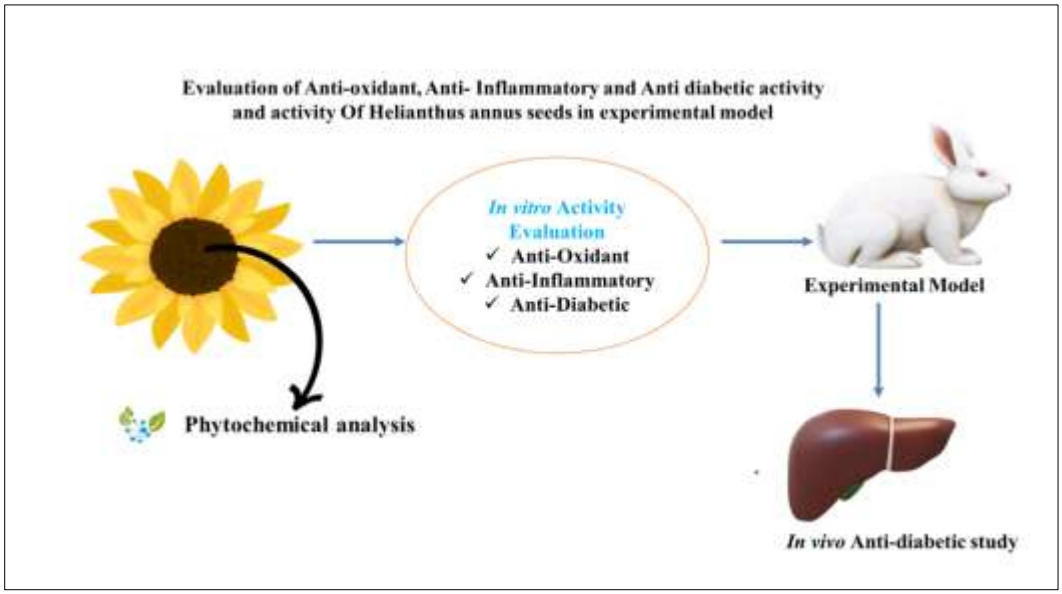
ABSTRACT

This study investigated the antioxidant, anti-inflammatory, and antidiabetic activities of Helianthus annuus L. (Sunflower) seeds. The plant material was collected from Murshidabad, West Bengal, and authenticated by the Botanical Survey of India. Seeds were processed through pulverization, defatting with n-hexane, and subsequent maceration with a 1:1 methanol-water mixture to obtain the extract. Phytochemical screening of the seed extract revealed the presence of various secondary metabolites, including alkaloids, glycosides, carbohydrates, tannins, flavonoids, and phenols.In vitro antioxidant assays, including reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and nitric oxide scavenging, demonstrated concentration-dependent antioxidant activity. The extract also exhibited anti-inflammatory effects by inhibiting heat-induced haemolysis and protein denaturation. The study further assessed the extract's impact on Nitric Oxide (NO) synthesis in liver and renal tissues, observing a reinforcing effect on NO production. Furthermore, In vitro studies on HepG2 and L6 cell lines indicated a concentration-dependent cytotoxic effect at higher concentrations and enhanced glucose utilization. Additionally, Lipid Peroxidation (LPO) assays in a high-fat diet and metformin-induced diabetic rat model demonstrated that while lower doses of the test substance increased kidney LPO, higher doses exhibited antioxidant properties by reducing LPO levels in both liver and kidney, approaching those of the normal control group. These findings suggest that Helianthus annuus L. seed extract possesses significant antioxidant and potentially antidiabetic properties, warranting further investigation into its therapeutic potential.

GRAPHICAL ABSTRACT:



KEYWORDS: *Helianthus annuus* L., Sunflower Seed, Hyperglycemia, Diabetes Mellitus, Glucose Uptake.

1. INTRODUCTION

The ongoing rise of chronic diseases significantly impacts global health, with diabetes mellitus at the forefront of this crisis. This metabolic disorder, characterized by persistent high blood sugar levels, has transcended geographical and socio-economic barriers, becoming a defining health issue of the 21st century [1]. Recent statistics paint a troubling picture. The International Diabetes Federation (IDF) estimates that in 2021, 537 million adults worldwide were living with diabetes, a number expected to increase to 783 million by 2045.This spectacular rise is as much a consequence of rising longevity and lifestyle change linked to urbanization and globalization as it points to the critical need for efficacious measures to prevent, manage, and finally, reduce the catastrophic impact of this disease. [2] The core issue driving this metabolic disorder is insulin resistance, where cells become less responsive to insulin, the hormone responsible for regulating glucose uptake and metabolism. This resistance is often linked to factors such as obesity, insufficient physical activity, and genetic predisposition, triggering a series of events [3]. Initially, the pancreas compensates by releasing more insulin, leading to hyperinsulinemia. However, as time goes on, the pancreas becomes less effective, insulin secretion decreases, and blood glucose levels begin to rise consistently, resulting in hyperglycaemia the defining characteristic of diabetes. [4]

Furthermore, chronic inflammation, oxidative stress, and the build-up of advanced glycation end products (AGEs) significantly contribute to the progression and complications of diabetes, perpetuating a damaging cycle of metabolic imbalance [5]. The hidden nature of diabetes reveals itself through a series of debilitating long-term complications that significantly diminish quality of life and contribute to premature death. Diabetic retinopathy, a leading global cause of blindness, results from damage to the blood vessels in the retina due to prolonged high blood sugar levels, leading to vision loss and ultimately, irreversible blindness. Diabetic neuropathy affects the peripheral nerves, causing symptoms like pain, numbness, tingling, and loss of sensation, particularly in the extremities, which increases the risk of foot ulcers and amputations [6]. Diabetic nephropathy, or kidney damage, can progress to end-stage renal disease, requiring dialysis or a kidney transplant. Furthermore, diabetes greatly heightens the risk of cardiovascular diseases, including coronary artery disease, stroke, and peripheral artery disease, driven by increased atherosclerosis and endothelial dysfunction [7]. While synthetic antidiabetic medications such as metformin, sulfonylureas, thiazolidinediones, and insulin form the cornerstone of diabetes management, their prevalence is not without drawbacks. Although these drugs can effectively manage blood sugar levels to varying degrees, they often come with a host of undesirable side effects. For instance, sulfonylureas may lead to hypoglycaemia, a dangerous condition where blood sugar drops too low, while thiazolidinediones are linked to weight gain, fluid retention, and cardiovascular risks.[8] Metformin is generally well tolerated but can cause gastrointestinal issues, and insulin therapy, while essential for many, requires careful monitoring and carries risks of hypoglycaemia and weight gain.[9] In addition to side effects, many synthetic medications are expensive, particularly the newer classes, creating significant barriers to access for patients in low- and middle-income countries. This situation underscores the pressing need for alternative and complementary therapies that are not only effective but also safer, more affordable, and potentially capable of addressing the complex mechanisms of diabetes [10]. The investigation of phytochemicals for their antidiabetic properties is not just a nostalgic nod to traditional methods; it is a scientifically valid and increasingly accepted strategy to tackle the diabetes crisis [11].

Among the botanical resources being studied for their therapeutic benefits, Helianthus annuus L., commonly known as the sunflower, emerges as a noteworthy candidate. While it is famous for its oil-rich seeds and bright flowers, the lesser-known phytochemicals found in its seeds offer significant promise for health and wellness [12-13]. Sunflower seeds are abundant in a variety of bioactive compounds, such as phenolic acids, flavonoids, terpenoids, and lignans. Research has shown that these phytochemicals exhibit a broad spectrum of pharmacological effects, including antioxidant, anti-inflammatory, and particularly antidiabetic properties. Notably, phenolic compounds like chlorogenic acid and caffeic acid, along with flavonoids such as quercetin and rutin, which are plentiful in sunflower seeds, have shown both In vitro and In vivo antidiabetic effects through various mechanisms. The rich array of bioactive compounds in Helianthus annuus seeds makes it a fascinating natural resource worthy of further exploration for its potential role in diabetes management [14-15]. The benefits of Helianthus annuus L. as a potential source of antidiabetic agents go beyond its rich phytochemical profile. Sunflowers are cultivated around the world, thriving in various climates and requiring minimal maintenance, which makes sunflower seeds both accessible and affordable. These seeds are already a popular food item, enjoyed globally for their nutritional benefits and pleasant flavour, indicating a level of safety and acceptance among consumers. Additionally, traditional medicine has long acknowledged the healing properties of different parts of the sunflower plant, including its seeds, although their specific role in managing diabetes may not be as well-documented as other uses. This combination of availability, cost-effectiveness, and potential for widespread use positions Helianthus annuus seeds as a promising candidate for developing new, accessible, and effective antidiabetic strategies, particularly in areas with high diabetes rates and limited resources. Consequently, this research project aims to thoroughly explore the antidiabetic potential of Helianthus annuus L. seeds [16-17].

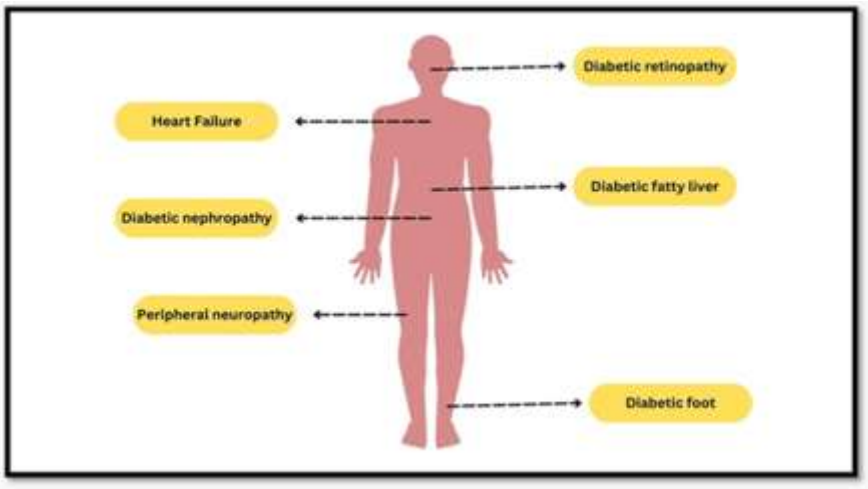


Figure 1: Systemic complications of Diabetes Mellitus

2. MATERIALS AND METHODS

2.1. Collection and authentication of plant

The whole fresh plant of Sunflower was collected in the month of August 2023 from village of Murshidabad district of West Bengal. The air-dried seeds were collected from UPL Limited, C/O Bharathi Bhramha Seeds Dist-Rangareddy, Telangana. The plant was identified and authenticated by the survey of Botanical Survey of India, Howrah with the authentication number JISU/2023/HA/001.

2.2. Extraction procedure

Helianthus annuus L. seeds were pulverized (300gm) and defatted with n-hexane (kept for 2 days) and the residual solvent was removed by air drying for 4-6 h at 40°C macerated with methanol (500ml) and with water(500ml) 1:1 for five days with continuous agitation and then the methanol was evaporated using rotary evaporator (40-45) °C and the final extract was collected.

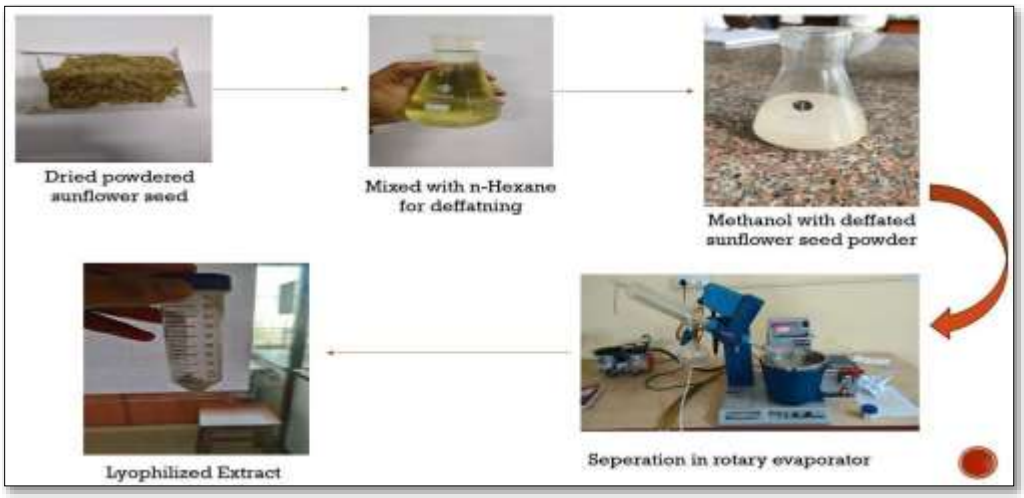


Figure 2: Extraction procedure of *Helianthus annuus* L. seed

2.3. Phytochemical analysis

The phytochemical screening of the seed and its extract involves a qualitative assay to identify various phytochemicals in the plant material. Phytochemical screening: Qualitative study of secondary metabolites. The qualitative phytochemical analysis [18] of the Helianthus annuus L. seeds extract was carried according to Kokate et al, 2005. The screening was conducted to identify the presence of various phytochemicals, including alkaloids, glycosides, carbohydrates, tannins, flavonoids, phenols, oils and fats were carried out using the protocol given by Raaman N. Raaman N.

2.4. Preparation of Test Sample
For conducting the following phytochemical tests, a 1mg/ml test solution was prepared. (10mg of crude extract was measured in a falcon tube and 10ml of distilled water was added to it).

2.5. Total Flavonoid content
The total flavonoid content of the crude extract was determined as described by Liu with some modifications. The TFC was determined from the linear equation y = 0.0086x + 0.071 of a standard curves prepared with quercetin and the amount of total flavonoid content in the extract was expressed in milligrams per gram of extract [19].

2.6. Total phenolic content
The total phenolic content of the crude extracts was determined as described by Javanmardi et al.The TPC was determined from the linear equation y = 0.0087x + 0.0645 of a standard curve prepared with Gallic acid and the amount of total phenolic content in the extract was expressed in milligrams per grams of extract [20].

3. IN VITRO ANTI-OXIDANT ACTIVITY
3.1. DPPH radical scavenging activity
The method described by Sánchez-Moreno et al. was used to assess the scavenging activity of DPPH free radicals. A 0.002% w/v DPPH solution was prepared in methanol. For the assay, 150 µl of each concentration of the extract was mixed with 150 µl of the DPPH solution, and control solutions for color correction were prepared without DPPH. The reaction mixtures were incubated in the dark for 30 minutes at room temperature, and UV-VIS spectrophotometer (Shimazu UV -1800) was used to observe absorbance at 517 nm. The antioxidant activity[21] was determined by this equation:
Percentage inhibition = [{absorbance of control – (absorbance of sample – colour factor)}/ (absorbance of control)]*100

3.2. Reducing power assay
The reducing power of the sample was assessed using the method developed by Oyaizu in 1986.The extract sample (1.0 mL) at various concentrations was combined with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [22]. This mixture was incubated at 50°C for 20 minutes. After the incubation, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 650 rpm for 10 minutes. Then, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% iron (III) chloride. Colour changes were monitored at 700 nm by a spectrophotometer. Ascorbic acid was used to compare the reducing power. The higher the absorbance, the better the reducing power of the sample [23]. The reducing power activity was determined by this equation:
Percentage inhibition = [{absorbance of control – (absorbance of sample – colour factor)}/ (absorbance of control)] *100

3.3. Nitric oxide radical scavenging assay
The inhibition of nitric oxide (NO) radical generation was estimated using Griess Illosvory reaction. The 3ml of reaction mixture contains 2ml of 10mM sodium nitroprusside, 0.5ml phosphate buffer saline and 0.5ml of the extract of leaves in different concentrations varying from 10-1000 µg/ml. product controls were taken for the different concentrations of to nullify the colour interference of the test sample. All the reaction mixtures were incubated in room temperature for 150 minutes. Thereafter 0.5ml of Griess reagent were added to each reaction mixture and again incubated for 30mins in room temperature. The concentration of nitrite was assayed at 540nm, calculated with the blank absorbance and percentage inhibitions were calculated using statistical formula [24].
Percentage inhibition = [{absorbance of control – (absorbance of sample – colour factor)}/ (absorbance of control)]* 100.

4. IN VITRO ANTI-INFLAMMATORY STUDY
4.1. Protein denaturation study using egg albumin
The reaction mixture included 0.2 mL of egg albumin (from a fresh hen's egg), 2.8 mL of phosphate-buffered saline (pH 6.4), and 2 mL of the test extract at varying concentrations (62.5 µg/mL, 125 µg/mL, 250 µg/mL, and 500 µg/mL). An equivalent volume of double-distilled water served as the control. The mixtures were incubated at 37°C ± 2°C in a biological oxygen demand incubator for 15 minutes and then heated at 70°C for 5 minutes. After cooling, the absorbance was measured at 660 nm using the vehicle as a blank. Diclofenac sodium was used as a reference, and traditional/herbal drugs were treated similarly to determine absorbance. The test extracts were selected to be as close as possible to the standard therapeutic mode. The percentage inhibition of protein denaturation was calculated by using the following formula [25] :-
Percentage inhibition = [(absorbance of control –absorbance of test sample)/ absorbance of control] ×100.

4.2. RBC membrane stabilization assay

The goat blood membrane stabilization method has been utilized to evaluate In vitro anti-inflammatory activity. Blood was collected from a goat and mixed with an equal volume of Alsever solution, which contains 2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl. The blood samples were stored at 4°C for 24 hours before use. The samples were centrifuged at 2500 rpm for 5 minutes, and the supernatant was discarded. The cell suspension was then washed with a sterile saline solution (0.9% w/v NaCl) and centrifuged again at 2500 rpm for 5 minutes. This washing process was repeated three times until the supernatant became clear and colorless. The packed cell volume was measured, and the cellular component was reconstituted to a 40% suspension (v/v) with phosphate-buffered saline (10 mM, pH 7.4) for use in the assays [26].

4.3 Heat induced haemolysis

A mixture consisting of 0.05 mL of goat blood cell suspension and 0.05 mL of hydro methanolic extracts of seeds was combined with 2.95 mL of phosphate buffer (pH 7.4). This mixture was incubated at 54°C for 20 minutes in a shaking water bath. Following incubation, the mixture was centrifuged at 2500 rpm for 3 minutes, and the absorbance of the supernatant was measured at 540 nm using a UV/VIS spectrometer. Phosphate buffer solution served as the control for the experiment [27].

Percentage inhibition = [{absorbance of control – (absorbance of sample – colour factor)}/ (absorbance of control)] *100

5. IN VITRO ANTI-DIABETIC STUDY

5.1. Cell Lines, Media, Reagents, and Assay Kits

The HepG2 liver cells and L6 myoblast cells were obtained from the American Type Culture Collection (ATCC). The G361 cell line was acquired from the National Centre for Cell Science (NCCS, Pune). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), 0.25 % Trypsin-EDTA, and Sodium pyruvate were procured from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 1% Penicillin- Streptomycin, Glutamine, Sodium Dodecyl Sulfate, MTT Cell Viability Assay kit, and FITC Annexin V/ Dead Cell Apoptosis Kit were obtained from Invitrogen, Thermo Fisher Scientific. Phosphate Buffer Saline (PBS) pH-7.4, Dimethyl Sulfoxide (DMSO), Sodium Bicarbonate, RPMI 1640, 2mM L-glutamine, 4.5 gm/L glucose, and Leibovitz's L-15 medium were purchased from HIMEDIA. Mouse Protein Kinase C Epsilon (PKCe) ELISA kit was procured from KRISHGEN BioSystems, Mumbai. Selegiline and Trypan Blue were purchased from Sigma-Aldrich (Saint-Louis, USA).

5.2. Maintenance of Cell Cultures

The culture of both of the cell lines were incubated at 37°C in a humidified environment with 5% CO2. The HepG2 cells were replenished with growth medium every 2-3 days, consisting of Eagle's Minimum Essential Medium (EMEM), supplemented with 10% of fetal bovine serum. The L6 cell lines were cultured in a medium consists of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% of fetal bovine serum.

5.2.1. MTT Cytotoxicity Study

The cytotoxicity assay was performed where HepG2 cells and L6 cells were cells were seeded at a density of 4×10(5) cells in 96-well plates with medium EMEM & DMEM respectively. The cells were allowed to get attached in the plate overnight and 100 µl of plant extract at various concentrations (10, 25, 50, 100, 200 µg/ml) were added to the specific well. The cells were allowed to have an incubation period of 48 hours with the extracts and then the spent medium was removed by aspiration and 100 µl of EMEM medium (HepG2) or DMEM medium (L6 myoblasts) containing 10% fetal bovine serum and 0.5 mg/ml MTT was added and further incubated for 3 hours at 37°C. After 3 hours the medium was removed and MTT crystals (Purple Formazan) were further dissolved into DMSO (Dimethyl sulfoxide) and absorbance was checked at 540 nm [29, 30].

5.2.2. Glucose Uptake Assay

The glucose uptake assay was done according to the method explained by Chanon S et al,2017 [31] HepG2 and L6 cells that were seeded in different culture plates at 1 × 105 cells/mL which were allowed to adhere to the plate and let that grow at 37°C for 24 hours in a humidified incubator supplemented with 5% CO2. The cells were preincubated at 37 °C for 24 hours with different concentrations (10, 25, 50, 75, 100 µg/ml) of plant extract. After that the spent medium was removed by aspiration and replaced with 25 µl of new culture medium like EMEM (HepG2) and DMEM (L6) and incubated for 3 hours at 37 °C. The both of the culture medium was diluted with 10-20 mM glucose, 0.1% Bovine Serum Albumin (BSA), and Phosphate-Buffered Saline (PBS) before it was incubated at 37 °C for 3 hours. Glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added to each well, incubated for 10-20 minutes and the absorbance was checked at 510 nm [32].

5.3. Estimation of cellular Phosphorylated PKC

The phosphorylated PKC estimation was done by commercially available sandwich ELISA kit using HepG2 cell lines. The HepG2 cells were cultured and grown in EMEM medium which was supplemented with 10% fetal bovine serum and incubated in humidified environment in 5% CO2. When the cells were grown up-to 90% confluence, the subculturing was done. Lipo-polysaccharide (LPS) was used to induce the intracellular inflammatory response to generate phosphorylated pkc. The sub- cultured cells were incubated for 24 hours at 37°C with the plant extract of various concentrations (10, 25, 50, 100 µg/ml). After incubation the inducing agent LPS was added at each well at concentration 25µg/ml [33].

5.4. DPP-IV Inhibition Assay

The experiment took place using 96-well microplates. A preincubation mixture (50 mL) was made with 35 mL Tris-HCl buffer and 15 mL DPP-IV enzyme. Various concentrations (mg/mL) ofextracts were also added. After waiting for 10 minutes at 37°C, 50 mL of gly-pro-p-nitroanilide was added as the substrate. The mixture was then incubated for another 30 minutes at 37°C. The absorbance was then measured every 10 seconds using a spectro-flurimetric reader at a wavelength of 405 nm [34].

6. *IN VIVO* ANTI-DIABETIC STUDY

6.1. Procurement and acclimatization of animals

The research was carried out at TAAB Biostudy Services, Kolkata, India using healthy adult male Albino Wistar rats weighing between 100 and 150 grams. The rats were housed in polypropylene cages lined with husk, maintained under standard environmental conditions: a temperature of 25 ± 2°C, relative humidity of 55 ± 10%, and a 12-hour light-dark cycle. They were on high fat diet with free access to water Animal experiments were approved by Institutional Animal Ethics Committee, TAAB Biostudy Services. Approval from Animal Ethical Committee for animal study (Reg No. 193/PO/Rc/S/17/CPCSEA).

Table 1: Composition of High Fat Diet [35].

Sl. No.	Ingredient	Quantity (g)	Energy (kcal)
1	Wheat Flour	100	362
2	Gram Flour	100	371
3	Sugar	100	400
4	Milk Powder	100	499
5	Coconut Oil	130	1170
6	Mineral and Vitamin Mix	5	0
Total		**535**	**2802**

6.2. Streptozotocin-High Fat Diet induced type 2 diabetes model

Animals were divided into two groups: the control group fed with standard Diet and the experimental group fed with high-fat diet (HFD). Indeed, to induce T2DM, the animals in the experimental group were fed an HFD containing 15% carbohydrate, 20% protein and 75% fat for 4 weeks followed by an intraperitoneal injection of STZ (35 mg/kg suspended in 0.1 mol/L citrate buffer at pH 4.5). Rats were fed with HFD for 4 weeks to induced insulin resistance, and thereafter administered with 35 mg/kg of STZ to induce diabetes. Seventy-two hours (72 h) after the injection of STZ, the blood glucose of the animals was measured and only the rats with a blood glucose greater than or equal to 200 mg/dL were used for the experiment. The diabetic rats were administered a 5% glucose solution orally to prevent hypoglycemia for the first 24 h after the STZ injection. Rats were starved for 16 h before receiving STZ [36].

6.3. Estimation of Fasting Blood Glucose (FBG) and Body Weight

Every week the fasting blood glucose (FBG) level of the rats were measured by using a glucometer (Accu-Chek Active, Roche, Mannheim, Germany) where the rats were kept at 12 hours of starvation. Fasting Blood glucose level, Body weight, food and water consumption of animals were evaluated on the 1st, 3rd , 7th, 14th, 21st and 28th days of the experiment. The blood was collected through tail snip method where the tail was anesthetized using local anaesthetic ointment.

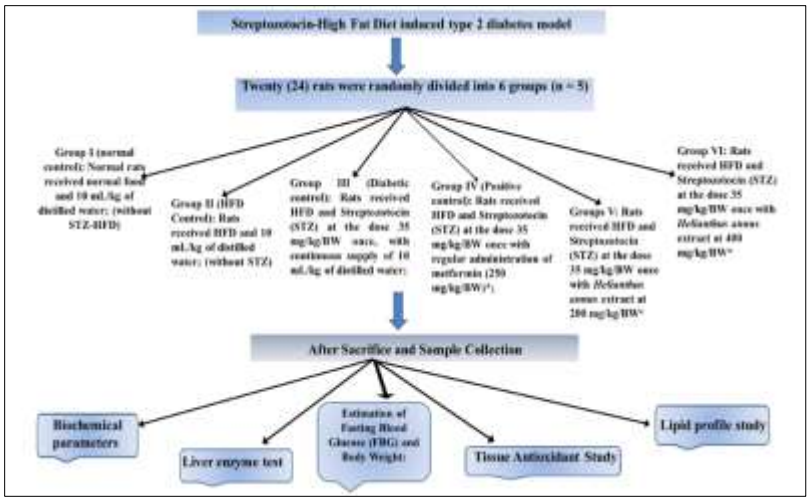


Figure 3: Group division and treatment schedule of STZ-HFD induced anti-diabetic study of the *Helianthus annus* L. seed extract

6.4. Biochemical parameters

For evaluating the various biochemical parameters, all animals were sacrificed after 28 days of treatment. Blood was collected by the method of cardiac puncture and the serum was collected by the centrifugation. Biochemical parameters, such as SGPT, SGOT, High density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol (TC) levels were also determined using the collected blood serum [37].

6.5. Tissue Antioxidant Study

6.5.1. Nitric oxide Assay

100 µL of 10% tissue (liver & kidney) homogenate in phosphate buffer was mixed with 500 µL of Griess reagent. It was incubated for 30 min at room temperature and absorbance was measured at 540 nm. Amount of nitric oxide was calculated with the help of the standard curve plotted with sodium nitrite in respect to the amount of protein present in tissue.

6.5.2. Lipid Peroxidation Assay

0.5 mL of 10% tissue (liver or kidney) homogenate in phosphate buffer was added to 1 ml of TBARS solution. The solution was heated in boiling water for 30 min and then cooled. It was then centrifuged at 3000 rpm for 15 min. The absorbance of supernatant measured at 532 nm against blank. Amount of malondialdehyde (MDA) was calculated with the help of the standard curve plotted with malondialdehyde (MDA) in respect to the amount of protein present in tissue [37].

6.6. Statistical Analysis

Experimental results were obtained as the mean value ± standard deviation (SD) (n = 5). Statistical analyses were performed using the SPSS statistical package (SPSS, Inc., Chicago, IL). A paired sample t test was applied. The statistical significances were achieved when $p < 0.05$.

7. RESULT

7.1. Phytochemical screening: Qualitative study of secondary metabolites

The preliminary phytochemical study of the extract of *H. annus* revealed the presence or absence of various secondary metabolite. The study has shown the presence of Carbohydrates, Phenol, Tannin, Alkaloids, flavonoids, in the methanolic extract of *H.Annus*.The extract did not show any positive result for glycoside.

Table 2: Identification of various secondary metabolites of the seed extract of *Helianthus annus*

Sl. No.	Secondary Metabolites	Result
1	Carbohydrates	+
2	Phenol	+
3	Glycoside	-
4	Tannin	+
5	Alkaloids	+
6	Flavonoid	+

7.2. Total Phenolic Content

The phenolic content was estimated according to the linear calibration curves of standard compounds Gallic acids standard curve. The total phenolic content was compared with the gallic acid standard and estimated 53.1 ug GAE/mg of crude extract using the formula y = 0.0087x + 0.0645 where R^2 value is 0.9812.

7.3. Total Flavonoid Content

Total flavonoids content (mg/gm) concentration was calculated in terms of quercetin equivalent using standard curve equation of quercetin. Y=0.0105X – 0.3686

Total Flavonoids phenolic content in terms of quercetin is 48.25mg/ml or 120.62mg/gm.

7.4. In vitro Antioxidant Activity

7.4.1. Reducing Power Assay

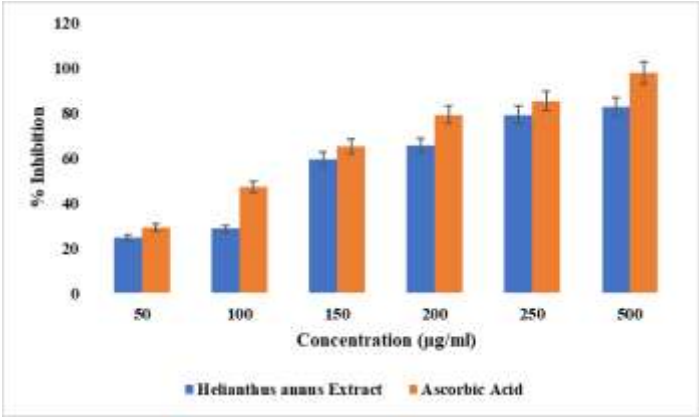


Figure 4: In vitro estimation of antioxidant potential of *Helianthus annuus* seed extract, using the method reducing power assay. Data were expressed as Mean ± Standard Deviation

The reducing power assay of Helianthus annuus seed extract demonstrated a concentration-dependent increase in antioxidant activity. As the concentration of the extract increased from 50 to µg/mL, the percentage of reducing power also progressively increased, indicating a stronger 500 ability to donate electrons and neutralize free radicals. At the lowest concentration tested (50 µg/mL), the reducing power was approximately 28–30%, whereas at the highest concentration (500 µg/mL), it reached around 95–100%. This trend suggests that the antioxidant capacity of the seed extract enhances with increasing concentration. The inhibitory concentration (IC_{50}), which is the concentration required to achieve 50% reducing power, can be estimated between 100 and 150 µg/mL based on the graph, where the % reducing power crosses the 50% mark. These results highlight the potent antioxidant potential of Helianthus annuus seed extract

7.4.2. DPPH Radical Scavenging Activity

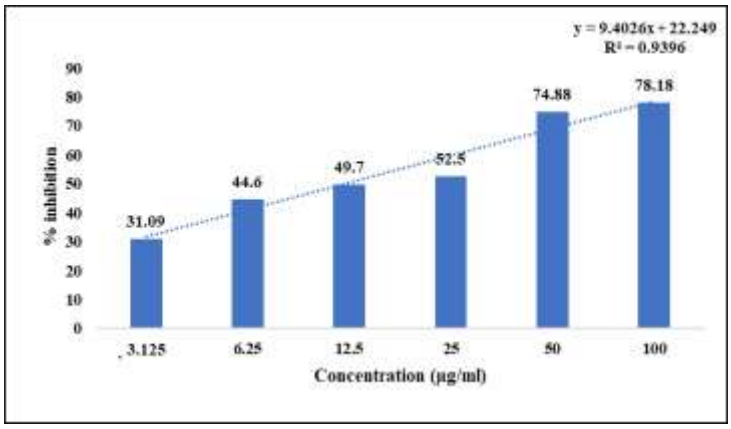


Figure 5: In vitro estimation of inhibitory potential of the seeds of *Helianthus annuus* by DPPH radical scavenging activity. Data were expressed as Mean ± Standard Deviation

The *Helianthus annuus* seed extract demonstrated a concentration-dependent increase in DPPH radical scavenging activity. The percentage inhibition increased from 31.09% at 3.125 µg/mL to 78.18% at 100 µg/mL, with the highest activity observed at 100 µg/mL. The linear regression analysis showed a good correlation between concentration and radical scavenging activity ($R^2 = 0.9396$), indicating a strong concentration-dependent antioxidant effect.

7.4.3. Nitric Oxide Scavenging Assay

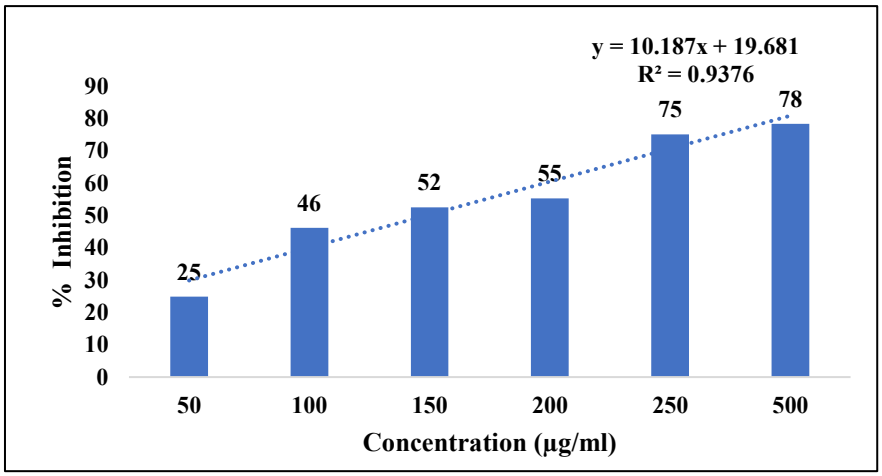


Figure 6: In vitro estimation of anti- oxidant potential of *Helianthus annuus* seed extract, using the method nitric oxide assay. Data were expressed as Mean ± Standard Deviation (n=3)

At the lowest concentration tested, 50 µg/mL, the extract demonstrates an inhibitory potential of approximately 24%. As the concentration increases to 100 µg/mL, the inhibitory activity nearly doubles to around 46%. Further increments in concentration continue to enhance the antioxidant potential. At 150 µg/mL, the inhibition is approximately 53%, and at 200 µg/mL, it reaches about A more substantial increase in inhibition is observed at 250 µg/mL, where it climbs to %55 approximately 75%. The highest concentration tested, 500 µg/mL, exhibits the most significant inhibitory potential, reaching around 80%

7.5. *In vitro* Anti-Inflammatory Study

7.5.1. Heat Induced Hemolysis

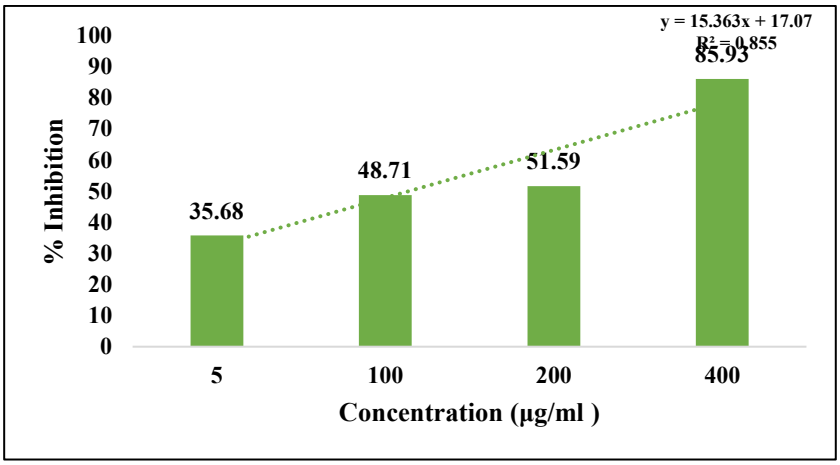


Figure 7: In vitro estimation of anti- inflammatory potential of *Helianthus annuus* seed extract, using the method heat induced haemolysis. Data were expressed as Mean ± Standard Deviation

At the lowest concentration of 50 µg/mL,the Helianthus annuus extract exhibits an inhibitory effect of approximately 37% on heat-induced haemolysis.As the concentration is doubled to 100 µg/mL,the inhibitory potential increases significantly to around 50%. Further increasing the concentration to 200 µg/mL results in a more pronounced anti-inflammatory action, with the inhibition reaching approximately 67%. The highest concentration tested,400 µg/mL, demonstrates the most substantial inhibitory effect on haemolysis, reaching approximately 87%.

7.5.2. Protein Denaturation Study Using Egg Albumin

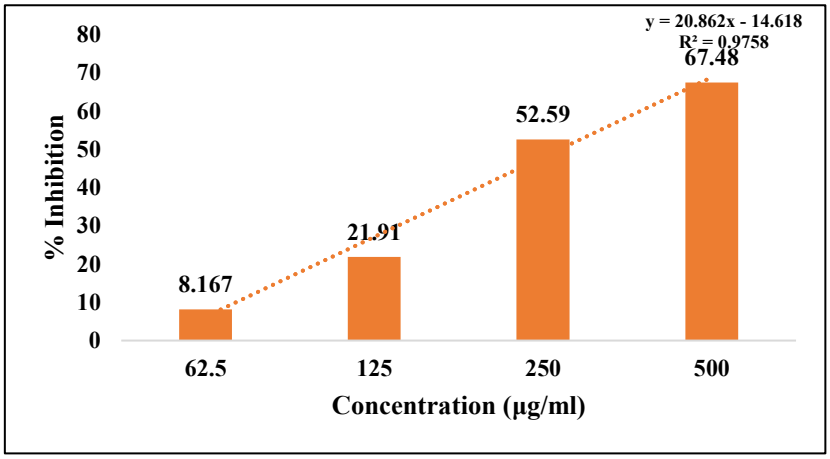


Figure 8: In vitro estimation of anti- inflammatory potential of *Helianthus annuus* seed extract, using the method protein denaturation study using egg albumin. Data were expressed as Mean ± Standard Deviation

The in-vitro anti-inflammatory potential of *Helianthus annuus* seed extract, as evaluated by the protein denaturation study using egg albumin, across various concentrations. At the lowest concentration tested, 62.5 µg/mL, the *Helianthus annuus* seed extract exhibits a relatively low inhibitory effect on protein denaturation, approximately 9%. As the concentration is doubled to 125 µg/mL, the inhibitory potential increases significantly to around 43%. A further increase in concentration to 250 µg/mL results in a more pronounced anti-inflammatory action, with the inhibition reaching approximately 54%. The highest concentration tested, 500 µg/mL, demonstrates the most substantial inhibitory effect on protein denaturation, reaching approximately 69%.

7.6. *In vitro* Cell Viability Study of HEPG2 Cell Line

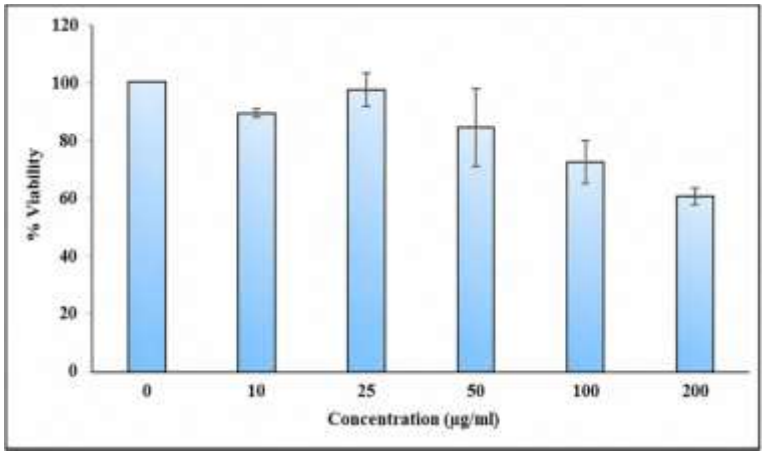


Figure 9: Cell viability screening of extract of *Helianthus annuus* seeds at different concentrations. Values are expressed as the mean ± Standard Deviation

The in-vitro cell viability of the HepG2 cell line following treatment with varying concentrations of a test drug i.e. extract of *Helianthus annuus* seeds. In the absence of the test substance (0 µg/mL), the cell viability is considered 100%, representing the normal growth of the HepG2 cell line. Upon exposure to a concentration of 10 µg/mL, a slight decrease in cell viability is observed, dropping to approximately 90%. Interestingly, at a concentration of 25 µg/mL, the cell viability recovers slightly, reaching around 98%, almost comparable to the control. However, as the concentration of the test substance further increases, a progressive decrease in cell viability is noted. At 50 µg/mL, the viability drops to approximately 85%, and at 100 µg/mL, it further declines to about 73%. The highest concentration tested, 200 µg/mL, results in the most significant reduction in cell viability, with only about 62% of the HepG2 cells remaining viable. This suggests that the test substance exhibits a concentration-dependent cytotoxic effect on the HepG2 cell line at concentrations above µg/mL 25

7.7. *In vitro* Cell Viability Study of L6 Cell Line

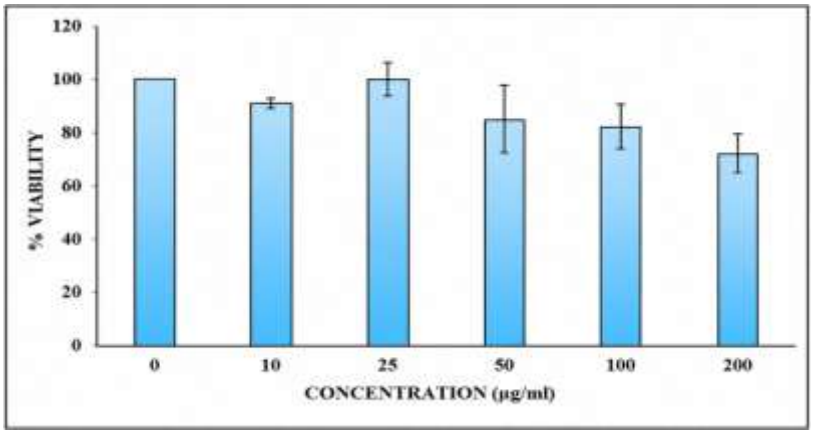


Figure 10: Cell viability screening of extract of *Helianthus annuus* seeds at different concentrations. Values are expressed as the mean ± Standard Deviation

The in-vitro cell viability of the L6 cell line following treatment with varying concentrations of an extract of *Helianthus annuus* seeds, in comparison to a control (0 concentration) which presumably represents untreated cells or cells treated with a vehicle. In the absence of the seed extract (0 µg/mL), the cell viability is at 100%, representing the normal viability of the L6 cell line under basal conditions. Upon exposure to the *Helianthus annuus* seed extract at a concentration of 10 µg/mL, a slight decrease in cell viability to approximately 91% is observed. Interestingly, at 25 µg/mL, the cell viability appears to recover and is recorded at approximately 100%, similar to the control. However, as the concentration of the seed extract increases further, a trend of decreasing cell viability is evident. At 50 µg/mL, the viability drops to around 85%, and at 100 µg/mL, it further decreases to approximately 82%. The highest concentration tested, 200 µg/mL, results in the most significant reduction in L6 cell viability, with approximately 73% of the cells remaining viable. This suggests that while lower concentrations (up to 25 µg/mL) of the Helianthus annuus seed extract do not significantly impact the viability of the L6 cell line, higher concentrations (50 µg/mL and above) exhibit a dose-dependent decrease in cell viability.

7.8. Glucose Utilization Assay of HepG2 Cell Line

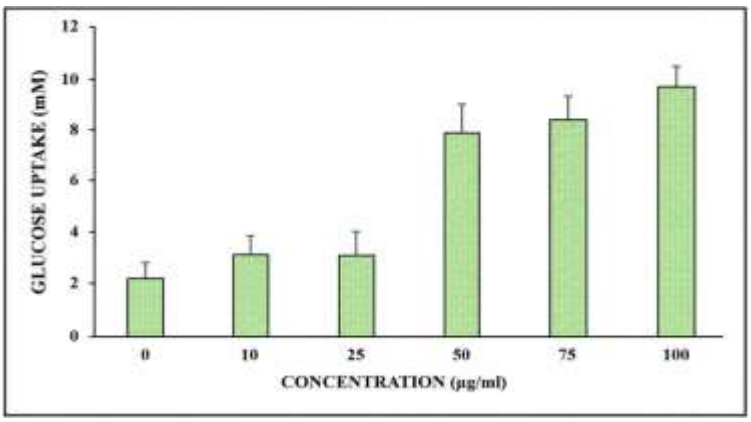


Figure 11: Effect of the different concentrations of extract of *Helianthus annuus* seeds in the glucose utilization assay. Values are expressed as the mean ± Standard Deviation

In the control group (0 µg/mL), the glucose uptake is approximately 3.7 units. At a concentration of µg/mL of the Helianthus annuus seed extract, the glucose uptake slightly increases to around 4.0 10 units. A similar level of glucose uptake, approximately 4.1 units, is observed at a concentration of µg/mL. However, as the concentration of the extract increases further, a more pronounced 25 increase in glucose utilization is evident. At 50 µg/mL, the glucose uptake rises to approximately units. This trend continues at 75 µg/mL, where the glucose uptake is approximately 7.9 units, 6.0 and reaches its highest point at 100 µg/mL, with a glucose uptake of approximately 9.2 units. These results suggest a concentration-dependent increase in glucose utilization by the HepG2 cell line in the presence of the Helianthus annuus extract.

7.9. Glucose Utilisation Assay of L6 Cell Line

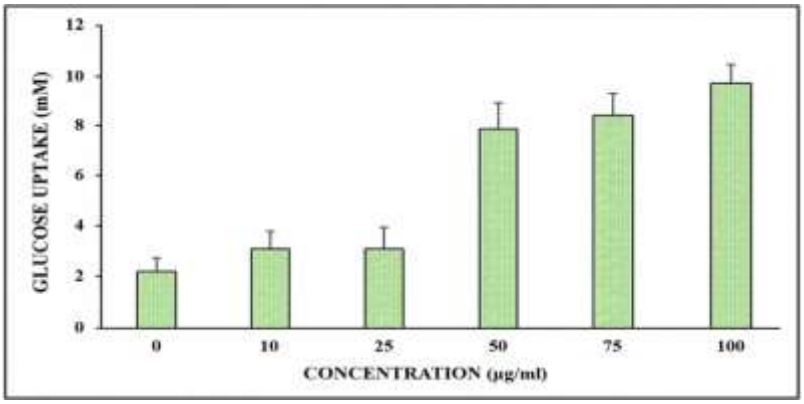


Figure 12: Effect of the different concentrations of extract of *Helianthus annuus* seed in the glucose uptake. Values are expressed as the mean ± Standard Deviation

The effect of varying concentrations (0, 10, 25, 50, 75, and 100 µg/mL) of Helianthus annuus seed extract on glucose uptake in the L6 cell line, as determined by a glucose utilization assay. The leftmost bar represents the control group with no extract (0 µg/mL), exhibiting the lowest glucose uptake at approximately 2.3 units. As the concentration of the Helianthus annuus extract increases from 10 to 25 µg/mL, a modest rise in glucose uptake is observed, reaching around 3.3 and 3.4 units, respectively. A more substantial increase in glucose uptake is evident at higher concentrations. At 50 µg/mL, glucose uptake significantly increases to approximately 8 units. Further increases in concentration to 75 and 100 µg/mL show a continued upward trend in glucose uptake, reaching approximately 8.6 and 9.9 units, respectively. The error bars indicate the standard deviation, suggesting variability within each concentration group. Overall, the data suggests a dosedependent increase in glucose uptake in L6 cells in the presence of Helianthus annuus extract, indicating its potential to enhance glucose utilization in these cells

7.10. Intra-Cellular PKC Estimation in HEPG2 Cell Line

To explore the molecular mechanism of the extract, the intracellular level of phosphorylated pkc was estimated in HepG2 cell line. The aim of this study was to identify the how much inhibition of phosphorylation of pkc can be obtained with the increasing concentration of the extract.

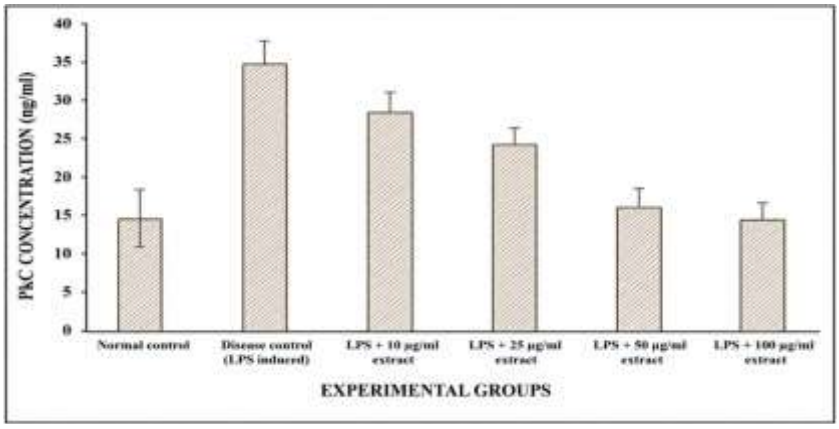


Figure 13: Intra-cellular pkc estimation of the methanolic extract seeds of *Helianthus annus* in HepG2 cell line. Data are expressed as Mean ± Standard Deviation

The intracellular protein kinase C (PKC) estimation in the HepG2 cell line under various treatment conditions involving a methanolic extract of *Helianthus annuus* seeds and an LPS-inducing agent. The first bar on the left represents the negative control, showing a baseline PKC value of approximately 14.5. The second bar illustrates the effect of the LPS-inducing agent alone, leading to a significant increase in PKC activity, reaching a value of around 34. The subsequent bars depict the PKC levels in cells treated with LPS in combination with different concentrations (10, 25, 50, and µg/mL) of the Helianthus annuus seed extract. At a concentration of 10 µg/mL of the extract, 100 the PKC value is reduced to approximately 28.5 compared to the LPS-induced group. As the concentration of the extract increases to 25 µg/mL, a further reduction in PKC activity is observed, down to about 24.5. Notably, at higher

concentrations of 50 and 100 µg/mL of the *Helianthus annuus* seed extract, the PKC levels are substantially lower, registering around 16 and 14.5, respectively. These values are approaching the baseline level observed in the negative control. The error bars indicate the standard deviation within each treatment group. Overall, the results suggest that the methanolic extract of Helianthus annuus seeds exhibits a concentration-dependent inhibitory effect on LPS-induced intracellular PKC activity in the HepG2 cell line.

8. *IN VIVO* STUDY

8.1. Lipid Profile Study

8.1.1. Estimation of Total Cholesterol, LDL Cholesterol, HDL Cholesterol Profile

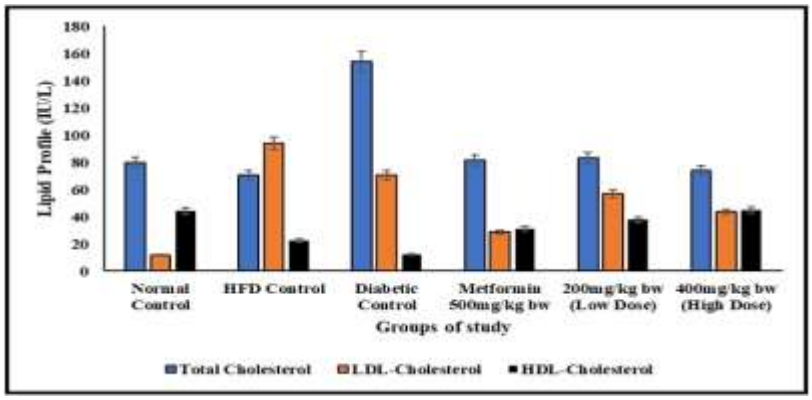


Figure 14: Estimation of Total Cholesterol, LDL Cholesterol, HDL Cholesterol of different group of rats

When compared to the diabetic control, treatment with the hydro-alcoholic extract of Helianthus annuus seeds for four weeks considerably decreased total cholesterol and LDL levels in a dose- dependent manner, according to estimates of the serum lipid profile in different groups of rats. Rats treated with Helianthus annuus seed extract had lipid levels that were comparable to those of the normal, healthy control group.

8.2. Tissue Antioxidant Study

8.2.1. Nitric Oxide (NO) Assay

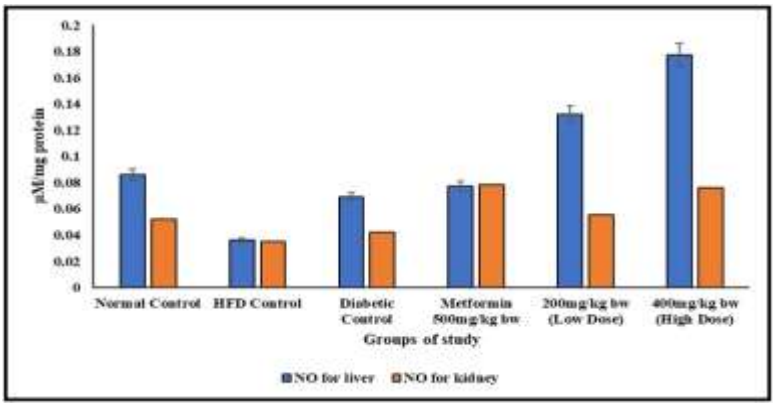


Figure 15: The estimation of the amount of Nitric oxide levels in Liver and kidney of different groups of experimental rats (n=5)

Baseline NO levels in the liver and renal tissues were found to be moderate in the Normal Control group. NO levels in both organs were significantly lower in the HFD (High-Fat Diet) Control group, suggesting potential oxidative stress and compromised NO production. Similar to the HFD group, the Diabetic Control group showed slightly higher liver NO but lower kidney NO, indicating tissue-specific responses to diabetes. NO levels in the liver and kidney tissues increased after receiving 500 mg/kg body weight of metformin, suggesting that oxidative equilibrium had been restored. Remarkably, liver NO levels significantly increased and kidney NO levels somewhat improved after treatment with test substance at a considerably a low dose (200mg/kg of body weight). Now reassuringly the Test substance at a higher dose (400mg/kg of body weight) was not only associated with the production of NO but achieved the highest levels of NO synthesizing in liver tissue and significantly in renal tissue as well, this effect was further reinforced

8.2.2. Lipid Peroxidation Assay

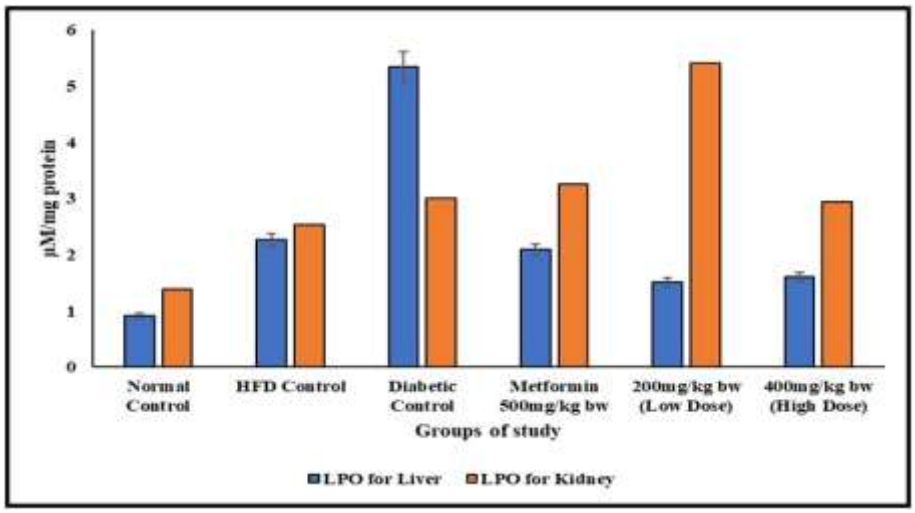


Figure 16: Estimation of the lipid peroxidation (LPO) in liver and kidney tissues of various groups of experimental rats (n=5)

LPO acts as a marker of oxidative stress and cellular membrane damage. Under typical physiological circumstances, the Normal Control group has the lowest amounts of LPO in the liver and kidney, indicating less oxidative stress. On the other hand, the LPO levels in the HFD Control and Diabetic Control groups are noticeably higher, particularly in the Diabetic Control group, where liver LPO peaks, suggesting considerable oxidative damage in diabetic settings. Compared to the Diabetic Control, treatment with metformin at 500 mg/kg body weight significantly lowers LPO levels in both tissues, indicating its protective and antioxidant properties. It's interesting to note that the test substance exhibits a dose-dependent reaction. It significantly raises kidney LPO at Lower doses (200 mg/kg of body weight), more than any other group, which may suggest a pro-oxidant effect at this dosage. However, at higher doses (400 mg/kg body weight), test substance treatment reduces LPO levels in both liver and kidney, approaching levels seen in the Normal Control group

9. DISCUSSION AND CONCLUSION

Herbal medicines made from plants have been the subject of more research in recent years because they are thought to be more effective than synthetic treatments and have fewer adverse effects. Given its possible therapeutic benefits and historical medical uses, research on *Helianthus annuus'* phytochemical components and pharmacological activities has attracted a lot of interest. Researchers seek to determine the bioactive substances that contribute to its therapeutic effects and clarify the underlying mechanisms of action by analyzing its phytochemical makeup and assessing its pharmacological activity. This study is essential to expanding our knowledge of *Helianthus annuus* and its possible uses in contemporary medicine. The phytochemical screening revealed that the hydro alcoholic seed extract has a total phenolic content is 53.1 ug GAE/mg and total flavonoid content 48.25mg/ml suggesting us that the extraction method used was effective in isolating phenolic and flavonoid compounds, demonstrating that our technique was appropriate for maximizing yield and maintaining the integrity of the compounds. This efficiency is essential for the reproducibility and scalability of extracting bioactive compounds for therapeutic applications[38].

The substantial presence of phenolics and flavonoids may be linked to the observed pharmacological activities, such as anti-inflammatory and antioxidant effects. These bioactive compounds are known to help reduce oxidative stress and inflammation, which are fundamental contributors to many chronic diseases [39]. The DPPH radical scavenging assay was used in the current work to evaluate the In vitro antioxidant activity of the sunflower seed hydroalcoholic extract, and the results showed great promise. The hydroalcoholic extract was found to significantly scavenge free radicals in a dose-dependent fashion with an IC50 of 150 µg/ml, the Nitric oxide assay also gave us sufficient proof that it possesses antioxidant qualities that may be advantageous. The IC50 value was found to be 12.5 µg/ml. The current study also demonstrated the antioxidant properties of the *Helianthus annuus* extract in comparison to ascorbic acid, a standard antioxidant. Higher reducing power was shown by increased absorbance in the reaction mixture. Glucose uptake serves as a vital biological indicator for evaluating the effects of activators or inhibitors on cell culture, assessing their influence on glucose utilization, and the cell's responsiveness to insulin. The described method has been validated as rapid and dependable, and it has been extensively utilized in numerous studies employing primary myotubes obtained from both healthy individuals and patients with metabolic disorders [40]. Certain phytochemicals enhance the sensitivity of non-pancreatic cells to insulin, thereby improving glycemic control. The possible

mechanism of action is in skeletal muscle and adipose tissue, increased insulin levels trigger a cascade of events that enhance glucose uptake. Insulin binding to its receptors initiates the phosphorylation of protein substrates, activating phosphatidylinositol 3-kinase (PI3K) and subsequent signalling via PKB/Akt and PKC-λ/ζ pathways. Consequently, GLUT4, an insulin-regulated glucose transporter protein, is mobilized to the cell membrane, facilitating the uptake of circulating glucose by muscle cells and adipose tissue through facilitated diffusion mediated by GLUT4 transporter proteins [41]. According to the study, there is a noticeable exponential increase in glucose absorption beginning at 50 µg/ml. The rise in glucose uptake was constant when comparing the outcomes of the two cell lines. But when comparing glucose uptake to the negative control (no medication was given), the glucose level in HepG2 cells increased by 61.13%, while in the L6 cell line, it increased by 77.31%, which is extremely encouraging. In comparison to diabetic control rats, diabetic rats who received oral HAE at doses of 200 mg/kg and 400 mg/kg in addition to Metformin showed a significant drop in blood glucose levels from the first to the fourth week. Additionally, diabetic rats exhibited reduced insulin levels compared to normal control rats, indicating potential STZ-mediated beta cell destruction or damage. These findings suggest that the hypoglycemic effect of HAE may be attributed to its protective action against STZ-induced damage to pancreatic beta cells, which could involve the regeneration of damaged beta cells and the inhibition of gluconeogenesis and glycogenesis. Diabetes induced by STZ was associated with a significant decline in body weight, indicating loss or degradation of structural proteins due to the condition. Structural proteins play a role in maintaining body weight. The biochemical parameters of the liver and kidney in diabetic rats indicate the potential of our extract as an effective anti-diabetic agent. This observation suggests that our extract not only helps in managing blood glucose levels but also contributes to maintaining the health and function of vital organs affected by diabetes. Further studies focusing on these biochemical markers can provide more insights into the therapeutic benefits and mechanisms of action of our extract.

In conclusion, our research underscores the potential of *Helianthus annuus* (sunflower) extract as a valuable natural remedy for managing diabetes mellitus and its associated complications. The study of phytochemical constituents and pharmacological activities of *Helianthus annuus*. extract has provided valuable insights into its therapeutic properties and traditional medicinal uses. Our findings reveal that the hydroalcoholic seed extract of Helianthus annuus. possesses significant antioxidant and anti- inflammatory properties, attributed to its high content of phenolics and flavonoids. These bioactive compounds are known for their ability to mitigate oxidative stress and inflammation, crucial factors in the development of chronic diseases. Furthermore, our study demonstrates the extract's remarkable capacity to enhance glucose uptake In vitro, indicating its potential as a therapeutic agent for improving glycemic control. The observed increase in glucose uptake, particularly in HepG2 and L6 cell lines, highlights the promising pharmacological activity of the extract in managing diabetes mellitus. Moreover, our research suggests that oral administration of the extract to diabetic rats results in a notable reduction in blood glucose levels and improvement in insulin sensitivity. These effects may be attributed to various mechanisms, including the protection of pancreatic beta cells and inhibition of gluconeogenesis and glycogenesis. Overall, our study contributes to the growing body of evidence supporting the medicinal properties of *Helianthus annuus* L. extract and its potential applications in modern medicine. Further research, including clinical trials and mechanistic studies, is warranted to fully elucidate its therapeutic potential and facilitate its development as a safe and effective treatment for diabetes mellitus and related complications. While our research provides compelling evidence of the pharmacological characteristics of *Helianthus annuus* (sunflower) seed extract, further in-depth investigation is essential to establish a deeper understanding of the possible mechanisms underlying its effects on diabetes. Specifically, there is a need for more comprehensive studies aimed at isolating and identifying the active compound(s) responsible for the pharmacological actions observed in our hydroalcoholic extract. By isolating and characterizing the bioactive components of the sunflower seed extract, we can gain valuable insights into their molecular structure and interactions within biological systems. In summary, while our research provides a solid foundation for the pharmacological potential of sunflower seed extract in diabetes management, further studies focused on isolating and characterizing its active constituents are necessary to advance our understanding and pave the way for the development of innovative therapies

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List of Abbreviations

HAE	*Helianthus annus*extract
WHO	World Health Organization
NCD	Non communicable diseases
DPPH	2, 2-diphenyl-1-picrylhydrazy l
STZ	Streptozotocin
TZDs	Thiazolidinediones
GLP1	Glucagon like Peptide 1
SGLT	Sodium Glucose co transporter
GLT	Glucoseco transporter
UV	UltraViolet
SGPT	Serum Glutamic-Pyruvic Transaminase
SGOT	Serum Glutamic-Oxaloacetic Transaminase
GAE	Galic Acid Equivalent
QE	Quercetin equivalent
PBS	Phosphate Buffer Solution
NaOH	Sodium Hydroxide

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