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Effects of Date Seed (Phoenix dactylifera) Extract in an Animal Model of Diabetes Mellitus

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A THESIS SUBMITTED BY

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Declaration of Authorship

I, Sara Refaat, declare that this thesis titled, "Effects of Date Seed (Phoenix dactylifera) Extract in an Animal Model of Diabetes Mellitus" and the work presented in it are my own. I confirm that:

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- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
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Abstract

Diabetes mellitus is a chronic illness and a global epidemic. The commercial diabetic medicines available are expensive and have several adverse effects. Alternative natural products are still being sought for. Date seeds (*Phoenix dactylifera*) are either thrown away as garbage or utilized as animal feed. The use of seed extract as an alternative therapy for diabetes offers a cost-effective and all-natural method for managing the disease.

Methods

Sprague dawley rats were randomly distributed into groups with a sample size of 4 rats per group. Date seed powder was boiled producing a 10% date seed extract which was administered in 10ml daily doses to each rat. Date seed extract was analyzed by high resolution UPLC-MS. Intraperitoneal Streptozocin injection was administered for diabetes induction at a concentration of 75mg/kg in sodium citrate buffer (pH 4.5). Diabetes was confirmed one week post injection with diabetic rats having a blood glucose above 250 mg/dL. Samples were collected biweekly for blood glucose and weight measurements. Stool pellets were flash frozen in liquid nitrogen and stored for sequencing. PowerSoil Kit was used for DNA extraction and further extraction used for Illumina 16s rRNA. QIIME2 and GraphPad Prism were used for bioinformatic and statistical analysis, respectively. Packages such as dada2, filterAndTrim, phyloseq in R and ggplot2 in R were used.

Results

Date seed extract had antidiabetic and antioxidant effects in rats, the diabetic group that received date seed extract showing a high prevalence of anti-diabetic and antioxidant benefits. The hypothesis was supported by the stark contrasts between the commercial remedy and the date seed extract. The blood glucose levels of the date seed group were 184.7 mg/dL, which was higher than the control group (159.7mg/dL), and lower than both the metformin and the diabetic untreated group with blood glucose levels of 271 mg/dL and 452.3 mg/dL respectively. Similar trends applied to oxidative stress tests, liver and kidney function tests.

The gut microbiome composition also revealed differences between the samples, with an increase in diversity and abundance of beneficial bacteria in the normal and date seed extract group and a decrease in diversity in the commercial treatment and the negative control group. This information gave us insight into the dysbiosis in the gut microbiome caused by the disease as well as the changes caused by both the commercial treatment and the date seed treatment.

Conclusion

This study sheds light on the usage of date seed extract as a diabetic treatment alternative and raises the prospect of additional research into the effects of date seed extract in human subjects to confirm its efficacy when used in place of conventional diabetic therapies.

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List of Abbreviations

ALT	Alanine transaminase
AM	Alternative medicine
AST	Aspartate aminotransferase
bp	Base pair
DC	Diabetes Control
ddH_2O	Double-distilled water
DM	Diabetes mellitus
dsDNA	double-stranded DNA
GDM	Gestational diabetes mellitus
GSH	Glutathione reduced
LPO	Lipid peroxidation
MDA	Malondialdehyde
NO	Nitric oxide
PAD	Peripheral arterial disease
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
SCFAs	Short-chain fatty acids
STZ	Streptozocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
xg	Relative gravitational force

Chapter 1 Introduction and Literature Review

1.1 Diabetes

Diabetes Mellitus (DM) is a chronic disease that affects 37.3 million individuals in the United States alone, with approximately 96 million pre-diabetic cases diagnosed (CDC, 2022). DM is characterized by the lack of blood glucose control in the body due to various genetic and environmental factors (Sapra & Bhandari, 2022). DM can be subclassified into different types based on the etiology of the disease itself. Namely, Type 1 DM, Type 2 DM, and Gestational Diabetes (Sapra & Bhandari, 2022). Type 1 Diabetes (T1DM) is characterized by the absence or low levels of insulin in the body (Sapra & Bhandari, 2022). T1DM patients require external insulin injections due to the lack of insulin production by the body (Lucier & Weinstock, 2022).

Type 2 Diabetes (T2DM) is the most common type, with approximately 90% of all cases. T2DM is mostly due to insulin resistance. T2DM is more common over the age of 45 and is associated with obesity statistics and poor lifestyles (Goyal & Jialal, 2022). Gestational Diabetes mellitus (GDM), is a form of diabetes that occurs during pregnancy and increases the risk of T2DM development in both the child born as well as the mother (Goyal & Jialal, 2022).

Diabetic patients often suffer from various complications, such as heart disease, stroke, Peripheral arterial disease (PAD), retinopathy, nephropathy, and peripheral neuropathy (Deshpande et al., 2008; Sapra & Bhandari, 2022).

1.2 Alternative and Traditional Medicine

The absence of effective treatments, the high-cost expense of pharmaceuticals, and the adverse effects of modern medicine have all revived public interest in alternative and traditional medicine (Akter et al., 2021).

Alternative medicine (AM) is a comprehensive strategy that is acknowledged as a diverse range of medications and medical procedures with significant therapeutic benefits. Two-thirds of the world's population prefers alternative medicine over modern medicine

when seeking support and assistance for their health (Akter et al., 2021).

Alternative and traditional medicine, including Ayurveda, herbal remedies, Yunani, homeopathy, acupuncture, naturopathy, chiropractic manipulation, and other methods, have all been shown to be effective in treating DM (Akter et al., 2021). Recent data show that half of the world's population depends on AM, including 42% of Americans, 48% of Australians, 70% of Canadians, and 49% of French people (Akter et al., 2021). Due to this situation, a knowledge bridge concerning AM therapies, safety usage, and toxicity or contraindications is needed to be developed between physicians, traditional practitioners, pharmacists, and patients. To improve the standards of AM and provide better patient care (Akter et al., 2021).

Interestingly many alternative approaches are encouraged in diabetic treatment plans. Diabetes patients are often treated with diet and exercise in addition to traditional medicine, especially the use of herbal medicine as an effective treatment of DM patients in the management and prevention of complications related to the onset of the disease. Probiotics, herbal remedies, homeopathic medicines, traditional medicines like traditional Chinese medicines, vitamins and minerals, and other natural health products are frequently used for the management of diabetes (Akter et al., 2021).

1.3 Dates (*Phoenix dactylifera*)

Millions of people around the world rely on dates (*Phoenix dactylifera L.*) as a staple diet. Dates come in approximately 2000 various shapes, sizes, and weights. As a result, the annual production of date seeds or pits is about 825,000 tonnes. This waste contains a significant amount of bioactive ingredients, which can be used both medically and in many industrial applications. Currently, seeds are still mostly utilized as animal feed or dried, roasted, and used to make a caffeine-free coffee-like product (Alkhoori et al., 2022).

Due to their high dietary fiber content, date seeds have a high nutritional value and may be used to reduce the risk of hypertension, coronary heart disease, high cholesterol, colorectal and prostate cancers, as well as diabetes, hyperlipidemia, and obesity (Alkhoori et al., 2022).

1.3.1 Chemical Composition of Date Seed

Dates seeds have been proven to be rich in active compounds such as phenolics and flavonoids, which have therapeutic properties such as antimicrobial, anti-inflammatory,

antioxidant, and anti-carcinogenic (Alkhoori et al., 2022). Oleic acid and other fatty acids, such as palmitic acid, linoleic acid, lauric acid, myristic acid, and stearic acid, were also found in date seeds (Alkhoori et al., 2022; Besbes et al., 2004).

1.4 Diabetes and the Gut Microbiome

The human microbiome consists of bacteria, archaea, and eukaryotic organisms that inhabit the bodies and significantly affect human physiology. Recent advances in the characterization of the microbiome's composition have identified some functional connections between the microbiota and the host. A full understanding of the role of microbiota still needs extensive research (Shreiner et al., 2015). Bacterial members of a microbial community are commonly identified by sequencing the 16S rRNA-encoding gene and comparing the sequences to databases containing known bacterial sequences (Shreiner et al., 2015).

Bacteroidetes and Firmicutes are the dominating phyla found in healthy adult humans. The microbiota of the gut varies considerably among healthy individuals (Shreiner et al., 2015). It is unclear whether microbiome alterations are linked with different diseases. However, it should be noted that the diseased state itself may lead to changes in the microbiota. Eating habits, as well as the use of antibiotics and other medications, may also alter bacterial composition (Shreiner et al., 2015).

Diabetes is strongly linked with gut dysbiosis. The gut of diabetic patients is distinguished by the prevalence of Bacteroidetes, the absence of butyric acid-producing bacteria, and the reduction in bacterial and functional diversity (Li et al., 2020). Obesity and type 2 diabetes are often accompanied by alterations in intestinal microbiota, intestinal inflammation, and intestinal barrier disturbance (Li et al., 2020).

Previous studies using mouse models indicated that the intestinal microbiota is responsible for the increased inflammatory response in obese and type 2 DM patients (Li et al., 2020). In addition, the gut microbiota may interact with dietary components to alter the host's insulin sensitivity, intestinal permeability, glucose, and lipid metabolism (Li et al., 2020).

The metabolic activity of the gut microbiota results in metabolites that may alter host physiological activities. The modification of gut microbiota by external interventions such as nutrition results in changes in intestinal microbial metabolites, which may influence the disease status in diabetic patients (Li et al., 2020).

1.4.1 Metformin and the Gut Microbiome

Metformin medication has been associated with the upregulation of glucagon-like peptide-1 (GLP-1) and peroxisome proliferator-activated receptors in both healthy persons and DM patients. Metformin may also lower lipid absorption and reduce inflammation in the gut, therefore making the gut microbiota seem more comparable to non-diabetic control levels (Li et al., 2020).

Recent investigations showed that anti-diabetes medications altered the gut microbiome of Chinese type 2 DM patients. Those using Metformin showed an increase in Turicibacter and Spirochaetes. Analyses of the makeup of the intestinal microbiota in metformin-treated diabetes individuals using Genomic analysis. Compared to non-diabetic patients, it showed that the abundance of A. muciniphila and several Short-chain fatty acids (SCFAs)-producing microbiota was low in diabetic patients (Li et al., 2020).

1.5 Animal Models of Diabetes

Experimental diabetes mellitus is often induced in animals because animal models are useful for elucidating the etiology of the illness (Kottaisamy et al., 2021). Diabetes may develop in experimental animals either naturally or with the use of chemical agents. The experimental animal models are categorized according to the type of diabetes they resemble and the mechanism of induction, which may be spontaneous or induced (Kottaisamy et al., 2021).

Streptozotocin (STZ) causes the selective damage of pancreatic islet -cells resulting in diabetogenic consequences (Furman, 2015). Due to this activity, mice develop insulin insufficiency, hyperglycemia, polydipsia, and polyuria, which are all symptoms of type 1 diabetes in humans. Mice, rats, and monkeys are animals that are susceptible to the cytotoxic effects of STZ on pancreatic -cells. STZ is now the most commonly used drug to induce diabetes in animal models (Furman, 2015).

1.6 Importance & Research Gap

Uncontrolled diabetes and hyperglycemia lead to the development of serious complications, such as heart attack, stroke, blindness, and damage to the kidneys and other organs, which can lead to early disability or premature death. Treatment of the complications is many times more costly than the cost of diabetes treatment (Ponomarenko, 2019).

The use of date seeds as an alternative medicinal treatment for diabetic patients would provide more accessibility to many individuals who are unable to withstand the financial burden of commercial drugs. In addition to the economic benefits of the use of date seeds as an anti-diabetic treatment or as an additional treatment, the environmental impact of using date seeds instead of discarding them will greatly positively affect the environment.

Therefore a thorough study of the effects of date seeds in an animal model of diabetes is a necessary step towards further human studies to validate the use of date seed extracts as an alternative anti-diabetic medication. Another aspect of the current study is to investigate the effects of date seed extract on gut microbiome changes in diabetic animals. A comparison between the effects of Metformin and date seed extracts is also essential to validate the efficacy of the extract as an antidiabetic agent.

1.7 Aim & Hypothesis

The hypothesis of the current study is that the date seed extract will have anti-diabetic properties as well as a high margin of safety in an animal model of diabetes.

The aim of this study is

- To evaluate the anti-diabetic properties of Date Seed tincture in an animal model of Diabetes Mellitus.
- To compare the gut microbiome of normal Sprague Dawley Rats and a diabetic animal model.
- To evaluate the changes in gut microbiome following metformin and date seed treatment in rats.

Chapter 2 Methodology

2.1 Animals

Male Sprague Dawley Rats, averaging 150 – 200g in weight, were randomly divided into control and treatment groups, each group consisting of 4 animals (total number of animals, n=16). All experiments were conducted in compliance with the guidelines established by the NIH for Animal Care and Use and were approved by October University for Modern Sciences and Arts (MSA) Animal Care and Use Committee.

Table 1. Experimental Groups distribution.

Group	Name
1	Normal Non-Diabetic Control
2	STZ Induced Diabetic Control – No treatment
3	STZ Induced Diabetic – Commercial Metformin Treatment
4	STZ Induced Diabetic – Date Seed extract (Algeria)

2.2 Date seed extract Preparation

Date seed powder (El Linah Foods, Algeria) was added to boiling the powder (100 grams / Liter) for 10 minutes, followed by filtration. 10 ml of the 10% extract was administered daily in the drinking water (Hasan & Mohieldein, 2016).

2.3 High-Resolution UPLC – MS

High-performance liquid chromatography (HPLC) was performed at Cairo University, Faculty of Agriculture, Food Safety, and Quality Control for the identification of different components within the tincture.

For the separation of metabolites in the date seed tincture, a chromatographic method was utilized by Waters Acquity UPLC system. 1.8m HSS T3 colum was used in a binary gradient of water/formic acid (99.9/0.1), and acetonitrile/formic acid (0.99/0.1) was used for optimum separation of eluted peaks (Farag et al., 2021). A flow rate of 150uL/min was used as

follows: 0-1 min, isocratic 95%, 5%, 1-16 min, linear from 5% to 95%, 16-18 min, isocratic 95%, 18-20min isocratic 5%. 3.1 uL were injected and compounds were detected using micro-TOF-Q quadrupole time-of-flight mass spectrometer (Bruker Daltonics Inc., Billerica, MA, US), in both positive and negative ion modes using Apollo II electrospray ion source. Conditions for the mass spectrometer were as follows: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 L min1, 190 C; capillary, 5500 V (+4000 V); end plate offset, -500V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; hexapole RF, 100Vpp; quadrupole ion energy 5eV; collision gas, argon; collision energy, 10eV; collision RF200/400Vpp (timing 50/50); transfer time, 70 1s; prepulse storage 51s; pulser frequency 10kHz; spectra rate 3Hz (Farag et al., 2021). Using a diverter valve, each analysis' internal mass calibration was done by infusing 20 L of 10 mM lithium formate in isopropanol/water, 1/1 (v/v), at a gradient duration of 18 minutes (Farag et al., 2021).

The UV–Vis spectra (220–600 nm), retention periods compared to external standards, mass spectra, and comparisons to our in-house database, the phytochemical dictionary of natural products database, and reference literature were used to describe the metabolites (Farag et al., 2021).

2.4 Diabetes Induction

Diabetes was induced by intraperitoneal injection of Streptozocin (STZ) (Sigma Aldrich, USA). Freshly prepared STZ 75 mg/kg in Sodium citrate buffer (pH of 4.5) was injected intraperitoneally. Confirmation of diabetes took place one-week post-injection. Animals with blood glucose levels above 250 mg/dL were considered diabetic and included in further experimentation. The body weight and blood glucose were measured biweekly.

2.5 Sample Collection

Stool pellets were collected into a sterile Eppendorf and flash-frozen in liquid nitrogen, then stored in a -80 freezer.

After four weeks of treatment, animals were anesthetized with an overdose of Ketamine/Xylazine (ketamine 80-100 mg/kg, xylazine 10-12.5 mg/kg) intraperitoneal injection and sacrificed. A cardiac puncture was used to collect blood samples for

biochemical analysis. Liver, Kidney, and pancreas tissue samples were collected and fixed in 4% formaldehyde for histological analysis.

2.6 Oxidative Stress Tests

Tissues were homogenized in phosphate-buffered saline (PBS) solution, followed by centrifugation (4.000 rpm, 14min, 4^{O}C), and the supernatant was used for further assays.

2.6.1 Reduced Glutathione (GSH)

Reduced glutathione (GSH) was estimated by a commercial kit (Biodiagnostics, Giza, Egypt). 0.5 ml of the sample and 0.5 ml of the reagent were mixed and incubated at room temperature for 5 minutes, followed by centrifugation (3,000 rpm, 15 min). The absorbance was then measured at 405 nm against a blank (Beutler et al., 1963).

GSH Calculation (Beutler et al., 1963):

Glutathione (GSH) Concentration in Tissue = $\frac{A\ of\ Sample*66.66}{g\ of\ Tissue\ used}\ mg/gram\ of\ tissue$

$= \frac{A\ of\ sample*2.22}{g\ of\ tissue\ used}\frac{mmol}{gram} of\ tissue$

2.6.2 Nitric Oxide Assay (NO)

For nitric oxide assay, a colorimetric assay was used (Biodiagnostics, Giza, Egypt). 0.1 ml of the tissue homogenate was added to 1.0 ml of the reagents. After the addition of all reagents, the plate is incubated at room temperature for 5 minutes, and then absorbance is read at 540 nm (Montgomery & Dymock, 1962).

NO Calculation (Montgomery & Dymock, 1962):

Nitrite in the sample (umol/L) = $\frac{A\ of sample}{A\ of\ Standard} *\ 50$

*A being the Absorbance.

2.6.3 Malondialdehyde (MDA)

For lipid peroxide assay, an MDA assay was used (Biodiagnostics, Giza, Egypt). 0.2 ml of the homogenate was added to 1.0 ml of chromogen and heated in a boiling water bath for 30 min. After cooling down, the absorbance was read at 534 nm (Satoh, 1978).

MDA Calculation (Satoh, 1978):

Malondialdehyde in Sample = $\frac{A\ of\ sample}{A\ of\ standard} * \frac{10}{grams\ of\ tissue}\ nmol/grams\ of\ tissue$

2.7 Histopathological Analysis

Tissue samples were rinsed with saline solutions and 5 mm x 5 mm pieces were fixed in 4 % paraformaldehyde for 24 hours before being dehydrated with an escalating sequence of ethanol (70%, 80%, 90%, 95%, and 100%). Tissues were washed in terpineol for at least 48 hours, infiltrated with Paraplast at 60ºC, and lastly embedded using an L-shaped metal mold. 5 um were cut using an electronic digital microtome (Histo-Line Yidi Italy, Model: YD 335), 5–6 m thick sections were cut, mounted to transparent glass slides using diluted egg albumin solution, and dried in an incubator at 38ºC for at least 24 hours. Tissues were deparaffinized, hydrated with distilled water, and stained using Mayer's hematoxylin and counterstained with 1% Eosin.

2.8 Biochemical Analysis

Serum collected was tested to assess liver function tests: AST and ALT, and lipid profile (Triglycerides, total cholesterol, and HDL). Kidney function tests were also performed for serum creatinine, blood urea nitrogen, and serum uric acid.

2.9 DNA Extraction

DNA was extracted from collected pellets according to the method described in the Qiagen DNeasy PowerSoil Kit (QIAGEN diagnostics, Germany). Each sample was weighed and homogenized, then lysed in lysis buffer. Samples were centrifuged (10,000 xg, 30 sec.), and the supernatant was transferred into a collection tube containing 250 ul inhibitor to aid in the precipitation of non-DNA inorganic and organic material. The sample was then centrifuged,

and the supernatant was extracted into a clean tube. A high-concentration salt solution was added, and the sample was transferred into an MB spin column, spun, and centrifuged (10,000 xg, 1 min). Flowthrough from the column was discarded, and the process was repeated, followed by the addition of ethanol wash and spinning at (10,000 x g, 30 sec). The flowthrough was used for further sequencing as it contained the DNA.

2.10 Sequencing

Sequencing took place after the extraction of the DNA from the Pellets collected. Sequencing was performed using Illumina 16s rRNA, which is a PCR-based protocol. The protocol included two PCR amplification steps followed by a cleanup step. Sequencing was performed at the Children's Cancer Hospital, Genomics research program in Egypt. Primarily, PCR was used to amplify the template out of the DNA extracted using interest specific primers. These primers contain overhang adaptors.

The degenerate primers used in the amplicon PCR were used to target V3 and V4 regions in bacterial rRNA, identifying the various microbial species. The primers used were 5'TCGTCGGCAGCGTCAGATTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC C as the 16s Amplicon PCR forward primer and reverse primer respectively. The amplicon PCR was followed by a cleanup step which used AMPure XP beads for the purification of 16s V3 and V4 amplicons from primer dimers and free primers. Index PCR was performed after, where dual indices and Illumina sequencing adapters were attached with the use of Nextera XT Index Kit. A second cleanup was performed after the Index PCR using AMPure XP beads, followed by quantification.

The library was validated by running 1ul of the final library on the Bioanalyzer DNA 1000 chip. This step verified the size then using V3, and V4 primer pairs, the final library size on Bioanalyzer trace was approximately 630bp. Quantification of the library, as well as normalization and pooling, were performed by fluorometric quantification using dsDNA binding dyes, and pooling and normalization were performed according to Illumina

manufacturing instructions. Raw Fastq files extracted are then used in the bioinformatic analysis using dada2, filterAndTrim, QIIME2, R packages (phyloseq & ggplot2).

2.11 Statistical Analysis and Bioinformatics

Data Processing and taxonomic identification was performed with the use of dada2 workflow after extracting the raw fastq files. filterAndTrim was used to merge and remove chimeras and to filter out as well as trim low-quality reads. With the use of nbases = 90000 error rates were learned, and filter reads were merged and then inferred using dada2 functions (dada, mergePairs). Sequences generated were imported into QIIME2, and feature-classifier was used to extract reads using silva 139-99 database. Parameters classifier-naïve-bayes were used, and classification was undertaken using classify-sklearn. Core-metrics-phylogenetic was used for the diversity metrics calculations. R packages (phyloseq & ggplot2) were used as well as QIIME2 for the visualization of the results. Other bioinformatic software used were microbiome and ComplexHeatmap.

Statistical analyses were carried out using GraphPad Prism software, version 7.04 (GraphPad, Inc., San Diego, CA, USA). All data were expressed as mean ± standard deviation. Post-hoc test of one-way ANOVA followed by Dunnets test was used to compare the differences in mean values between different groups. P-values less than 0.05 were considered statistically significant.

Chapter 3 Results

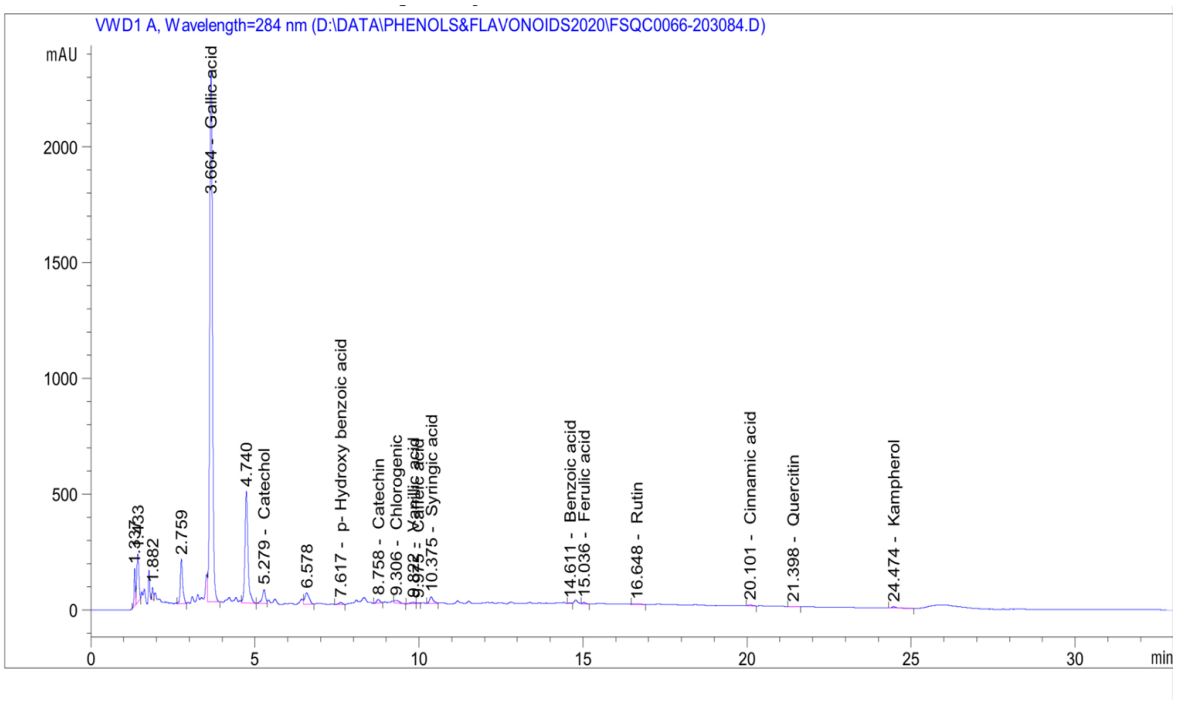

Figure 1. Peak Chromatogram of High-Performance Liquid Chromatography of Date Seed extract.

3.1 High-Performance Liquid Chromatography (HPLC) of DS extract showed high abundance of gallic acid.

High-performance liquid chromatography was performed on the date seed extract with a wavelength of 284 nm yielding a peak chromatogram indicating the various compounds found in the extract and their relative abundance within the extract. The most abundant compound found, as shown in the peak chromatogram (Figure 1), was gallic acid with a value of over 2000 mAU. The second most abundant compound was catechol, followed by hydroxybenzoic acid, catechin, chlorogenic, syringic acid, benzoic acid, ferulic acid, rutin, cinnamic acid, quercitin, and Kampherol.

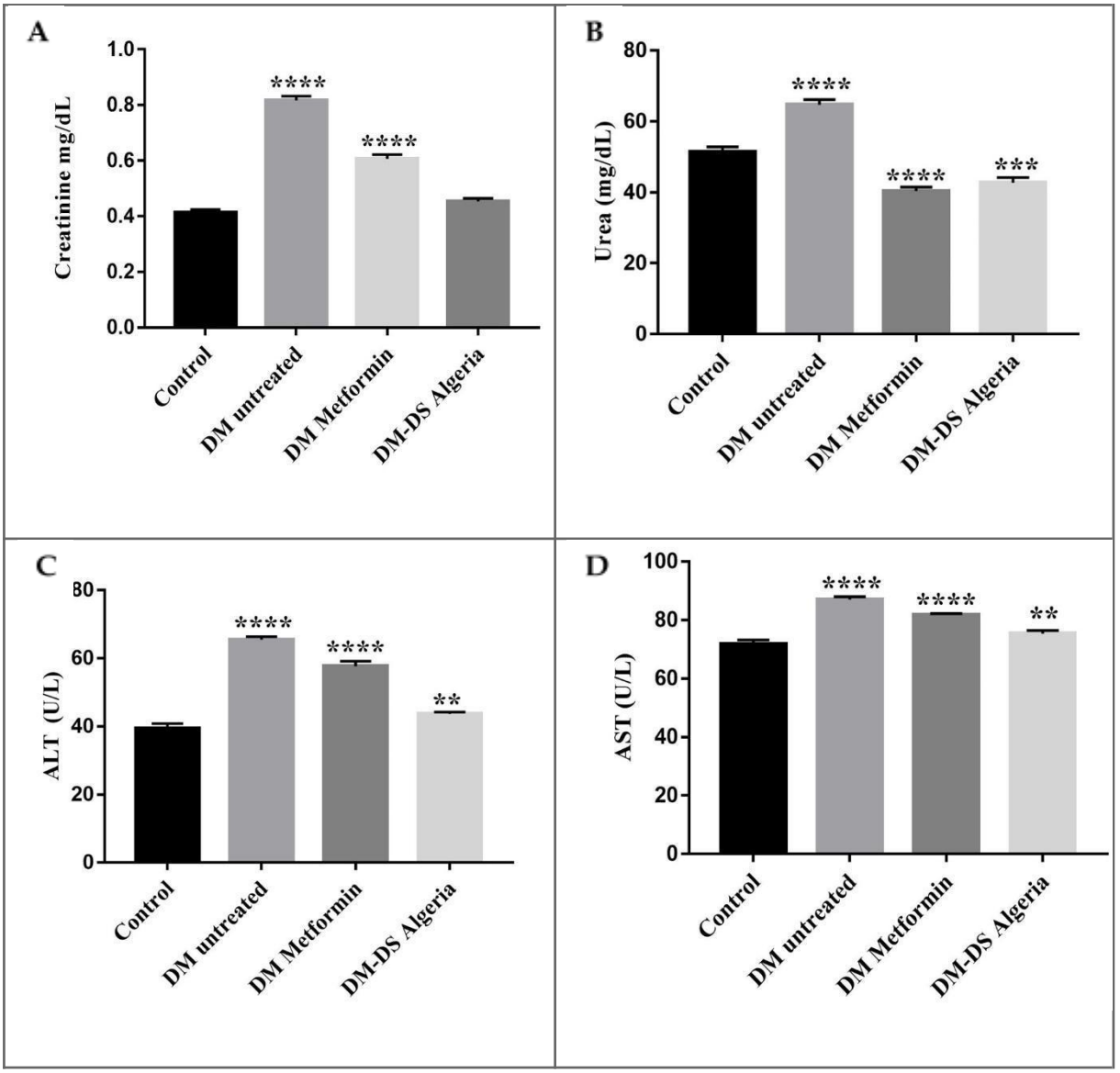

Figure 2. Biochemical Analysis of liver and renal functions.

Liver ALT, AST, and kidney creatinine and Urea of different groups of diabetic animal models with various treatments. **A**. Creatinine results of the control group with no diabetic induction and no treatment, DM untreated, DM metformin, and DM-DS Algeria treatment. **B**. Indicates the urea content in control, Dm untreated, DM metformin, DM-DS-Algeria groups. **C**. ALT measurements of the control group, DM untreated, DM metformin, and DM-DS Algeria. Subfigure **D** shows the AST contents of the control group, DM metformin, DM untreated, and DM-DS Algeria groups. ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001 (One-way ANOVA followed by Dunnets test)

3.2 Elevated liver and kidney function results in diabetic and metformin rats with reduced reduced values in date seed group and control group.

Creatinine and Urea levels (Figure 2) increased significantly in diabetic rats. Treatment with date seed extract lowered the creatinine levels back to the control levels compared to the

Metformin treatment. Urea levels were similar in the Metformin and date extract-treated groups. Liver enzymes (AST and ALT) increased in diabetic rats. Metformin treatment did not reverse this increase in ALT and AST levels. Date extract treatment reduced the enzyme levels, albeit they were still higher than the control values.

Diabetes caused a significant increase in serum Glucose, HDL, Triglycerides, and cholesterol (Figure 3). Metformin treatment showed slightly lower levels of serum Glucose, HDL, Triglycerides, and cholesterol compared to the untreated diabetic animals. These levels were still significantly higher than normal control values. Date extract, on the other hand, caused a significant reduction in Glucose and triglycerides levels. While the levels of cholesterol and HDL were significantly lower than those of the Metformin-treated group but still higher than the control.

3.3 Reversal of oxidative stress in date seed extract group.

Oxidative stress tests (Figure 4) showed a significant reduction in GSH levels and an increase in NO and MDA levels in diabetic rats. Metformin treatment caused a slight reversal of the GSH and NO levels that were still significantly different from the control group. On the other hand, Metformin treatment corrected the MDA levels. Date seed extract-treated animals showed levels that were different from those of the normal control group.

Table 2. Biochemical Analysis values of Kidney (creatinine, Urea) and Liver (ALT and AST) functions ± Standard Deviation.

	Creatinine	**Urea**	**ALT**	**AST**
Control	0.4133 ± 0.011	51.33 ± 1.52	39.33 ± 1.52	71.67 ± 1.52
DM untreated	0.8167 ± 0.015	64.67 ± 1.5	65.2 ± 0.86	87 ± 1.1
DM Metformin	0.606 ± 0.015	40.33 ± 1.155	57.67 ± 1.52	81.67 ± 0.577
DM-DS Algeria	0.453 ± 0.011	42.67 ± 1.52	43.67 ± 0.577	75.33 ± 1.155

Table 3. Biochemical Analysis values of blood glucose and lipid profile (cholesterol, triglycerides, and HDL) ± Standard Deviation.

	Cholesterol	**Triglycerides**	**HDL**	**Glucose**
Control	62.33 ± 1.52	55.67 ± 1.52	32.33 ± 2.51	159.7 ± 10.02
DM untreated	97.33 ± 2.5	86.67 ± 1.5	76.67 ± 1.52	452.3 ± 10.69
DM Metformin	87.33 ± 2.08	75.67 ± 1.5	52 ± 2	271 ± 7
DM-DS Algeria	79.67 ± 2.08	50.33 ± 1.52	42.33 ± 1.528	184.7 ± 5.5

Table 4. Oxidative stress tests (MDA, GSH, and NO) ± Standard Deviation.

	MDA	GSH	NO
Control	24.69 ± 1.75	0.369 ± 0.019	31.9 ± 1.4
DM untreated	47.08 ± 1.23	0.176 ± 0.014	47.2 ± 1.32
DM Metformin	31.36 ± 1.95	0.281 ± 0.017	40 ± 1.58
DM-DS Algeria	27.11 ± 1.57	0.383 ± 0.014	35.05 ±1.4

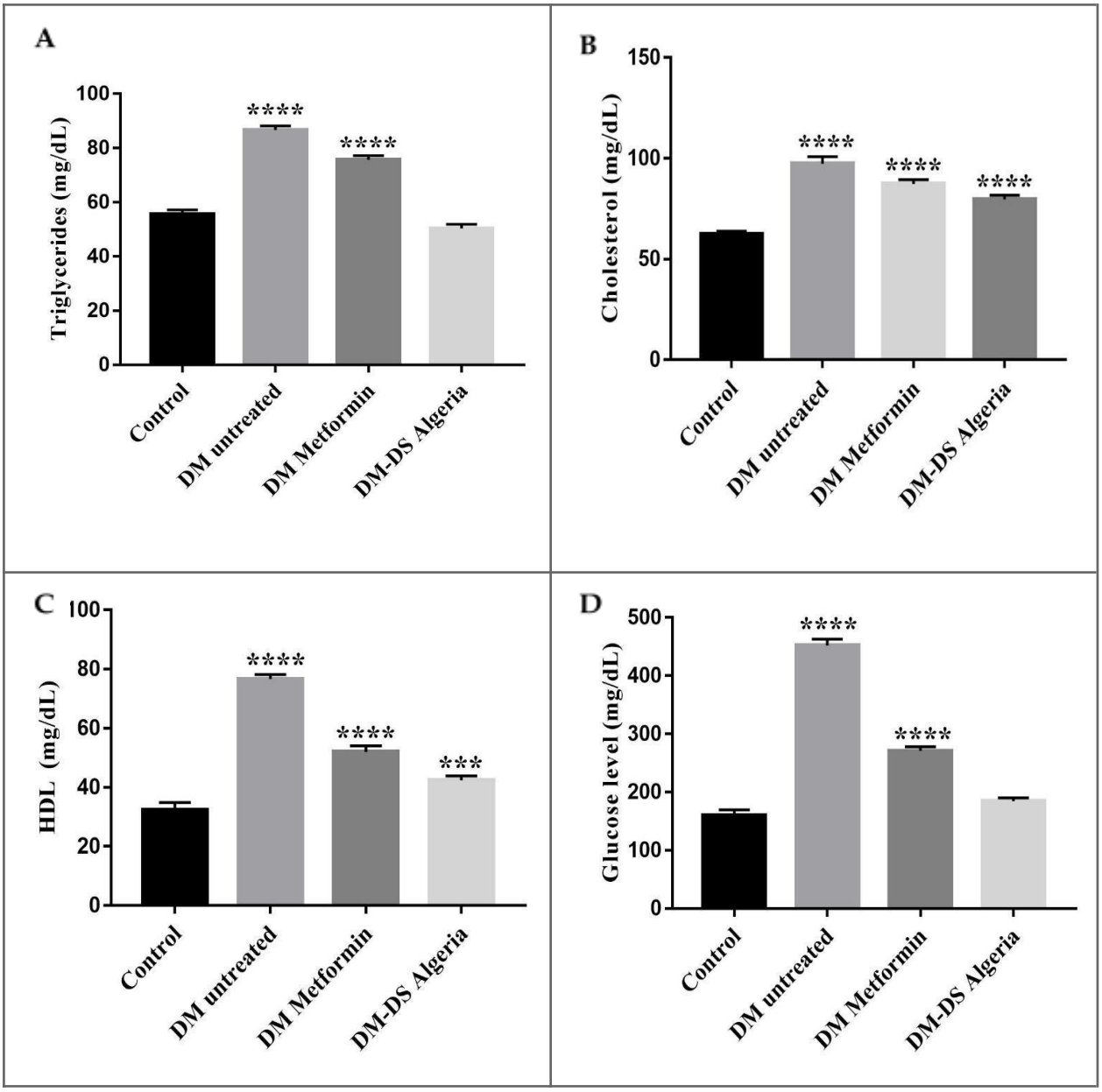

Figure 3. Lipid profile and glucose levels.

Biochemical Analysis of triglycerides, cholesterol, HDL, and glucose. **A**. Triglyceride concentrations in blood of control, DM untreated, DM Metformin, and DM-DS Algeria. **B**. Concentrations of cholesterol for control, DM Metformin, DM untreated, and DM-DS Algeria. **C**. HDL concentrations in control, DM untreated, DM Metformin, DM-DS Algeria groups. **D**. Glucose level measurements of control, DM untreated, DM Metformin, and DM-DS Algeria groups. **p-value <0.01, ***p-value <0.001, **** p-value <0.0001 (One-way ANOVA followed by Dunnets test)

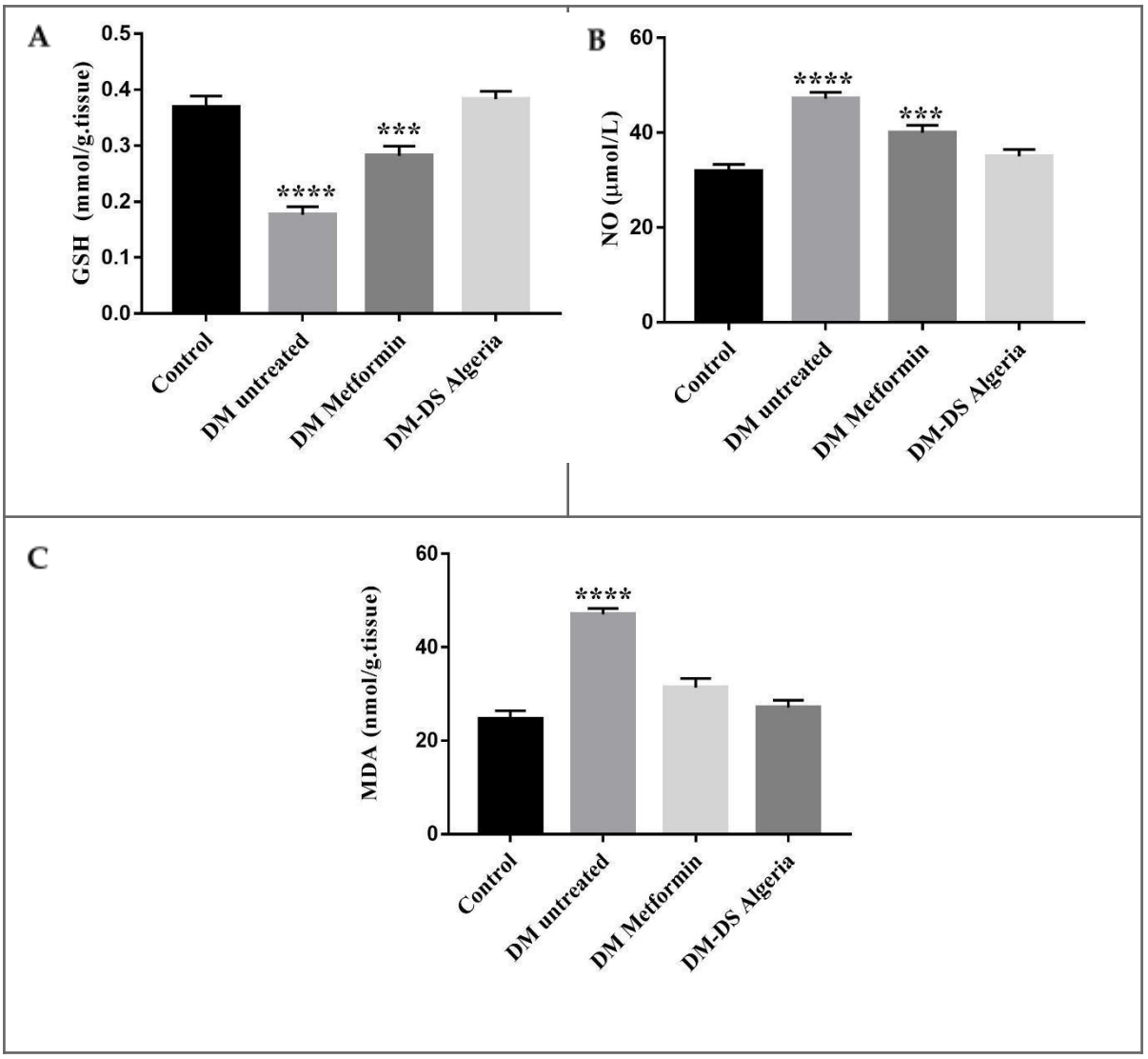

Figure 4. Oxidative stress tests.

Oxidative stress parameters MDA, GSH, and NO were performed on tissues from the control group, the DM untreated group, the DM metformin group, and the DM-Date Seeds (Algeria) group. *p-value <0.05, **p-value <0.01, ***p-value <0.001, **** p-value <0.0001 (One-way ANOVA followed by Dunnets test)

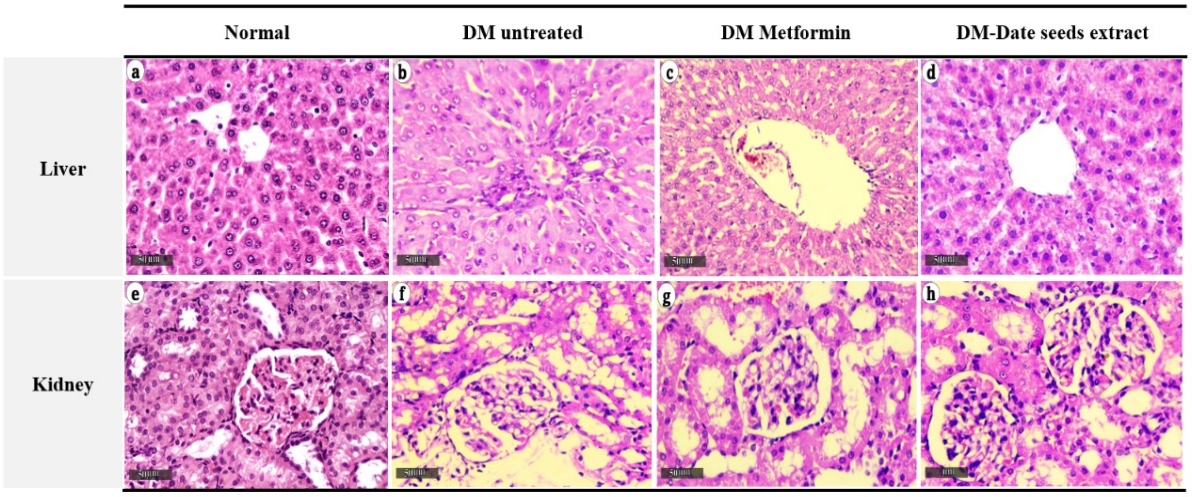

Figure 5. Histological analysis of liver and kidney tissues.

Photomicrographs of liver and kidney sections of control rats (a, e) stained with Hematoxylin and Eosin (H & E) showing normal liver and renal architecture. Untreated diabetic group (b, f) shows necrotic hepatocytes with fused blood sinusoids filled with degenerated cells. The kidney shows severe glomerular damage. Metformin-treated diabetic group (c, g) showed mild hepatic degeneration with moderately dilated sinusoid in the liver. The kidneys showed interstitial inflammation and moderate glomerular and tubular damage. Date seeds treated group (DM Algeria) (d, h) showed almost normal hepatic strands aligned with normal sinusoids, which are lined with some active Kupffer cells. Kidneys showed slight tubular and glomerular damage compared to DM untreated group.

3.5 Necrosis in histopathological structure of untreated diabetic rats, and metformin group, and improvement in histological structure

Examination of liver sections (Figure 5) of control rats showed normal histological structure. The untreated diabetic group showed necrosis of hepatocytes adjacent to the central vein associated with inflammatory cell infiltration. Liver sections from the metformin group showed similar histopathological changes to the untreated diabetic group. Treatment with date seed extracts showed improvement in the histological structure of the liver with minimal infiltration of inflammatory cells.

Normal histology of the kidney (Figure 5) was seen in the healthy control group. Diabetic rats showed vacuolar degeneration and interstitial nephritis. Treatment with metformin showed similar degenerative changes in renal tubules. Date seed extract treatment improved the histological structure of the kidney, and no inflammation or necrosis was observed.

3.6 Analysis of the gut Microbiome

A rarefaction curve (Figure 6) was produced, showing a plateau of all samples' curves at around 10000 reads, indicating the stabilization of species composition generation, which shows the quality of the sequencing is good enough for analysis of the samples.

The results (Figure 7) of the alpha diversity measurements indicate that the commercial treatment (Metformin) shows a diversity measure that ranged between 4.15 – 4.225 approximately, the no-treatment group's diversity measurement ranged between 3.96 – 4.14, the normal control group ranged between 3.71 – 4.18, and finally, the DM-date seeds group (labeled treatment) showed a range of 3.94 - 4.59. The results indicate an extensive difference between the DM-DS group samples, sample D with relatively high diversity, and sample C with relatively low diversity; the diversity measurement also shows that the normal range has relatively lower values of alpha diversity in comparison to the other groups. The metformin group, as well as the DM no treatment group, have smaller ranges with higher values.

The beta diversity (Figure 8) using Bray-Curtis PCoA showed a clear separation between the commercial treatment (metformin), the DM no treatment and the normal control group, and DM-date seed group (labeled treatment). The DM-date seed group and the normal control group were grouped together, showing a similarity in diversity, and a clear separation shows a difference between the commercial treatment and the DM no treatment in comparison to the normal control and DM-date seed.

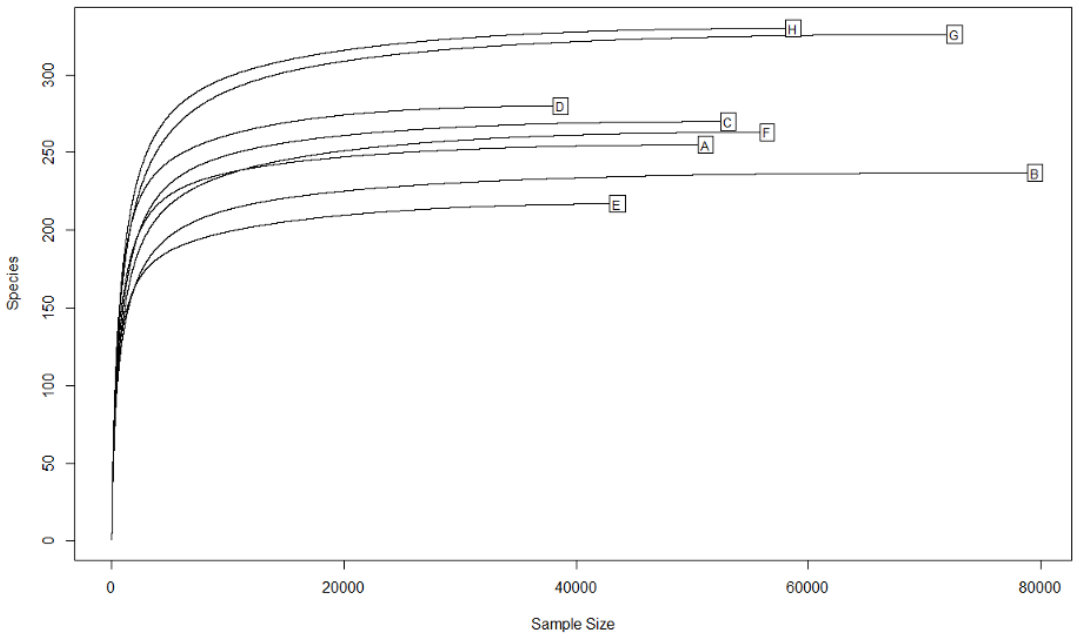

Figure 6. Rarefaction Curve of gut microbiome samples after extraction and sequencing. **A & B** DM No treatment. **C & D** DM-Date seeds. **E & F** Normal control. **G & H** DM Metformin treatment.

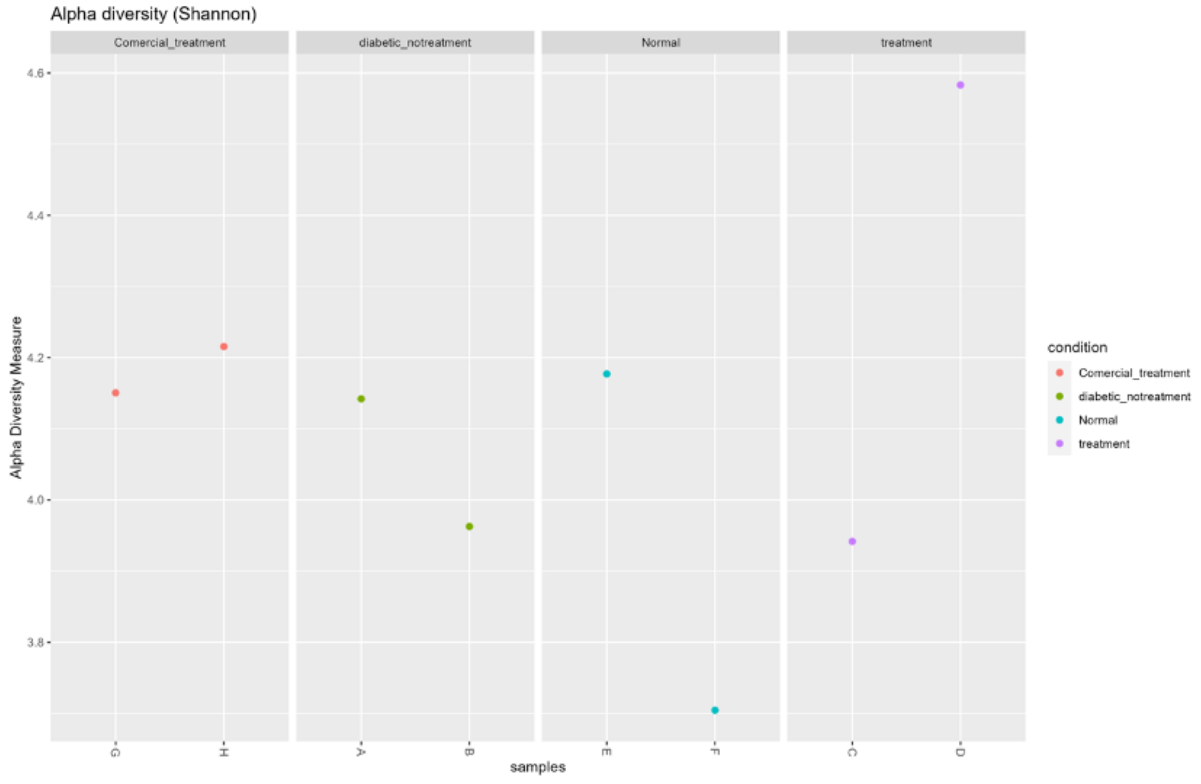

Figure 7. Alpha diversity measurement using shannon of all sequenced gut microbiome samples.

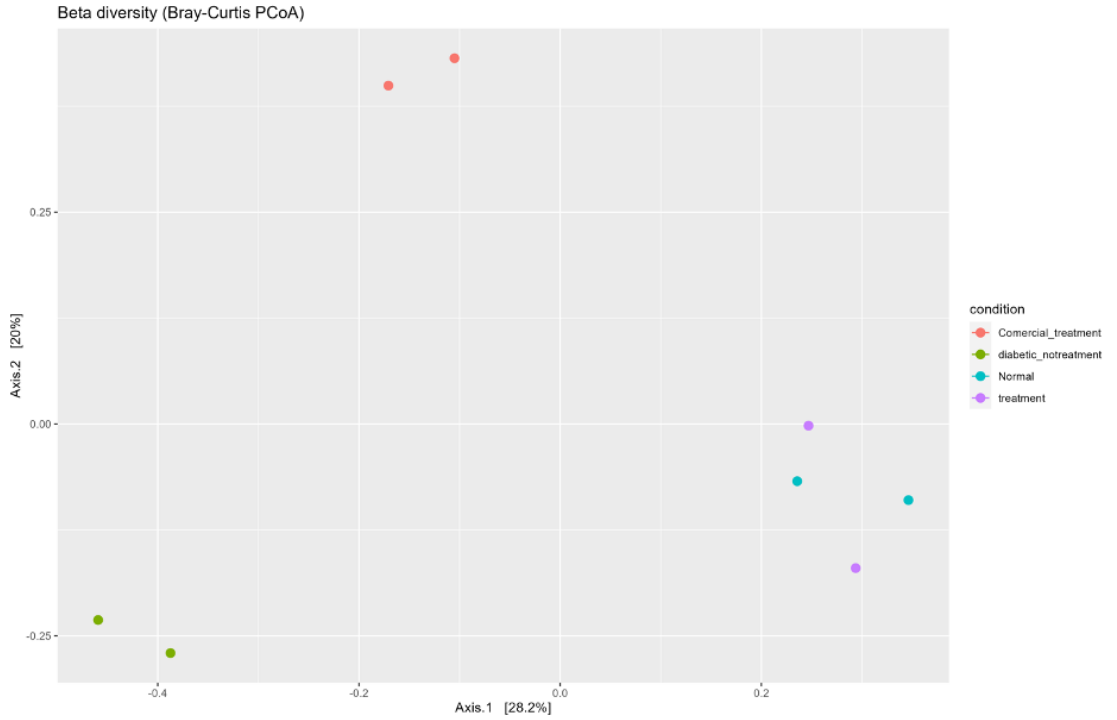

Figure 8. Beta diversity graph of all sequenced gut microbiome samples.

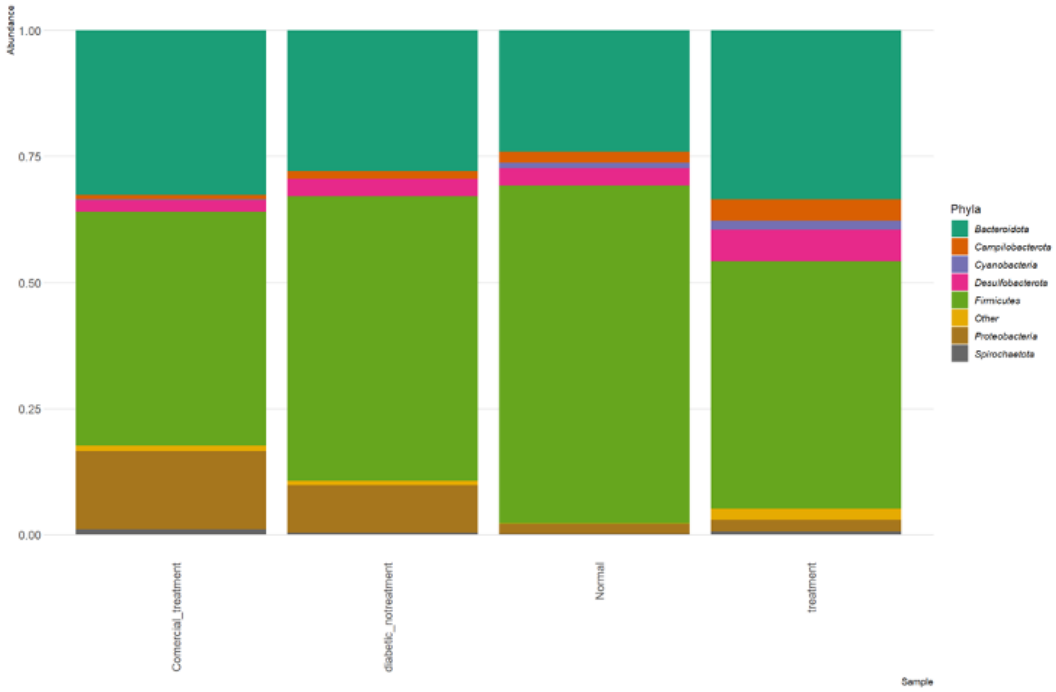

Figure 9. Stacked Bar chart of phyla abundances in different groups of gut microbiome.

The bar graph (Figure 9) shows an abundance of *Firmicutes* in the normal control group with decreased abundance in all other groups. Additionally, a low abundance of *Proteobacteria* in the normal control group was shown with an increased abundance in both the DM No treatment group as well as the commercial treatment (metformin group), the DM-Date seed group had a relatively low abundance of *Proteobacteria* similar to the normal control group. The abundance of *Bacteriodota* is relatively similar between all groups with the DM-Date seeds group as well as the commercial treatment having slightly greater abundances and the normal group having the lowest abundance with a slight difference between the other groups. The depletion of *Cyanobacteria* and *Campilobacterota* can be seen clearly in both the commercial treatment and the DM no treatment.

Figure 10 is a visual representation of the abundance of families in the gut microbiota of different groups. A variety of abundances are portrayed in the stacked bar chart, with the most prominent difference shown in the difference in abundance of the *Moraxellaxeae* and *Lactobacillaceae* families, where there is an obvious depletion in the abundance of *Lactobacillaceae* and *Moraxellaceae* in the DM-date seeds group in comparison with the normal control group while the commercial treatment and DM no treatment group show a similar abundance of both families.

There is also an abundance of *Clostridiaceae* in the DM no treatment group while there is low visible abundance in other groups, which showed a great abundance of the family as well as the lack of any Clostridia family in the DM No treatment group with a slight abundance in all other groups. *Eubacterium* and *Ruminococcaceae* family were also very abundant in the DM-date seeds group and reduced in all other groups.

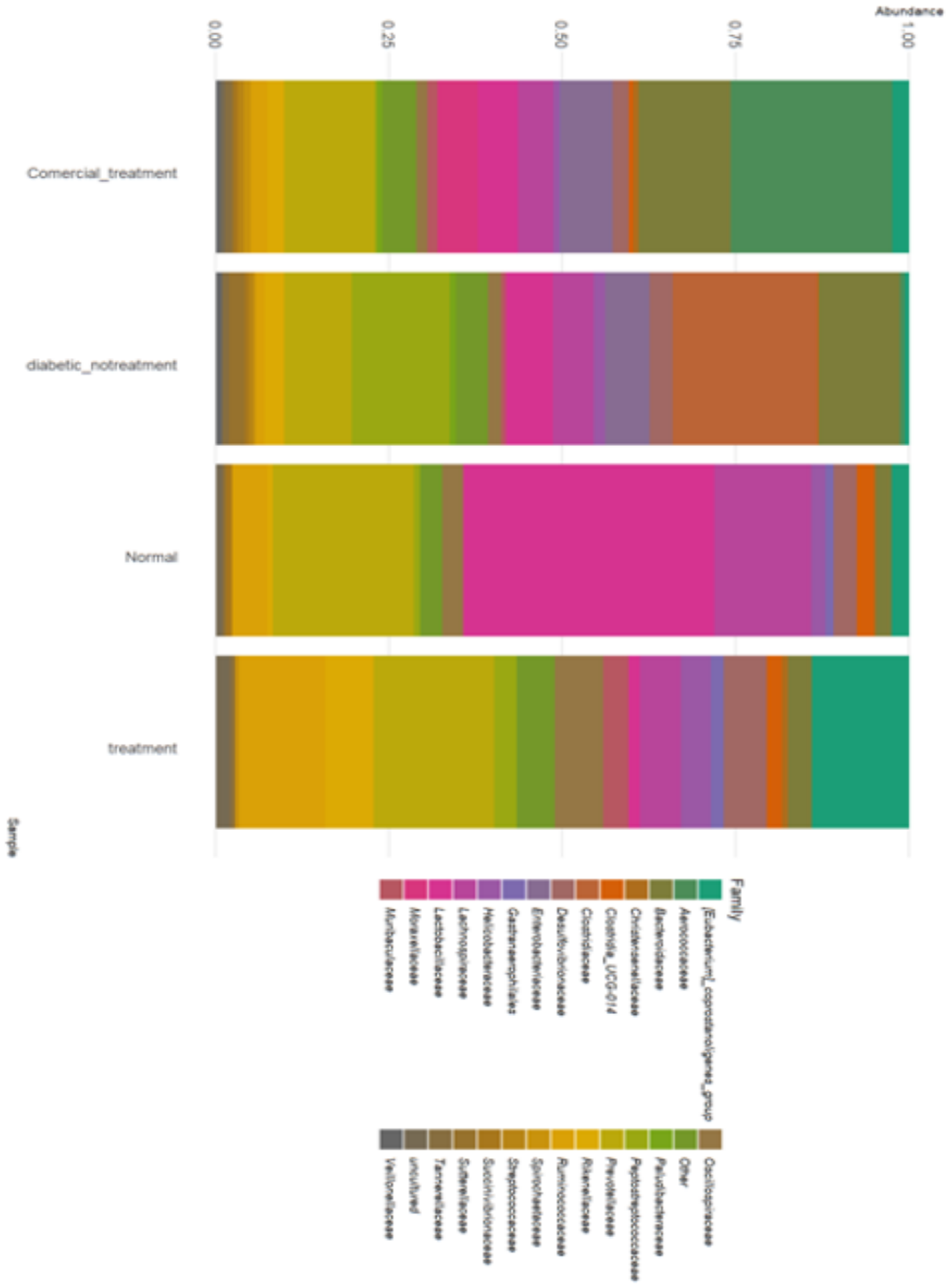

Figure 10. Stacked Bar Chart of Family abundances in different groups' gut microbiome.

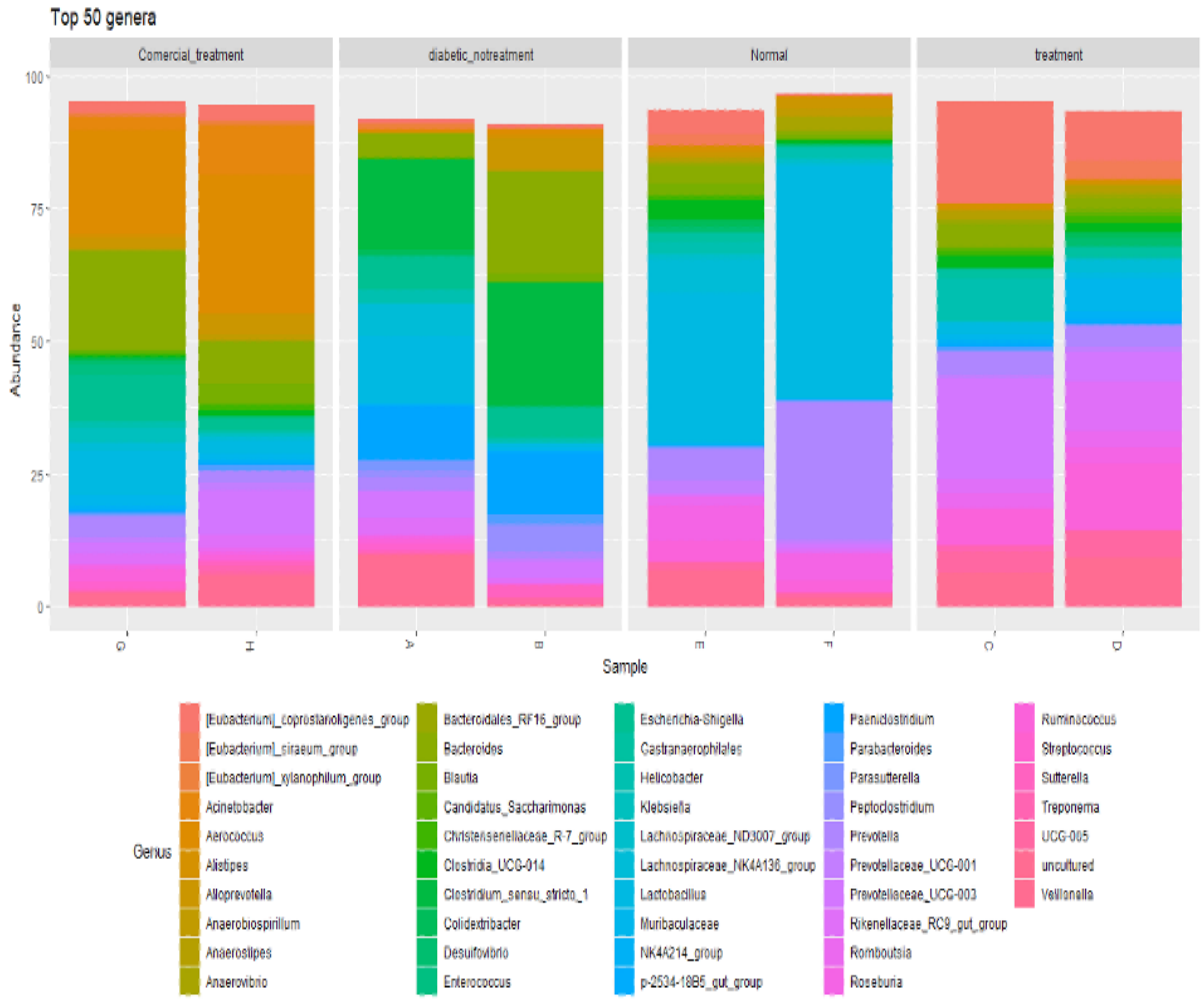

Figure 11. Stacked bar chart of genus abundance in different groups' gut microbiome.

The different compositions of gut microbiota can be observed in the abundance of the different genera in different groups (Figure 11). There is an observable difference between the different compositions of gut microbiome genera in each group. There is greater abundance of *Acinetobacter* and *Aerococcus* in the commercial treatment than all other groups. An increase in the abundance of *Bacteroides* in both the commercial treatment and the DM no-treatment group can be seen in comparison to its lack of abundance in both the normal control and the DM-Date Seeds group. The normal control group portrays an abundance of *Lactobacillus* and *Prevotella* more than all other groups.

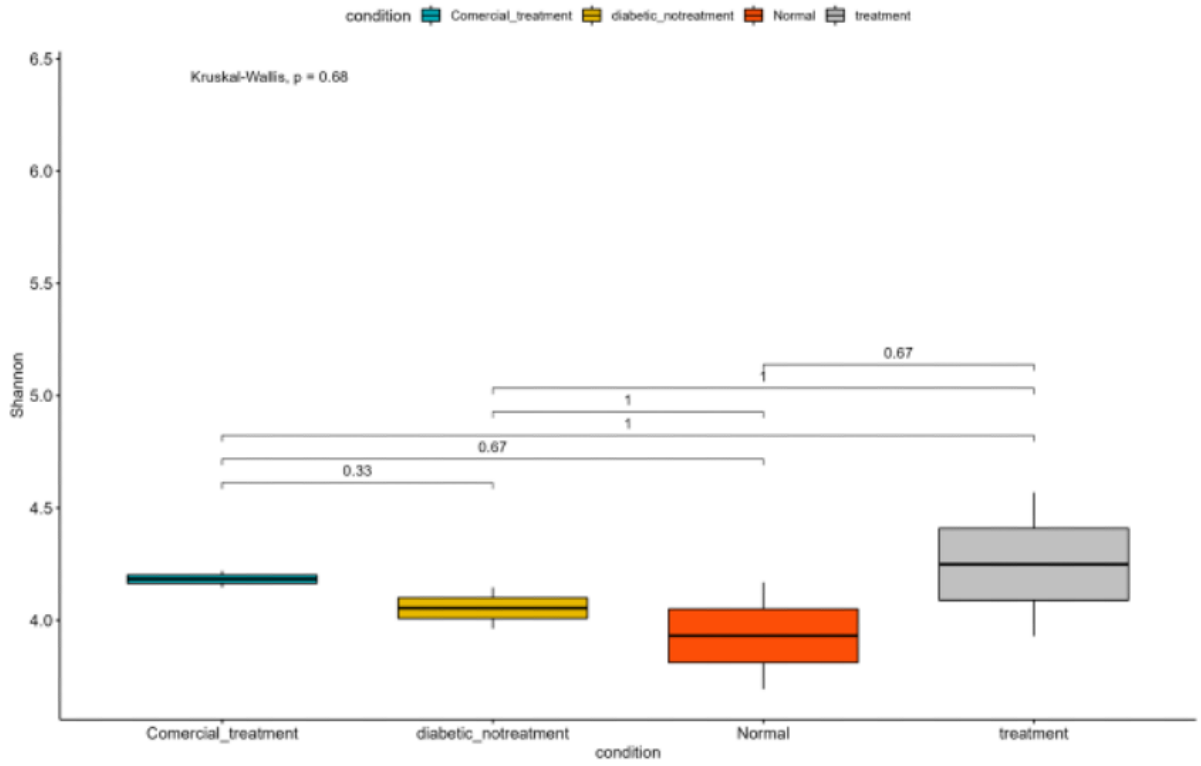

Figure 12. Boxplot of Shannon alpha diversity including p.value.

The Boxplot (Figure 12) shows a greater average in the diversity of the DM-date seeds group in comparison to the commercial treatment group, the DM no treatment group, and the normal control group. The normal control group showed the lowest average diversity with the DM no treatment following and the commercial treatment with the largest value in diversity right under the DM-date seeds group. The p.values show no statistical significance with all p. values over the value of 0.05.

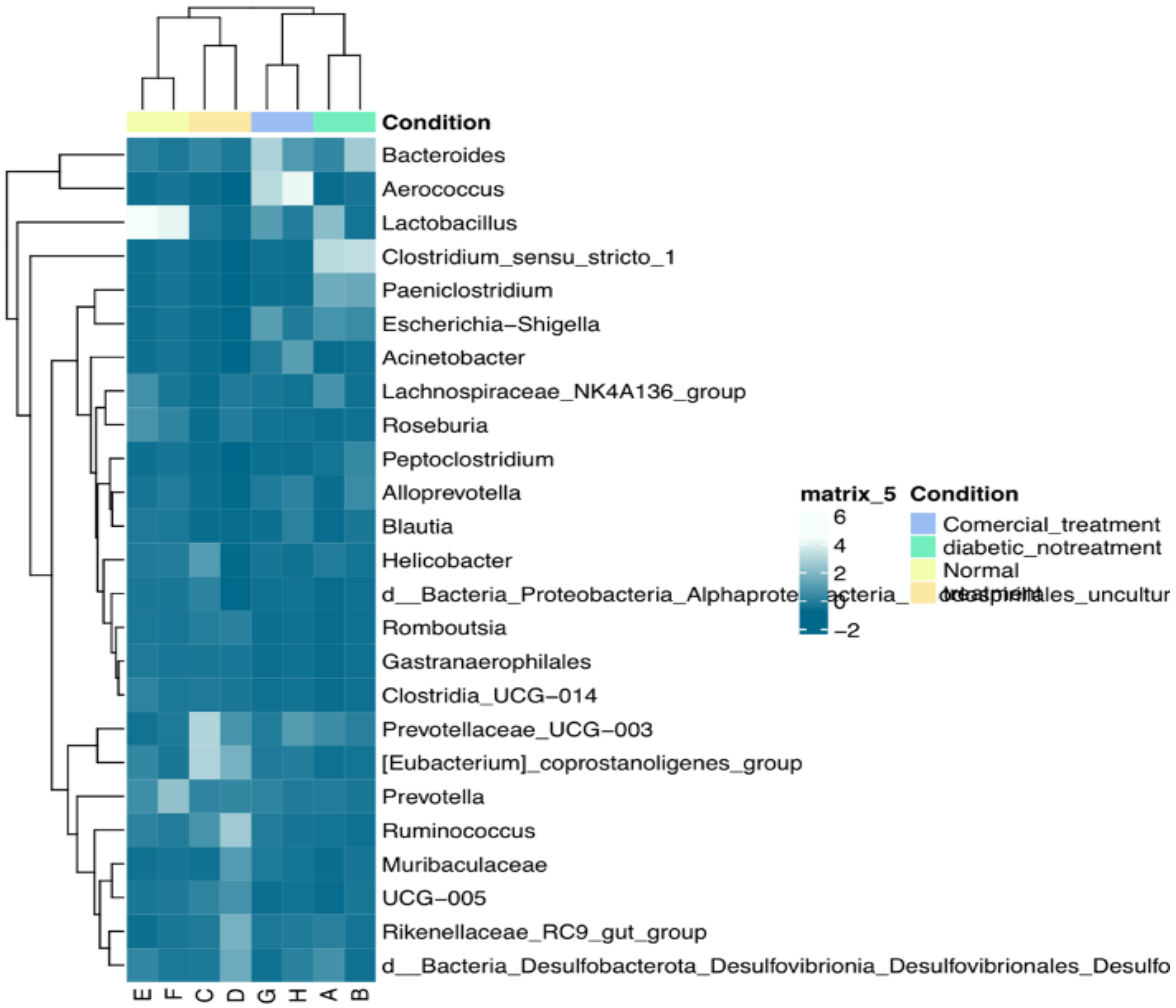

Figure 13. Heatmap of genera abundance in different conditions.

The heatmap displays the genus abundance variation in each group (Figure 13). The heatmap clustered the commercial treatment (blue) and DM no treatment (green) together and clustered the normal control group (yellow) and the DM-date seeds group (orange) together, indicating a similarity between the normal control group and the date seed extract group. The heatmap portrays higher abundance in lactobacillus in the Normal control group. There is also higher abundance of *Bacteroides* and *Aerococcus* in the DM no treatment group and the commercial treatment.

The DM-date seeds group portrays higher abundance of *Prevotellaceae* and *Eubacterium*. There was also higher *Clostridium* abundance in the DM no treatment group. *Escherichia Shigella* showed higher abundance in both the commercial treatment group as well as the DM

No treatment group, with low abundance in both the normal control group and the DM-date seeds group. All other genera do not show a significant difference in abundance between groups.

Chapter 4 Discussion

DM is a chronic illness that hinders the ability of the patient to live a normal life. DM is one of the most common diseases worldwide and is classified as a global epidemic. The search for effective diabetes treatment is an ongoing challenge. The economic impact and the financial strain for the patients due to the high cost of treatment cause many patients to neglect the treatment (Cutler et al., 2018).

In the search for an alternative natural treatment for diabetes, research showed that date seeds have potential as an anti-diabetic agent but require more research to confirm their anti-diabetic abilities. Many bioactive compounds with anti-diabetic activities have been identified in medicinal plants and herbal remedies. Glycosides, flavonoids, and tannins have been shown to be abundant in date seeds. Therefore implying the potential anti-diabetic activity of date seeds. Date seed extracts, unlike insulin, did not cause hypoglycemia in normal rats (El-Fouhil et al., 2010).

The current study shows that HPLC analysis of date seed extract showed high gallic acid content and high hydroxybenzoic acid and catechin content. Gallic acid has many beneficial effects, such as its anti-inflammatory and antioxidant properties. The presence of gallic acid content aids in validating the possible uses of date seeds as an alternative treatment. Previous reports showed similar phytochemicals in Saudi Ajwa/Sukkari date seed extracts (Hasan & Mohieldein, 2016).

The biochemical analysis also provided more validation of the possible uses of date seed extract as a safe anti-diabetic treatment. Date seed extracts were superior to the Metformin treatment. The glucose levels supported our hypothesis. Date seed extract lowered the blood glucose levels and returned them closer to the normal control value better than the commercial treatment (Metformin) with minimal side effects (El-Fouhil et al., 2010).

High urea and creatine levels are a sign of deteriorating kidney function. Treatment with date seeds reversed the levels of urea and creatine to almost normal levels. This supports our hypothesis that the date seed extract has anti-diabetic properties and is a promising alternative for commercial treatments. The liver function tests (ALT and AST) provide an indication of liver damage (Lala et al., 2022). Higher AST and ALT values are a sign of liver damage, while lower AST and ALT values indicate a relatively healthier liver condition. Treatment

with date seeds showed values similar to those of the normal control group. Hasan et al. (2016) also showed a reversal of renal damage after the use of Saudi Ajwa/Sukkari date seed extract (Hasan & Mohieldein, 2016).

Previous studies assessed the anti-diabetic, hypolipidemic, and antioxidative effects of Saudi Ajwa/Sukkari date seed extract on streptozotocin-induced diabetic rats' livers and kidneys. Treatment for eight weeks considerably decreased the blood glucose and HbA1c levels in treated rats (Hasan & Mohieldein, 2016).

Hypertriglyceridemia and hypercholesterolemia are the most prevalent lipid disorders in diabetics. High cholesterol and triglycerides are associated with an increased risk of stroke and heart disease, and heart disease. Additionally, high triglycerides can be an indication of pancreatitis (Kota et al., 2012). Untreated diabetic rats showed the highest values of cholesterol and triglycerides. The lipid profile was improved with date seed extract. HDL levels were lowest in control and the date seed treated groups. Some phytoconstituents of the extract, notably saponins and steroids, limit intestinal fat absorption by means of a resin-like effect and inhibition of lipase activity (Hasan & Mohieldein, 2016).

Multiple researchers have found a considerable drop in the body weight of diabetic rats induced by STZ (Zafar & Naeem-ul-Hassan Naqvi, 2010). The decrease in body mass of diabetic animals might be explained by the loss of structural proteins since structural proteins contribute to body mass (Hasan & Mohieldein, 2016). The administration of date seed extract to diabetic rats resulted in considerable weight maintenance. This may be due to the enhancement of insulin secretion and glycemic control generated by the active component in date seed extract (Hasan & Mohieldein, 2016).

Oxidative stress is an important factor in the pathophysiology of diabetes. Evidence showed that DM induces alterations in tissue antioxidant enzyme concentration and activity in both human and experimental diabetes. Decreased levels of antioxidants have been seen in the blood and renal tissues. Additionally, oxidative stress may impact nucleic acids, resulting in altered DNA bases (Singh et al., 2019).

In the present work, oxidative stress assessment was performed using GSH, NO, and MDA. High levels of GSH are found during the regeneration of the liver. The decrease or deficiency in GSH can lead to the progression of cancer (Traverso et al., 2013). Additionally, GSH increases antioxidant levels and therefore aids in the resistance of oxidative stress in cancer

cells. Date Seed treated group showed the highest GSH concentration, followed by the normal Control. Metformin and no treatment had the lowest GSH concentration. The results support the claim that date seeds possess antioxidant abilities. MDA is an indicator of lipid peroxidation. Higher MDA indicates an increase in oxidative stress. Date seed treated and normal control group shows low oxidative stress levels in the DM-date seeds group, with values closer to the normal control and lower than the metformin group. Hasan et al. (2016) showed similar results, therefore, supporting our hypothesis that date seeds can replace or become a viable alternative to commercial medication for DM (Hasan & Mohieldein, 2016).

Nitric Oxide (NO) levels are another factor used to assess oxidative stress. Increased NO levels indicate increased inflammation (Sharma et al., 2007). The NO concentrations show the same trend with DM no treatment and DM metformin having the highest concentrations, therefore, indicating an increase in the inflammatory process in the body of diabetic rats. Date seeds successfully reduced NO levels to the normal control levels.

Microbiome Analysis

The microorganisms that live within and on top of our bodies have the power to substantially alter human physiology. The study of the human microbiome has increased thanks to the use of non-culture-dependent techniques (Bik, 2016). Evaluation of the genetic potential of the microbial population is another advantage of metagenomic study, which involves sequencing all of the microbial DNA in a complex community.

The study of the gut microbiome of diabetic patients (and animal models) shows a change in the gut microbiome. These changes affect the overall health of the individual. The increase in the abundance of opportunistic pathogenic bacteria causes an overall decline in health, while an increase in beneficial bacteria aids in the improvement of health. The ability of different treatments to affect the gut microbiome and alter it, either returning it to its original normal state or decreasing the overall diversity, increasing the abundance of pathogenic bacteria as well as decreasing the abundance of beneficial bacteria.

The changes in the gut microbiome give us an insight into the effect of DM on the gut microbiome and the effect of each treatment, and its possible ability to alter the gut microbiome and return it to its natural state. Our results indicate a similarity between the abundance of *Roseburia* and *Streptococcus* in both the normal control and the date seeds

groups. On the other hand, the non-treated group showed an increase in the *Clostridium* genus, indicating the abundance of pathogenic opportunistic bacteria. The Normal control also shows an abundance of *Lactobacillus*, a beneficial bacteria in comparison to all genre abundances, *Lactobacillus* was the most upregulated.

Previous studies showed that in non-obese diabetic (NOD) mice, the incidence of type 1 diabetes is dependent on the gut microbiome and lipopolysaccharides-mediated gut signaling involving toll-like receptor 4 (TLR4) and myeloid differentiation primary response 88 (MyD88). MyD88 is a crucial signal transduction factor in the interleukin-1 and TLR signal transduction pathways (Li et al., 2020). A deficiency of MyD88 modifies the microbiota of the distal intestine. Studies indicate that NOD mice missing the MyD88 protein will not develop type 1 diabetes. When the gut microbiota of MyD88-deficient NOD mice was transplanted to wild-type NOD female mice, it protected against diabetes, decreased the severity of pancreatitis, and considerably delayed the onset of autoantibody-mediated glycosuria (Neuman et al., 2019). Short-chain fatty acids (SCFAs) are also implicated in the preventative mechanism of type 1 diabetes in humans (Kim, 2018; Li et al., 2020).

Metformin disturbs the microbial properties linked with diabetes, including the makeup of the intestinal microflora (Zhang & Hu, 2020). A double-blind, placebo-controlled investigation involving type 2 diabetic patients revealed that metformin affected the intestinal microflora balance in human patients, while germ-free mice exhibited enhanced glucose tolerance after receiving metformin-modified microbiota (Li et al., 2020). Metformin was also administered to mice receiving a high-fat diet (HFD) in a controlled experiment, and the findings showed that the quantity of the mucin-degrading bacterium *Akkermansia muciniphila* (*A. muciniphila*) was greater than that reported in the control group (Li et al., 2020; Shin et al., 2014).

Recent investigations comparing the gut microbiome of Chinese type 2 diabetic patients on various anti-diabetes medications with those receiving metformin showed enrichment of *Turicibacter* and *Spirochaete* (Zhang & Hu, 2020). Analyses of the makeup of the intestinal microbiota in metformin-treated diabetes individuals using genomic analysis compared to non-diabetic patients showed the abundance of *A. muciniphila* and several SCFAs-producing microbiotas was low in diabetic patients (Shin et al., 2014).

Numerous studies have shown a strong correlation between the development of diabetes and changes in the gut microbiota's composition profile (Iatcu et al., 2022). In instance, disrupted

Bacteroidetes/*Firmicutes* phylum eubiosis has been associated with increased intestinal permeability, with bacterial byproduct infiltration across a leaky gut barrier prompting inflammatory reactions typical of diabetes (Iatcu et al., 2022).

It has been demonstrated that a number of bacteria play a protective function by lowering the risk of developing diabetes through a decrease in proinflammatory indicators and by maintaining the integrity of the intestinal barrier (Iatcu et al., 2022). For instance, it has been demonstrated that *Lactobacillus fermentum*, *plantarum*, and *casei*, *Roseburia intestinalis*, *Akkermansia muciniphila*, and *Bacteroides fragilis* all enhance insulin sensitivity and glucose metabolism while reducing pro-inflammatory cytokines (Iatcu et al., 2022). Notably, some medications, like the diabetes drug metformin, have been demonstrated to change the makeup of the gut microbiota. (Iatcu et al., 2021) This suggests that metformin interacts with the gut microbiota by modifying inflammation, glucose homeostasis, gut permeability, and bacteria that produce short-chain fatty acids (Iatcu et al., 2022). Metformin also increases the formation of butyrate and propionate in individuals with gut dysbiosis brought on by diabetes, which enhances the patient's capacity to catabolize amino acids (Iatcu et al., 2022). The effects of metformin on glucose metabolism could be attributed to these alterations as well as higher levels of *Akkermansia* in the stomach (Iatcu et al., 2022). It seems that the same metabolic mechanisms that link gut microbiota dysbiosis and type 2 diabetes and are related to chronic low-grade inflammation and oxidative stress also play a role in the development and progression of diabetic complications. This connection supports the idea that altering the gut microbiota may be a promising method for treating diabetes and its consequences, as described in the following sections (Iatcu et al., 2022).

Gut microbiome has been linked to metabolic illnesses including type 2 diabetes. Reports differ on taxonomic groupings and illness. Gurung et al. (2020) evaluated 42 human research showing microbial relationships with illness and found supportive probiotic preclinical or clinical trials. *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia*, and *Roseburia* were negatively correlated with T2D, whereas *Ruminococcus*, *Fusobacterium*, and *Blautia* were positively correlated with T2D (Gurung et al., 2020).

Many species specific similarities in the gut microbiome, by studying fecal matter in humans, rats, and mice (Nagpal et al., 2018). This indicates the ability of using Rats as an experimental model. Rats have been used as a model experimental organism in many biomedical fields, and are more frequently used in gut microbiota studies. The similarities

between Rats and Humans gut microbiome indicate the ability of using rats as a model to assess the effects of different diseases such as diabetes on the gut microbiome and the possible progression into human pre-clinical trials (Čoklo et al., 2020).

Conclusion and Future Work

Diabetes is a chronic illness and a global epidemic which requires more research in order to properly treat the disease. The current commercial treatments are not only costly but are accompanied by various side effects other than the lack of ability to completely return the individual's biochemical data as well as gut microbiome to its original state.

Studying the effects of date seed extract (*Phoenix Dactylifera*) as an alternative natural medication for diabetic patients is essential in the development of diabetes treatments. The exploration of the effects of Date Seed Extract proved to have a positive effect on the diabetic animal model, with anti-diabetic and antioxidant effects being prevalent in the diabetic group administered the Date Seed Extract. Significant differences were also portrayed between the commercial treatment and the Date Seed extract supporting the hypothesis.

The gut microbiome composition also indicated differences between the samples, with higher diversity and abundance of beneficial bacteria in the normal and Date Seed extract group and lower diversity in the commercial treatment and the negative control group, providing us with insight on the dysbiosis in the gut microbiome caused by the disease as well the changes caused by both the commercial treatment as well as the Date Seed treatment.

This study provides insight into the use of Date Seed Extract as an alternative to commercial treatments for Diabetes and opens up the possibility of studying the effects of date seed extract further using human subjects to ensure the efficacy of date seed extract being used as an alternative to commercial treatments for diabetic patients.

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