Proceedings of Science and Mathematics
Faculty of science, universiti teknologi malaysia
https://science.utm.my/procscimath
Volume 29 (2025) 88-100

Regulatory Role of miR-29 in Mice Supplemented with Moringa Extract

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Abstract
The purpose of this study is to investigate the regulatory effects of miR-29 in response to *Moringa oleifer*a supplementation and to explore the molecular mechanisms underlying its health benefits. MiR-29 plays a crucial role in gene expression regulation, significantly impacting cellular processes such as proliferation, apoptosis, and differentiation. Given the increasing interest in natural supplements for health improvement, *M. oleifera*, a plant known for its potent medicinal properties, has been selected for this research to know its effects on miR-29a and associated gene regulation. This study aims to accomplish two primary objectives: (1) To analyse and compare the expression level of miR-29 in response to *M. oleifera* extract and placebo in mice using RT-PCR, and (2) To identify the target genes of miR-29 using target prediction tool software such as MicroRNA Target Prediction Database (miRDB) and TargetScan. Bioinformatics analysis was performed to predict potential targets of miR-29, employing tools such as miRDB. These tools facilitated the identification of key genes involved in critical cellular pathways that are potentially regulated by miR-29. Subsequently, an in vivo study was conducted wherein mice were supplemented with *M. oleifer*a extract for a specified period. The expression levels of miR-29 in various tissues were then quantified using RT-PCR. This approach allowed for the precise measurement of miR-29 modulation in response to Moringa supplementation. This research provides valuable information on the regulatory functions of miR-29 and its interaction with *M. oleifera*.
Keywords: miR-29; *Moringa oleifera*; gene expression.

Introduction

Small non-coding RNA molecules called microRNAs (miRNAs) are essential modulators of gene expression. MiRNAs can either degrade or inhibit the translation of target genes by binding to messenger RNA (mRNA), which regulates the production of proteins. Understanding the functions of miRNAs, as well as their potential uses in clinical practice and research for both healthy individuals and those with medical conditions, has advanced significantly in recent years (Choi et al. 2010; Samad et al., 2023). Some research has demonstrated that the duration and intensity of exercise have a direct impact on the levels of specific circulating miRNAs that are involved in angiogenesis, inflammation, and cardiac muscle contractility. This suggests that these miRNAs may play a role in mediating the physiological cardiac adaptation to exercise (Condrat et al., 2020). miR-29 is recognized as a miRNA involved in numerous biological processes, including fibrosis and inflammation (Horita et al., 2021; Sasso et al., 2024).

To completely understand the complex relationship between miR-29 and its target genes, as well as how both impact the human body, particularly in diverse cellular environments, more research is necessary. In the meantime, due to its therapeutic qualities and health benefits, *Moringa oleifera*, also known as Moringa, has been used for centuries. Its antifungal, antidepressant, anti-inflammatory, and antiviral qualities are well known (Khan et al., 2022; Franklin et al., 2022). The high vitamin, mineral, and bioactive compound content of *M. oleifera*'s leaves, seeds, and pods makes it a promising intervention for enhancing general health outcomes (Trigo et al., 2020). Examining the regulatory activity of *M. oleifera* on specific miRNAs and target genes may identify new mechanisms of its traditional medicinal use and reveal new treatments.

Materials and methods

Animal source and housing

This study aims to demonstrate the biological effects of miR-29 and its response to *M. oleifera* powder supplementation in an albino, laboratory-bred strain of house mice (BALB/c). Twelve female, laboratory-bred strains of house mice (BALB/c) at the age of 5 weeks are used for this study. It was bought from the Rat Breeder Farm, Malaysia. The mice are housed under standard laboratory conditions with unrestricted access to food and water. Their environment is controlled, with bedding changed every two days and food and water replenished as needed.

Target prediction

To find potential targets of miR-29, bioinformatics tools were employed, of which the tools used were miRDB (https://mirdb.org/) and TargetScan (https://www.targetscan.org/vert_80/) (Chen & Wang, 2020, Lin et al., 2025; McGeary et al., 2019; Yang et al., 2024). These databases utilize computational algorithms to forecast miRNA-target interactions, considering sequence complementarity and other pertinent characteristics. Initially, miRDB was employed to verify the exact order of miR-29 within the organism we are studying. Subsequently, by entering the gene symbol or ID of the target gene and specifying the organism, a list of predicted target sites within the 3' UTR of the target mRNA was obtained. TargetScan was deployed by inserting the desired miRNA to the tools with default parameters.

Experimental design

For the wet lab supplementation protocol, mice were randomly divided into two groups, with each group consisting of 6 albino, female, laboratory-bred strains of house mice (BALB/c) at the age of 5 weeks. One group was labelled as the control group (NM) and the other as the treatment group (M). The NM group received an unrestricted standard feed of pellets, water, and placebo, while the M group received an unrestricted standard feed of pellets and water with the addition of *M. oleifera* solution, supplemented for 12 days. *M. oleifera* powder was obtained from commercial sources. The specific amount of powder was based on the weight of the mice. The calculations are done to achieve the desired concentration of 300 mg/kg, and the weighted powder is dissolved in distilled water. The solution was freshly prepared before each administration as the weight of the mice changes daily.

The method of supplementation for the M group and the NM group was through oral gavage. The instruments consist of a gavage needle (round tip diameter: 1.3 mm, needle outer diameter: 0.8mm, needle inner diameter: 0.5mm, length of needle that can be inserted: 3.3 cm, total length: 4.5 cm) and a 3 ml syringe. The mouse was gently restrained to immobilize the head and body using the experimenter's hands. The gavage needle was carefully inserted into the mouth of the mouse by guiding the needle along the roof of the mouth and into the esophagus. Then, the prepared solutions were administered slowly and steadily. After administration, the gavage needle will be slowly withdrawn. This will be done to both the M and the NM groups once a day in the morning.

Sample collection and RNA extraction

At the end of the 12-day supplementation period, blood samples were collected from the submandibular vein and heart of each mouse by the attending veterinarian. Mice were anesthetized by the method of intraperitoneal injections (IP), in which they administered the drug was administered into the peritoneal cavity. The obtained blood samples are inserted into a 3ml EDTA-coated tube to prevent coagulation and placed in an ice box. After obtaining the blood sample, RNA extraction was done according to the MiPure Cell/Tissue miRNA kit instructions. 1ml of RNA isolator was added to the sample. The cell pellet was treated by vortexing. To allow for sufficient lysis of the cells, the tube containing the homogenate was placed at room temperature for 2-3 minutes. Then, 200 µl of chloroform was added to the lysis buffer, and it was shaken for 15 seconds. It was then placed at room temperature for 3 minutes. The sample was centrifuged at 4℃ and 12,000 rpm (13,400 × g) for 15 minutes. 500 µl of supernatant was transferred into a new 1.5 ml RNase-free centrifuge tube. Then, 160 µl of absolute ethanol was added to the supernatant and vortexed for 10 seconds to mix well. Care was taken to

transfer only the supernatant aqueous phase, avoiding the middle layer and bottom organic phase, to ensure the subsequent extraction results were not affected. The MiPure RNAspin Column was placed in a 2 mL collection tube. The above mixture was transferred into the MiPure RNAspin Column and centrifuged at 12,000 rpm (13,400 × g) for 30 seconds. 0.9 × volume of absolute ethanol was added to the filtrate and pipetted up and down 3-5 times.

The MiPure miRNA Column was placed into a 2 mL collection tube. Half the volume of the mixture was transferred into the MiPure miRNA Column and centrifuged at 12,000 rpm (13,400 × g). The filtrate was discarded, and the MiPure miRNA Column was returned to the 2 mL Collection Tube. The remaining mixture was transferred into the MiPure miRNA Column and centrifuged at 12,000 rpm (13,400 × g) for 30 seconds. The filtrate was discarded, and the MiPure miRNA Column was returned to the 2 mL Collection Tube. 500 µl of Buffer miRW1 (it was checked that absolute ethanol had been added in advance) was added to the MiPure miRNA Column. The column was incubated at room temperature for 1 minute and centrifuged at 12,000 rpm (13,400 × g) for 30 seconds. The filtrate was discarded, and the MiPure miRNA Column was returned to the 2 mL Collection Tube. 500 µl of Buffer miRW2 (it was checked that absolute ethanol had been added in advance) was added to the MiPure miRNA Column. The column was incubated at room temperature for 1 minute and centrifuged at 12,000 rpm (13,400 × g) for 30 seconds. The filtrate was discarded, and the MiPure miRNA Column was returned to the 2 mL Collection Tube. 500 µl of 80% ethanol (freshly prepared with RNase-free ddH2O) was added to the MiPure miRNA Column. The column was incubated at room temperature for 1 minute and centrifuged at 12,000 rpm. The filtrate was discarded, and the MiPure miRNA Column was returned to the 2 mL Collection Tube. The empty column was centrifuged at 12,000 rpm (13,400 × g) for 2 minutes to dry the MiPure miRNA Column membrane. This step thoroughly removed the residual ethanol in the MiPure miRNA Column. The MiPure miRNA Column was transferred to a new 1.5 ml RNase-free centrifuge tube. It was dried at room temperature for 2-5 minutes. 30-50 µl of RNase-free ddH2O was added to the center of the MiPure miRNA Column membrane. The column was incubated at room temperature for 2 minutes and centrifuged at 12,000 rpm (13,400 × g) for 1 minute to collect the filtrate. The MiPure miRNA Column was discarded, and the miRNA was stored at -70℃.

cDNA synthesis

cDNA synthesis will be done according to the protocol of the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. To prepare the reaction, the specified reagents were added to a sterile, nuclease-free tube, which was kept on ice. First, the Template RNA was added, with amounts ranging from 0.1 ng to 5 µg for total RNA. Following this, the chosen Primer was added, 1 µL of Oligo(dT)18 primer. Finally, nuclease-free water was added to bring the total volume of the mixture to 12 µL. The remaining components were then added in the following order: 4 µL of 5X Reaction Buffer, 1 µL of RiboLock RNase Inhibitor (20 U/µL), 2 µL of 10 mM dNTP Mix, and 1 µL of RevertAid M-MuLV RT (200 U/µL), bringing the total volume to 20 µL. The solutions were mixed gently and briefly centrifuged. For oligo(dT)18, the mixture was incubated for 60 minutes at 42°C. The reaction was terminated by heating at 70°C for 5 minutes. The resulting reverse transcription reaction product could then be stored at -70°C. For preserving the integrity of the cDNA, dilution was carried out in nuclease-free water in a clean setting. All reagents and cDNA samples were kept on ice while preparing. The results of the Nanodrop spectrophotometer, which provide an instant nucleic acid read, were used to calculate the dilution formula.

The dilution is carried out by initially labelling a new, nuclease-free microcentrifuge tube. The measured volume of nuclease-free water is next pipetted into the tube, with the measured addition of the calculated volume of cDNA stock. To adequately mix, the tube was vortexed for a short time. A brief spin-down for 7 seconds in a centrifuge is required to collect all liquid at the bottom of the tube for total homogenization. The diluted cDNA was placed at -20°C for daily use and, for storage in an extended period of time, it was divided into smaller amounts. This organized approach ensures the generation of normally concentrated and pure cDNA samples, which are crucial for producing reproducible and consistent results in subsequent PCR.

The product of the first strand cDNA synthesis can be directly used in PCR for amplification. For each PCR reaction, individual components were mixed prior to use, and all reaction components were assembled on ice. For a 25 µL reaction, the following volumes were combined. 12.5 µL of One Taq Quick-Load 2X Master Mix with Standard Buffer, 0.5 µL of 10 µM Forward Primer, 0.5 µL of 10 µM

Reverse Primer, as well as 1 µL template DNA. This is to ensure the final concentration was less than 1,000 ng. Finally, 10 µL nuclease-free water was added to bring the total reaction volume to 25 µL.

PCR protocol

The following thermal cycling regimen was carried out on a gradient mastercycler for target sequence amplification. 35 cycles of amplification were conducted according to the following sequence: denaturation at 94°C for 30 seconds, primer annealing at 45°C for 15 seconds, and extension at 68°C for 1 minute. The final extension step was then carried out at 68°C for 5 minutes, after which the reaction was incubated at 10°C. To prepare the 1% agarose gel, one gram of agarose powder was weighed and placed into a conical flask. Next, one hundred millilitres of TAE buffer was poured into the same flask, and the mixture was then microwaved for 1 minute and 30 seconds to dissolve the agarose. After heating, four microliters of gel stain were added to the solution. The prepared solution was then cooled down before being poured into a gel tray, where it was allowed to solidify for 20-30 minutes. Once solidified, the gel was carefully placed into the RNA tank in preparation for gel electrophoresis.

For the gel electrophoresis run, the ladder required extra preparation. The ladder was prepared by combining 1 µL of loading dye with 5 µL of ladder for 1% agarose gel on parafilm. Following preparation, the ladder mixture and all sample mixtures (20 µL each) were carefully pipetted into the designated wells of the gel, with the first row containing the ladder and the subsequent rows containing the samples. Finally, the gel was run for 60 minutes at 425 milliamperes and 90 volts to separate the RNA fragments. Quantitation of specific PCR product band intensity was performed using ImageJ software (version 1.53t, National Institutes of Health, USA). Images were cropped to include relevant lanes and bands with no non-linear brightness or contrast correction. For each lane, square boxes were drawn around the target PCR product band and the corresponding reference gene amplicon. The 'Integrated Density' was measured for each chosen area. Background signal was subtracted for each band by measuring an equally sized area just off to the side of the band in the same lane. The background-subtracted Integrated Density of the target PCR product band was then divided by the background-subtracted Integrated Density of the housekeeping genes from the same lane. This provided a 'Normalized Relative Intensity' for each sample, in arbitrary units (AU), which was exported to Microsoft Excel for statistical analysis.

Statistical analysis was performed on normalized miR-29 expression levels from ImageJ densitometry data using Microsoft Excel (version 365) with the Data Analysis ToolPak. A paired t-test was used to compare the mean miR-29 expression before and after supplementation of MO in the same mice to account for the within-subject design. Data are expressed as the mean ± SEM, and statistical significance was set at $p < 0.05$.

Results and discussion

To determine the capability objectives of miR-29, we employed the TargetScan target prediction tool, a widely recognized bioinformatics resource for miRNA target prediction. TargetScan utilizes a combination of sequence complementarity, evolutionary conservation, and thermodynamic balance to predict miRNA-mRNA interactions. Through inputting the miR-29 collection and specifying the organism of interest, TargetScan generates a list of ability mRNA targets, along with their predicted binding sites in the three' untranslated regions (UTR). The TargetScan output provides a comprehensive list of miR-29 targets, each annotated with key capabilities such as the expected binding site, context, and a score reflecting the likelihood of the interaction. Figure 1 below showcases a portion of the TargetScan output, highlighting the anticipated miR-29 binding sites within the 3' UTR of a selected gene across numerous mammalian species. The extent of conservation across one-of-a-kind species is indicated through the coloration-coding of the binding web sites, with darker colourings representing better stages of conservation. This statistic is crucial for evaluating the functional significance of the predicted interactions, as conserved websites are more likely to be functionally relevant. The TargetScan evaluation found several capacity miR-29 target websites in the 3' UTR of the gene of interest. Those websites show off varying stages of conservation across exclusive mammalian species, suggesting practical relevance. The predicted binding sites with higher conservation ratings are considered more

likely to be authentic miR-29 targets, as they're much less likely to be under selective pressure to maintain the interaction.

Figure 1 Prediction outcome of miR-29 target in TargetScan

To complement the TargetScan analysis, we utilized miRDB, a widely used bioinformatics tool for miRNA target prediction. miRDB utilizes a comprehensive set of algorithms and experimental data to predict miRNA-mRNA interactions, taking into account factors such as sequence complementarity, evolutionary conservation, and binding free energy. Just like TargetScan, we input the miR-29 collection

and distinguished the organism to retrieve a list of potential mRNA targets and their expected binding sites in the three' UTR.

The miRDB output gives a list of anticipated miR-29 targets, in conjunction with precise facts on the predicted binding sites, which include their place inside the 3' UTR. Figure 2 showcases a part of the miRDB output, displaying the anticipated miR-29 binding sites inside the 3' UTR of a particular gene. The 3' UTR sequence is displayed in conjunction with the predicted binding sites highlighted, imparting a visual illustration of the capacity of miR-29-mRNA interactions. This record is crucial for additional analysis and experimental validation of the predicted goals.

Evaluating the outcomes obtained from miRDB and TargetScan, we aimed to identify a consensus set of miR-29 targets. The intersection of goals expected via both databases is likely to represent more robust and dependable predictions, as unbiased algorithms and datasets may support them. This comparative evaluation will assist in prioritizing goals for further experimental validation, including luciferase reporter assays or gene expression analysis.

MicroRNA and Target Gene Description:

miRNA Name	mmu-miR-29b-3p	**miRNA Sequence**	UAGCACCAUUUGAAAUCAGUGUU
Previous Name	mmu-miR-29b		
Target Score	98	**Seed Location**	825, 1252, 3088, 3658, 3727, 4921
NCBI Gene ID	13435	**GenBank Accession**	NM_001271753
Gene Symbol	Dnmt3a	**3' UTR Length**	6696
Gene Description	DNA methyltransferase 3A		

3' UTR Sequence

```
   1 gggacatggg ggcaaactga agtagtgatg ataaaaaagt taaacaaaca aacaaacaaa
  61 aaacaaaaca aaacaataaa acaccaagaa cgagaggacg gagaaaagtt cagcacccag
 121 aagagaaaaa ggaatttaaa gcaaaccaca gaggaggaaa acgccggagg gcttggcctt
 181 gcaaaagggt tggacatcat ctcctgagtt ttcaatgtta accttcagtc ctatctaaaa
 241 agcaaaatag gcccctcccc ttcttcccct ccggtcctag gaggcgaact ttttgttttc
 301 tactcttttt cagaggggtt ttctgtttgt ttgggttttt gtttcttgct gtgactgaaa
 361 caagagagtt attgcagcaa aatcagtaac aacaaaaagt agaaatgcct tggagaggaa
 421 agggagagag ggaaaattct ataaaaactt aaaatattgg ttttttttct tttccttttc
 481 tatatatctc tttggttgtc tctagcctga tcagatagga gcacaaacag gaagagaata
 541 gagaccctcg gaggcagagt ctcctctccc acccccgag cagtctcaac agcaccattc
 601 ctggtcatgc aaaacagaac ccaactagca gcagggcgct gagagaacac cacaccagac
 661 acttttctac agtatttcag gtgcctacca cacaggaaac cttgaagaaa accagtttct
 721 agaagccgct gttacctctt gtttacagtt tatatatata tgatagatat gagatatata
 781 tatataaaag gtactgttaa ctactgtaca tcccgacttc ataatggtgc tttcaaaaca
 841 gcgagatgag caaagacatc agcttccgcc tggccctctg tgcaaagggt ttcagcccag
 901 gatggggaga ggggagcagc tggagggggt tttaacaaac tgaaggatga cccatatcac
 961 cccccacccc tgccccatgc ctagcttcac ctgccaaaaa ggggctcagc tgaggtggtc
1021 ggaccctggg gaagctgagt gtggaattta tccagactcg cgtgcaataa ccttagaata
1081 tgaatctaaa atgactgcct cagaaaaatg gcttgagaaa acattgtccc tgattttgaa
1141 ttcgtcagcc acgttgaagg ccccttgtgg gatcagaaat attccagagt gagggaaagt
1201 gacccgccat taaccccacc tggagcaaat aaaaaaacat acaaaatgta ctggtgcttt
1261 ctgtctaagt tgccttttgt gtgttctttt ataaggcccc accatcccct ctgcacatgg
1321 cagctccggt cctggaatgt gatgtttttg gtcatctcta aagactgcag tttcatactt
1381 gggaggctga tgacaccttt attataatta ttcttatggt tctggctata attgttttaa
1441 gattttcttt cagaaaacaa aaacccaaca cccttccctt taggtttcaa accaaggtgc
1501 ggggggggtgg caggtgcttt tttaaggacc agtggctctg gtgccctggc tcccacccct
1561 caggccaggt gagccactgg gcaacaagct aggcagccag ggagtttgag gcccaccctc
1621 cgggccagtc actcttctct tcttcttccc ttcctcgtga gtccggtgtg tcagggctgg
1681 agggaggccg gggcagcctc cctccttgtg tgtgtggttg gagtggcgtg tgtttctttt
1741 ctagtgtttg ctctgatggc tgtgctctca cttgagtcag cttcacctgg gccatgtgct
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Figure 2 Images of miR-29 and its target's information in miRDB

Figure 3 illustrates the average weight of mice in grams for the treated (M) and control (NM) groups over a 12-day period. Both groups showed a gradual increase in weight up to day 4, reaching a peak of approximately 19 g. After this point, the treated group (M) exhibited a progressive decline in weight, reaching about 15 g by day 12. In contrast, the control group (NM) maintained relatively stable weights after day 6, fluctuating slightly but remaining within the range of 16 - 17 g until day 12. Overall, treatment resulted in a noticeable reduction in average body weight compared to the control group, which sustained higher and more stable weights throughout the study.

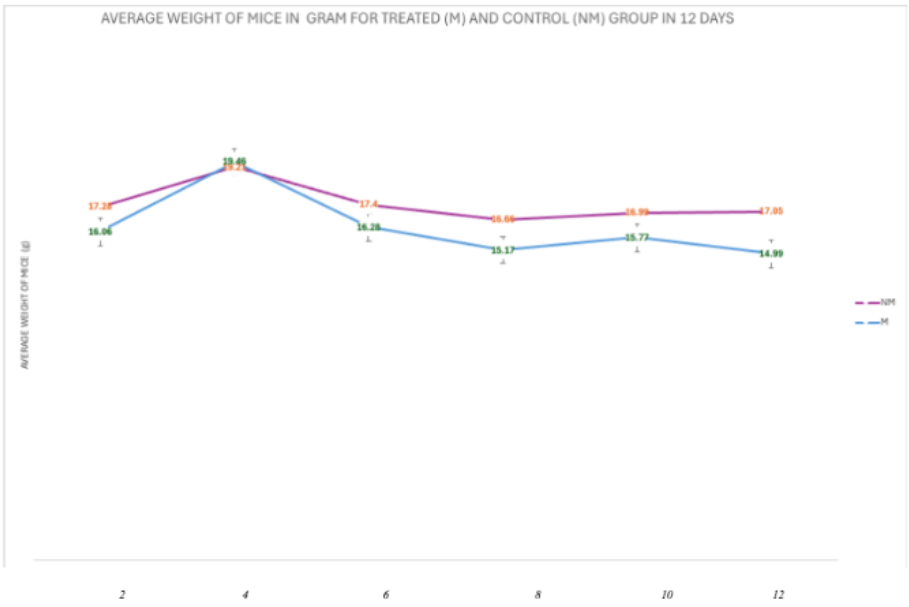

Figure 3 Average weight of mice in grams for the treated (M) and control (NM) groups in 12 12-day graph.

Quantitative and qualitative RNA analysis of the extracted RNA are necessary to offer reliable downstream molecular applications. Regarding RNA concentration, an optimal value of 100-1000 ng/μL is usually adequate to provide sufficient starting material for downstream analyses, such as cDNA synthesis. As indicated in Table 1, NM1 and M1 samples possessed RNA concentrations below this value (<100 ng/μL), while the remaining samples offered >100 ng/μL of concentrations, which was indicative of sufficient starting material. The lower concentrations of samples NM1 and M1 are perhaps the result of a variety of factors, including heterogeneity of the original tissue or blood input sample, loss of RNA through the extraction process from incomplete lysis or binding to the column purification medium, or RNA breakdown from poor handling of the sample prior to or during extraction.

Table 1: Nanodrop Result for each sample

Sample	RNA concentration (ng/L)	A 260/280
NM1	70	1.92
NM3	162.8	2.01
NM4	154	2.2
NM5	188.9	1.99
NM6	213.5	1.99
NM8	113.9	2.02
M1	66.8	1.79
M2	114	1.83
M6	152.2	1.68
M9	336.7	1.98
M10	185	2

RNA purity is measured by the A260/A280 absorbance ratio. A ratio of 1.80 or higher is considered good-quality RNA. In our current test (Table 1), the A260/A280 ratios of samples M1 and M6 were below 1.80, whereas those of the remaining samples were above this value. A value below 1.80 definitely indicates protein carryover, which can directly hinder enzymatic processes, such as PCR, and jeopardize the accuracy of gene expression measurements. A260/A280 ratio and potentially disrupt downstream enzymatic activities. Further, the pH of the final elution buffer used in the last purification step of the RNA could influence this ratio. In this study, a DNA ladder was always included on each gel as an essential molecular weight marker for estimating amplicon sizes and assessing the quality of the electrophoretic run. As observed in Figure 4, the DNA ladder exhibited sharp and clear banding, resolving uniformly through the top half of the gel with minimal smearing. This indicates optimal gel preparation, good running conditions, and clear DNA visualization.

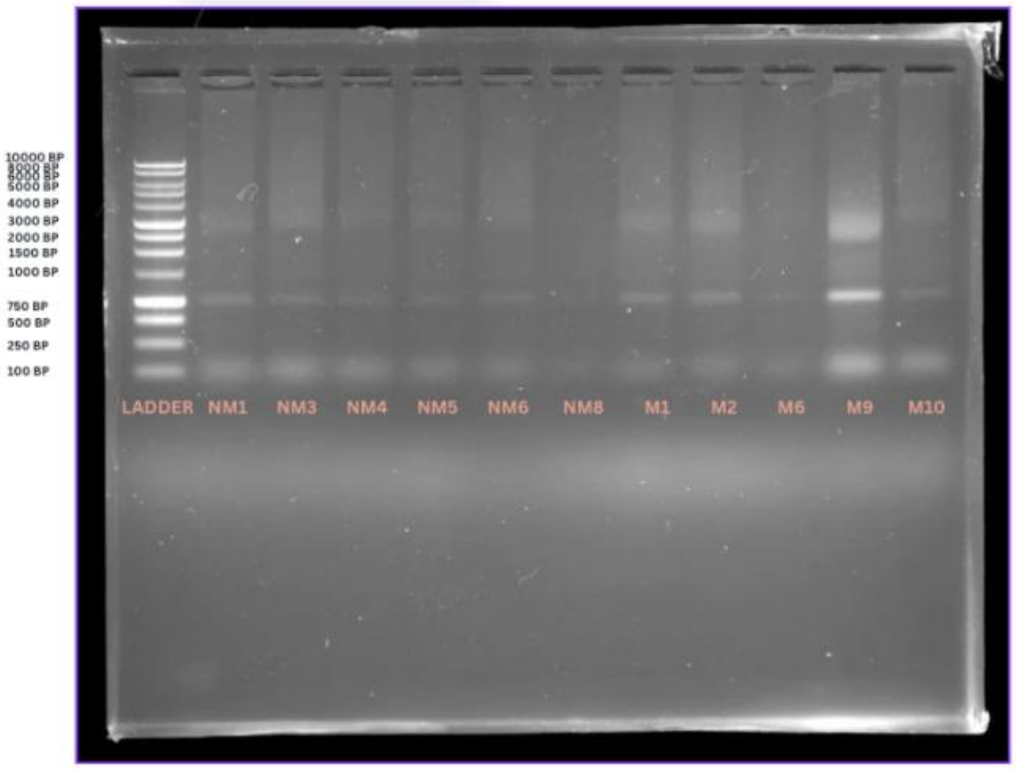

Figure 4 Gel electrophoresis for all the samples

In this study, a DNA ladder was always included on each gel as an essential molecular weight marker for estimating amplicon sizes and assessing the quality of the electrophoretic run. As observed in Figure 4, the DNA ladder exhibited sharp and clear banding, resolving uniformly through the top half of the gel with minimal smearing. This indicates optimal gel preparation, good running conditions, and clear DNA visualization.

For single-amplicon products, the 100 bp marker of the ladder was used as a critical point of reference, confirming proper sizing for smaller PCR products. For experimental amplicons, most samples yielded bands of the correct size, indicating successful amplification. In some samples with suboptimal amplification, such as NM8, potential problems are suggested. This could result from PCR inhibition, where contaminants interfere with the polymerase or primer binding activity. Inhibition can result from chelating agents (EDTA) or proteins from the RNA extraction process.

Apart from the molecular study, physical contaminants were observed in the gel matrix, dust particles and strands of hair. Such contamination, likely introduced during transfer or gel pouring, can retard DNA migration, complicate band visibility, and compromise data analysis. To prevent this, strict

adherence to sterile laboratory practices is necessary. This includes cleaning the gel casting equipment and work surface carefully before use with dust-free laboratory tissues. Additionally, cover the gel tank carefully with a clean barrier, such as a paper towel or plastic wrap, when the gel is in the process of hardening or when the tank is exposed for loading or running procedures.

In addition to DNA analysis, total RNA sample integrity was also evaluated through visualization of rRNA bands on an agarose gel prior to cDNA synthesis. For eukaryotic samples, two distinct and sharp bands corresponding to the 28S and 18S rRNA subunits are observed, with the 28S band being approximately twice the width of the 18S band (a 28S/18S ratio of approximately 2:1), indicating good-quality, intact RNA. A smear or a prominent 18S (or 16S) band typically reflect RNA degradation.

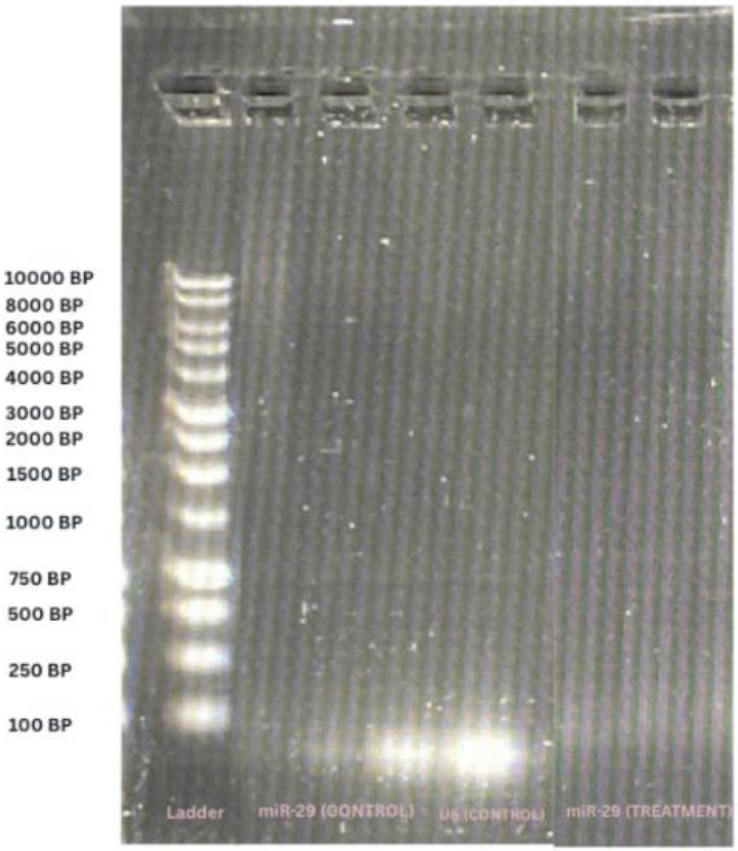

Figure 5 Image of gel electrophoresis for miR-29 and U6 in the control (NM) sample and miR-29 in the treatment (M) sample

As evident from Figure 5, the DNA ladder exhibited distinct and sharp banding with even resolution through the top half of the gel and no significant smearing. For the miR-29 and its reference gene U6 targeted amplicon products, the 100 bp marker in the ladder served as a crucial reference point for confirming correct amplicon sizing. Visualization of the experimental amplicons revealed that U6 consistently displayed the brightest band, as expected of a highly expressed and stable endogenous reference small RNA. Bands for miR-29 were also clearly visible in both control and treatment samples, albeit less intense than U6 bands. This difference in intensity is consistent with U6's role as a highly expressed small nuclear RNA, which tends to be present at significantly higher levels than any individual microRNA, such as miR-29. The lack of visibility can point to issues such as partial PCR inhibition, template degradation, or variability in primer efficiency, which are a few other parameters that can result in suboptimal amplification. To avoid such problems in future, steps such as further dilution or re-purification of the cDNA template can be taken.

Based on the bar chart (Figure 6), the data shows a visually dramatic increase in the normalized value for the Treated (M) group compared to the Control (NM) group. The mean value for the treated group is significantly higher, appearing to be around 33, whereas the mean for the control group is close to 1. However, the presence of a very large error bar on the treated group's column is a critical point of concern. This large error bar suggests a high degree of variability or wide dispersion of data points within the treated group. This indicates that some individual values in the treated group were significantly higher than the mean, while others may have been significantly lower.

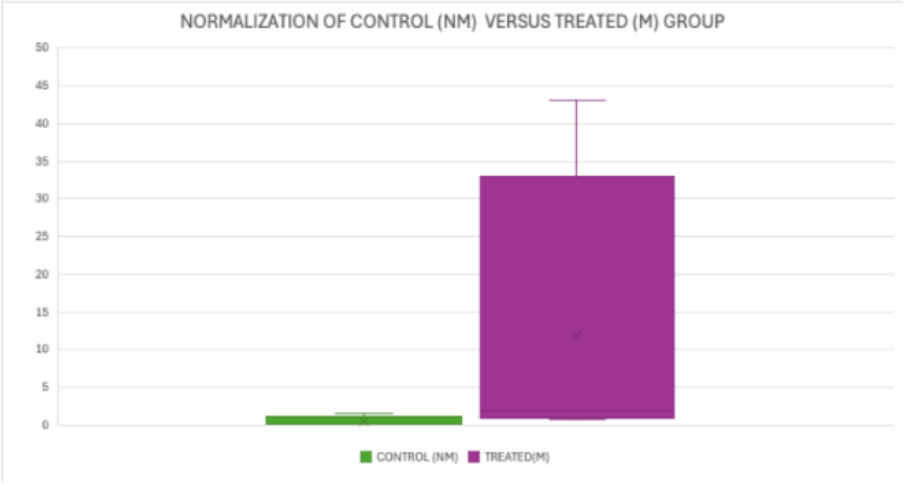

Figure 6 Images of normalization of the control (NM) versus the treated (M) group bar chart

Based on the statistical analysis (Table 2), the study found no significant change in the normalized miR-29 expression in mice plasma after *M. oleifera* supplementation. A paired-samples t-test yielded a t-statistic of -1.1224991 and a p-value of 0.37830. As this p-value is well above the conventional significance threshold of 0.05, we fail to reject the null hypothesis. Therefore, the observed difference in miR-29 levels before and after supplementation is not statistically significant and could have occurred by chance. The results indicate that the supplementation did not have a measurable effect on miR-29 expression under the conditions of this study. Given the extremely small sample size (n=3) and high variability, as indicated by the large standard deviation relative to the mean, these findings should be interpreted with caution. Future studies with a larger sample size are needed to draw more definitive conclusions regarding the effect of *M. oleifera* on miR-29 expression.

Table 2: Table of t-Test: Paired Two Sample for Means

Parameter	**0.0880261**	**0.8143302**
Mean	0.59737654	15.5831668
Variance	0.52409949	568.701996
Observations	3	3
Pearson Correlation	0.99998399	
Hypothesized Mean Difference	0	
df	2	
t Stat	-1.1224991	
P(T<=t) one-tail	0.18915283	
t Critical one-tailed	2.91998558	
P(T<=t) two-tail	0.37830566	
t Critical two-tailed	4.30265273	

Conclusion

In general, the outcomes of this research successfully identified the target genes of miR-29 using target prediction tool software, such as the MicroRNA Target Prediction Database (miRDB) and TargetScan, and analyzed the expression level of miR-29 in response to *M. oleifera* extract and a placebo in mice. The results were then validated using PCR, confirming the mechanisms identified. Despite the insignificance in terms of statistical test, these findings therefore point towards a potential positive health impact of *M. oleifera* via modulation of miR-29 and merit further investigation into the specific mechanisms and broader therapeutic potential of this observed upregulation of microRNA. It should be the goal of future studies to advance beyond current experimental limitations and determine the long-term effects of *M. oleifera* more fully, as well as define the specific health consequences of miR-29 modulation in this model.

Acknowledgement

First and foremost, I would like to express my sincere appreciation to my main thesis supervisor, Dr. Abdul Fatah A. Samad, for his invaluable guidance, encouragement, and constructive critiques. Without his continued support and interest, this thesis would not have reached its present form. Additionally, I would like to extend my heartfelt thanks to my co-supervisor, Dr. Mohd Aizuddin Mohd Lazaldin, fellow undergraduate students, as well as master students, for their unwavering support. Their assistance has been a source of strength and motivation. My sincere appreciation also extends to all my colleagues and others who have assisted on various occasions. Their views and tips have been incredibly useful, and though it is not possible to list all of them in this limited space, their contributions are deeply appreciated.

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