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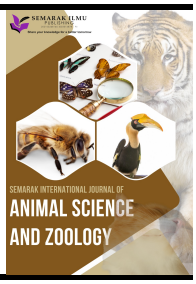

Bioinformatics and Experimental Analysis of miR-21-5p-Mediated Gene Regulation in Moringa-Supplemented Mice

Lee Chiao Min[1], Azlina Zulkapli[2], Mohd Aizuddin Mohd Lazaldin[2], Abdul Fatah A. Samad[1,*]

[1] Department of Biosciences, Faculty of Sciences, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
[2] Special Resource Unit, Laboratory Animal Research Unit, Institute for Medical Research Jalan Pahang 50588 Kuala Lumpur, Malaysia

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ABSTRACT

Modulation of microRNAs (miRNAs) through miRNA-based therapeutics faces several challenges, including in vivo instability and activation of the innate immune response. *Moringa oleifera* offers a promising natural alternative for regulating miR-21-5p expression due to its rich phytochemical profile and lower immunogenic risk. This study investigated the impact of Moringa extract on miR-21-5p expression in BALB/c mice, followed by bioinformatic prediction and analysis of the downstream effects on its identified target gene. Mice were split into two groups, with the treatment group receiving 300 mg/kg of Moringa extract via oral gavage for 12 days in a row, while the control group was given the same amount of distilled water. Gene expression levels were analysed using RT-PCR. Bioinformatic analysis using TargetScan predicted a total of 303 potential miR-21-5p's target genes, among which SMAD7 was chosen for further RT-PCR analysis due to its strong predicted interaction and known role in disease regulation. The results showed that the treatment group's expression of miR-21-5p was significantly downregulated ($p = 0.028$), with the average normalised band intensity decreasing from 0.55 ± 0.059 in the control group to 0.21 ± 0.009 in the treatment group ($n = 3$ mice per group). This reduction was accompanied by an increase in SMAD7 mRNA levels, from 2.61 ± 0.979 to 3.29 ± 1.374 ($p = 0.7083$), indicating that miR-21-5p directly targets SMAD7. Consequently, the potential of Moringa extract as a natural modulator of miRNA expression is supported by these findings.

1. Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs that are single stranded and short, comprising approximately 22 nucleotides [1]. Thousands of miRNAs have been discovered in a variety of organisms, including animals, plants and microorganisms [2]. The post-transcriptional stage of gene expression is significantly regulated by these molecules, primarily by binding to messenger RNAs (mRNAs) and repressing their expression, thereby influencing various biological processes and disease-related pathways [3,4]. In most cases, this regulation entails the interaction of the target

* *Corresponding author.*
E-mail address: abdulfatah@utm.my

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mRNA's 3′ untranslated region (3′ UTR) with the miRNA seed region, leading to reduced translation efficiency or mRNA degradation [5]. However, miRNAs can also interact with regions other than 3′ UTR, including the coding sequence, gene promoters, and 5′ UTR [6]. Notably, binding of miRNA to the coding regions and 5′ UTR has been shown to suppress gene expression, while interactions with promoter regions have been associated with transcriptional activation [7].

As one of the most thoroughly investigated miRNAs, miR-21 is acknowledged for its vital role in controlling gene expression in numerous biological functions, such as cell development, differentiation, inflammation, proliferation, apoptosis and metabolic homeostasis [8]. In both murine and *Homo sapiens*, miR-21 is situated within the 11th intron of the *TMEM49* (*VMP1*) gene, located on chromosome 11 and chromosome 17 at position 17q.23.1, respectively [9,10]. Like most miRNAs processed via the canonical pathway, miR-21 biogenesis is initiated by RNA polymerase II-mediated transcription of the primary transcript, pri-miR-21 [11,12]. The Drosha-DGCR8 complex later processes this transcript into a precursor miRNA (pre-miR-21), which exportin-5 carries to the cytoplasm. After that, the precursor is trim into a miRNA duplex by the RNase III enzyme Dicer, from which the guide strand is incorporated into the miRNA-induced silencing complex (miRISC) and the passenger strand is typically broken down [9,13]. The mature miRNA may arise from either arm of the precursor: the 3′ strand (miR-21-3p) or the 5′ strand (miR-21-5p) [14]. Among these, miR-21-5p is the dominant isoform and has been widely implicated in pathological conditions including fibrosis, inflammation, cancer and cardiovascular diseases [15].

Moringa oleifera, commonly referred to as ‘pokok kelor’ in Malaysia, is a nutrient-rich plant widely valued for its medicinal and nutritional properties [16]. Belonging to the family Moringaceae, *Moringa* originates from South Asia, with its native range spanning northern West Bengal, India and northeast Pakistan [17]. Most of the Moringa plant’s parts, including the flowers, seeds, leaves and pods are suitable for consumption and have been traditionally used in India’s ancient Ayurvedic medicine, where the plant is believed to help treat over 300 diseases [18,19]. Toxicological assessments have shown that aqueous leaf extracts of Moringa are generally safe at doses ≤1000 mg/kg, while acute toxicity has been observed at supra-supplementation levels (≥3000 mg/kg) in Sprague-Dawley rats [20]. Recent studies have identified a range of bioactive phytochemicals in *Moringa*, including phenolic acids, flavonoids and polyphenols, which contribute to its anti-inflammatory, antioxidant, antibacterial and anti-diabetic activities [21-23].

miR-21-5p is consistently and aberrantly upregulated in a wide spectrum of pathological conditions, where it suppresses essential regulatory genes such as PDCD4, PTEN, and SMAD7, thereby driving pathological processes including fibrosis, inflammation, and oncogenesis [24–26]. While RNAi-based therapeutics have demonstrated efficacy in inhibiting miR-21, their clinical translation has been severely constrained by immune-related toxicities arising from the intrinsic immunostimulatory properties of RNA molecules [27,28]. This limitation exposes a critical and unresolved gap in the development of safe and effective strategies for modulating pathogenic miRNA expression. In this context, *Moringa oleifera*, a phytochemical-rich plant with potent anti-inflammatory and antioxidant activities, presents a compelling candidate for investigation [29]. Evidence from other bioactive compounds such as resveratrol, genistein, and quercetin indicates that plant-derived molecules can modulate miR-21 expression by targeting upstream transcriptional regulators and signalling pathways [30,31]. This study aims to critically evaluate the capacity of *M. oleifera* leaf extract to modulate miR-21-5p expression in a murine model. The findings are expected to advance the concept of phytochemical-based miRNA modulation as a potentially safer, plant-derived alternative to conventional RNAi therapeutics, with broad implications for managing miR-21–driven pathologies.

2. Methodology

2.1 Application of Ethical Approval

Before conducting the research, ethical approval was first obtained from the UTM Research Ethics Committee. The research design and animal handling procedures were strictly adhered to the ethical rules and guidelines for Animal Care and Use for Research Ethics at UTM. (Approval No.: UTMREC-2025-114)

2.2 Oral Supplementation of Moringa extract on Mice

Resource equation approach was utilised for the determination of sample size as it is suitable for the study whenever assumptions about standard deviation and effect size are not feasible [32]. As a result, a total of 20 normal and wild type BALB/c mice aged 5 weeks were purchased from Sapphire A Enterprise. Female mice were selected because they exhibit less unstructured variability compared to males, even without controlling for oestrous cycles [33]. The mice first underwent a 3 days acclimatisation period to ensure proper adaptation and reduce handling stress. They were kept in cages within the animal facility at Block T02, UTM, with unrestricted access to food and water, under controlled conditions at 25 ± 5°C, following an alternating 8-hour light/16-hour dark cycle. After acclimatisation, each mouse's body weight was measured and documented. The animals were subsequently assigned randomly into two groups, each consisting of ten mice. The treatment period lasted for twelve days and the dosage of Moringa extract given to the treatment group was 300 mg/kg [34]. The extract used in this study was obtained in the form of health supplement capsules. Oral gavage was performed each morning at approximately 10 a.m. The control group was administered 200 µL of distilled water, whereas the treatment group received Moringa extract dissolved in 200 µL of distilled water, with the dosage calculated according to each mouse's body weight. A 1 mL syringe and a reusable 45 mm bulb-tipped, stainless steel curved oral gavage needle with an inner diameter of 0.5 mm were used for each feeding group. Both groups had unrestricted access to food and water and maintained under the same caging conditions as previously.

After the 12-day feeding period, anaesthesia was induced in the mice via intraperitoneal injection of a ketamine and xylazine mixture (Ilium Ketamil, Xylazil-100, 100 mg/mL, Troy Laboratories, Glendenning, New South Wales, Australia) at a dosage of 0.1 mL/100 g of body weight. A volume of 0.5 to 1 mL of blood was collected through facial vein puncture or cardiac puncture and stored in a 3 mL EDTA tube. A 100 µL whole blood sample was thoroughly mixed with the RNA isolator (1 mL) and stored at 4°C in a refrigerator for subsequent RNA extraction. The remaining blood samples were kept at -80°C in a freezer.

2.3 RNA Extraction and Evaluation of RNA Purity and Concentration

Total RNA was extracted from whole blood using the MiPure Cell/Tissue miRNA Kit (RC201-01; Vazyme, Nanjing, China) following the manufacturer's instructions [35]. In short, RNA isolator and chloroform were mixed with the blood samples, followed by phase separation via centrifugation. The supernatant was collected, mixed with absolute ethanol, and loaded onto the MiPure RNAspin Column in two steps. The column was sequentially washed with Buffer miRW1, Buffer miRW2, and 80% ethanol, then dried and eluted with RNase-free water. The total RNA was stored at –80°C for later use. After that, the extracted RNA's concentration and purity were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Massachusetts, USA), with OD260/230 and OD260/280 ratios used to assess purity [36]. RNA integrity was evaluated by 1% agarose gel

electrophoresis, with SYBR™ Safe DNA Gel Stain (S33102; Thermo Scientific, Massachusetts, USA) incorporated into the gel for visualisation. For size reference, the first lane was loaded with a marker consisting of 1 µL of Blue/Orange 6X Loading Dye (G190A; Promega, Madison, WI, USA) and 5 µL of 1 kb DNA Ladder (G571A; Promega, Madison, WI, USA). The remaining lanes were loaded with RNA samples, in which 200 ng of RNA mixed with 1 µL loading dye and nuclease-free water (to 6 µL). Finally, electrophoresis ran at 90 V for 60 minutes, and band visualisation was performed using the Bio-Rad Gel Imaging System.

2.4 cDNA Synthesis

The RevertAid First Strand cDNA Synthesis Kit (K1621; Thermo Fisher Scientific, Waltham, MA, USA) was employed to synthesised complementary DNA (cDNA) in accordance with the manufacturer's protocol, with a concentration of 700 ng total RNA being used [37]. A volume of 12 µL reaction mixture was first prepared by mixing appropriate volume of RNA samples with the oligo$(dT)_{18}$ primers and nuclease-free water. Subsequently, 5X Reaction Buffer (4 µL), RiboLock RNase Inhibitor (1 µL), RevertAid M-MuLV Reverse Transcriptase (1 µL) and dNTP Mix (2 µL) were added, bringing the total reaction volume to 20 µL. After gentle mixing and brief centrifugation, the mixture was incubated at 42 °C. After 60 minutes, the mixture was heated for 5 minutes to terminate the reaction at 70 °C. The synthesised cDNA was subsequently used for PCR analysis or stored at –70 °C for long-term storage and –20 °C for short-term use.

2.5 RT-PCR Analysis: miR-21-5p Expression

Following the instructions provided by the manufacturer, the OneTaq® Quick-Load 2X Master Mix with Standard Buffer (M0486S; New England Biolabs, Massachusetts, USA) was utilised to perform PCR [38]. Three cDNA samples were randomly selected from both control and treatment groups. Each 25 µL reaction mixture consisted of the cDNA template (1 µL), OneTaq® Quick-Load 2X Master Mix (12.5 µL), nuclease-free water (10.5 µL) and 10× diluted forward and reverse primers specific to miR-21-5p (0.5 µL each). The reaction mixture was briefly centrifuged to eliminate air bubbles, which could interfere with thermal transfer and increase evaporation during amplification [39]. The same procedure was repeated using the reference gene U6 for normalisation and the sequences of all primers involved are listed in Table 1.

The following thermal conditions were used for PCR amplification: 30 seconds of initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C (30 seconds), annealing at 45°C for 15 seconds, and extension at 68°C for 1 minute. A final extension step lasting five minutes at 68°C brought this to a close. As previously mentioned in Section 2.3, the amplified products were then resolved on a 1% (w/v) agarose gel. A pre-stained 1 kb DNA ladder was used as a molecular size reference, while 20 µL of each PCR sample was loaded into the remaining wells.

2.6 Identification of Genes regulated by miR-21

The miR-21-5p's target genes prediction was accomplished using bioinformatics approaches. Specifically, databases like TargetScan (https://www.targetscan.org/mmu_80/) was utilised to identify potential target genes. This computational platform identifies potential miRNA targets by detecting specific binding sites such as 8mer, 7mer and 6mer sequences, which are complementary to the miRNA's seed region [40]. TargetScanMouse version 8.0 was utilised to predict miR-21-5p's target genes with all parameters remained at their default settings. Finally, the research was done by

inserting the name of the miRNA, "miR-21" into the search bar of TargetScanMouse and the analysis was run. The predicted miR-21-5p target genes were then further validated through RT-PCR to assess their expression levels.

2.7 RT-PCR Analysis: Expression of the Identified Target Genes

PCR and agarose gel electrophoresis were conducted according to the same protocols as described in the section of 2.5, with different primers used (SMAD7 and GAPDH, the reference gene for normalisation) [38].

Table 1
Supplemental table (primers used)

Genes	Forward (5'-3')	Reverse (5'-3')
miR-21-5p	TGTTGAGTCGTATCCAGTGCAA	GTATCCAGTGCGTGTCGTGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
SMAD7	TTCCTCCGCTGAAACAGGG	CCTCCCAGTATGCCACCAC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

2.8 Statistical Analysis

Relative expression levels were determined by quantifying band intensities using ImageJ software (version 1.54p) [41]. GAPDH was used as the reference gene for mRNA analysis, while U6 served as the reference for miRNA quantification. The mean ± standard error of the mean (SEM) was used for presenting all numerical data. Comparisons between control and treatment groups were assessed using Welch's t-test, which is appropriate for small sample sizes (n = 3) and unequal variances often observed in biological data [42]. With a p-value of less than 0.05 considered statistically significant, GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, California, USA) was used for statistical analysis and graphical visualisations.

3. Results

3.1 Body Weight of Mice Throughout the Twelve Days Feeding Period

The body weight of mice was recorded and subsequently plotted as a line graph in Figure 1.

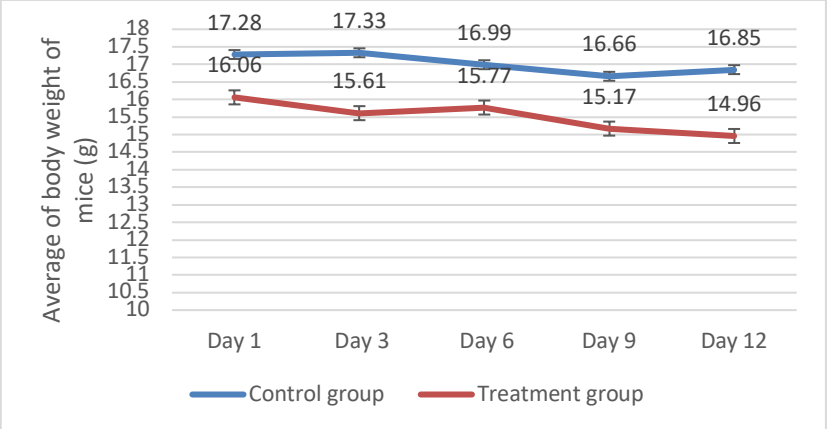

Fig. 1. Mice's body weight throughout the twelve days feeding period; Data were presented as mean ± SEM, n = 10 mice/group

Unfortunately, a total of six mice died before the blood withdrawal session was carried out, with five mice from the treatment group and one mouse from the control group. The occurrence of mortality was most likely due to incidents such as gastroesophageal reflux disease, accidental introduction of fluid into the trachea and lungs, chronic inflammation, bleeding and hepatic apoptosis [43]. Additionally, progressive weight loss was observed in most of the mice models. This is because oral gavage can induce a stress response and may cause oesophageal tears due to repeated gavage, which lead to feeding difficulties and loss of appetite [44].

A greater reduction in body weight was perceived in the group of mice administered with Moringa extract which may be attributed to its anti-obesity properties [45]. A notable reduction in body weight, along with decreased epididymal, perirenal, and mesenteric fat tissues, was observed following oral administration of *Moringa oleifera* leaf petroleum ether extract (0.125, 0.25, and 0.5 g/kg) to high-fat diet-induced mice over a 14-week period [46].

3.2 RNA Quality Assessment by NanoDrop and Agarose Gel Electrophoresis

As shown in Table 2, most of the extracted RNA samples exhibited high purity as indicated by 260/280 absorbance ratios close to 2.0, suggesting minimal protein contamination [47]. However, sample 6 from the treatment group showed a lower 260/280 ratio of 1.68, implying the potential presence of protein, phenol or other substances that absorb near 280 nm. Moreover, all samples were found to have notably low 260/230 ratios, which may suggest contamination by salts or other organic compounds with strong absorbance near 230 nm. In order to enhance RNA purity and eliminate residual impurities such as phenol and salts, an additional ethanol wash step is recommended [48]. The RNA concentrations across all samples were within an acceptable range, with the highest concentration observed at 336.7 ng/µL and the lowest at 70 ng/µL. These values indicate that the extraction protocol the kit used were generally effective in yielding sufficient RNA for downstream applications.

Table 2
Purity and concentration of extracted total RNA

Control group				Treatment group			
	A_{260}/A_{280}	A_{260}/A_{230}	RNA Concentration (ng/µL)		A_{260}/A_{280}	A_{260}/A_{230}	RNA Concentration (ng/µL)
C1	1.92	0.74	70.0	T1	1.81	1.04	70.7
C3	1.91	0.89	178.9	T2	1.87	0.79	117.0
C4	1.82	0.83	130.4	T6	1.68	0.95	152.2
C5	1.98	0.90	188.1	T9	1.98	1.38	336.7
C6	2.00	0.69	214.5	T10	1.96	0.99	188.0
C8	2.01	1.67	115.1				

Distinct bands were observed on the 1% agarose gel as shown in Figure 2, corresponding to the 28S and 18S ribosomal RNA (rRNA) components that typically found in total RNA extracts. The 28S rRNA band appeared around 3000 bp, while the 18S rRNA band was located at approximately 1000 bp. In addition, faint bands beneath the 18S rRNA suggest the presence of smaller RNA fragments. Notably, no high molecular weight bands were detected, indicating the absence of genomic DNA contamination [49]. Since the eighth control sample loaded in Lane 7 did not exhibit distinct 28s rRNA band and 18s rRNA band, it was excluded from further RT-PCR analysis due to severe RNA degradation [50].

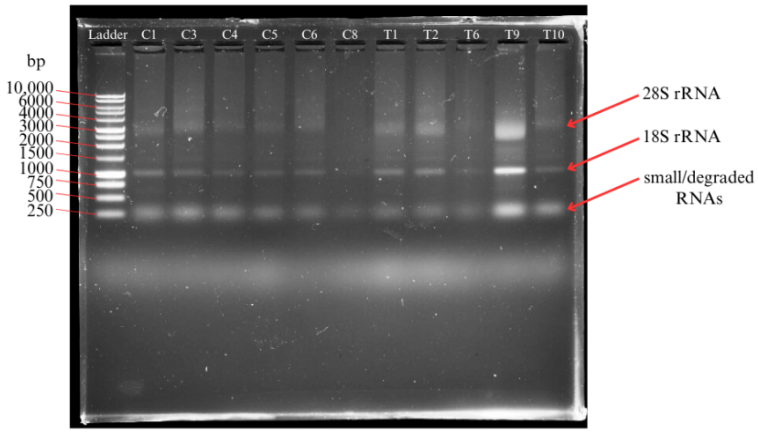

Fig. 2. Gel image of extracted RNA. Lane 1: 1 kb DNA ladder; Lanes 2–12: extracted RNA samples

rRNA serves as a useful reference because small RNA species are often difficult to detect clearly on agarose gels. It makes up approximately 80% of total RNA, with the 28S and 18S subunits being the most prominent and thus more easily detected [51]. In gel electrophoresis, high RNA integrity is typically indicated by a 28S to 18S band intensity ratio of approximately 2:1 or greater [52].

Based on these criteria, the extracted RNA samples (excluding Lane 7) demonstrated acceptable integrity, with most lanes showing distinct and well-resolved rRNA bands. Although slight smearing was observed in several lanes, the overall band pattern indicates that the RNA was sufficiently intact for downstream RT-PCR analysis. This partial degradation is likely due to RNA's natural instability and the widespread presence of RNases in the environment [53]. Therefore, maintaining an RNase-free environment is crucial. This includes using gloves, sterile and nuclease-free pipette tips and tubes and treating buffers with diethyl pyrocarbonate (DEPC) to inactivate RNases [54].

3.3 RT-PCR Products of miR-21-5p and U6 Analysed by Agarose Gel

The RT-PCR process was successful as a single clear band was observed around 100 bp in all loaded lanes as shown in Figure 3. There was no significant smearing and no high molecular weight secondary structures appearing in the upper region of the gel. These observations suggest that the primers used were highly specific and the annealing temperature of 45 °C was appropriate for effective amplification [55]. The bands representing miR-21-5p expression appeared relatively faint, which is consistent with the generally low abundance of miRNAs within cellular environments [56]. In contrast, strong, sharp and intact bands were observed in the lanes containing PCR products amplified using U6 primers. U6 is a reference gene which can also be considered as a housekeeping gene, characterised by its stable expression and minimal variation under diverse experimental conditions [57]. Therefore, it is essential for reducing systematic bias and ensuring accurate normalisation of gene expression data [58].

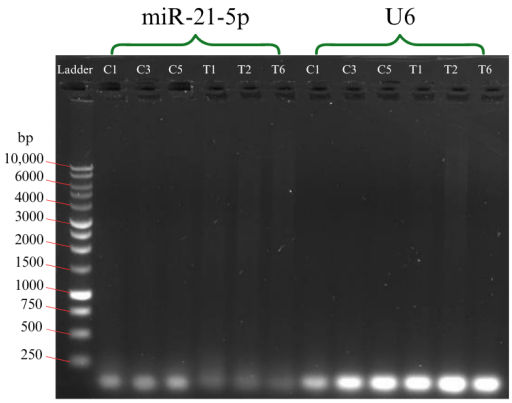

Fig. 3. PCR gel image. Lane 1: 1 kb DNA ladder; Lanes 2–4: miR-21-5p (control); Lanes 5–7: miR-21-5p (treatment); Lanes 8–10: U6 (control); Lanes 11–13: U6 (treatment)

3.4 Target Gene Prediction of miR-21-5p

Table 3 shows a portion of the results generated from TargetScanMouse 8.0 for miR-21-5p target prediction. The complete list of predicted targets is accessible via the provided link. (https://data.mendeley.com/datasets/cgvj9jd8c4/1)

Table 3
Output from TargetScanMouse 8.0 for miR-21-5p target prediction

Target gene	Representative transcript ENSMUST0000...	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Conserved sites				Poorly conserved sites				6mer sites	Representative miRNA	Predicted occupancy			Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}	Previous TargetScan publication(s)
					total	8mer	7mer-m8	7mer-A1	total	8mer	7mer-m8	7mer-A1			low miRNA	high miRNA	trans-fected miRNA				
Gpr64	0112408.3	G protein-coupled receptor 64	177	Sites in UTR	2	2	0	0	0	0	0	0	0	mmu-miR-21a-5p	0.0291	0.1983	0.9588	-0.71	-0.71	0.37	2007, '09, '11, '15
Cpeb3	0079754.5	cytoplasmic polyadenylation element binding protein 3	708	Sites in UTR	1	1	0	0	1	0	0	1	0	mmu-miR-21a-5p	0.0297	0.1904	0.7465	-0.31	-0.31	0.20	2005, '07, '09, '11, '15
Rnf24	0059372.5	ring finger protein 24	141	Sites in UTR	1	0	0	1	1	0	1	0	3	mmu-miR-21a-5p	0.0103	0.0744	0.4788	-0.23	-0.25	0.44	2009, '11, '15
Unkl	0039734.6	unkempt-like (Drosophila)	42	Sites in UTR	1	0	0	1	1	0	1	0	1	mmu-miR-21a-5p	0.0103	0.0744	0.4741	-0.19	-0.19	0.33	2007, '09, '11, '15
Fgf18	0109363.2	fibroblast growth factor 18	26	Sites in UTR	1	1	0	0	0	0	0	0	0	mmu-miR-21a-5p	0.0109	0.0730	0.3253	-0.48	-0.75	0.73	2015
Cux1	0004097.10	cut-like homeobox 1	195	Sites in UTR	1	0	1	0	1	0	1	0	3	mmu-miR-21c	0.0100	0.0730	0.4952	-0.06	-0.07	0.46	2015
Spry1	0108109.2	sprouty homolog 1 (Drosophila)	291	Sites in UTR	1	1	0	0	0	0	0	0	2	mmu-miR-21a-5p	0.0105	0.0727	0.3759	-0.49	-0.50	0.63	2005, '07, '09, '11, '15
Gm8773	0101627.2	predicted gene 8773	7	Sites in UTR	1	1	0	0	0	0	0	0	0	mmu-miR-21a-5p	0.0105	0.0724	0.3658	-0.56	-0.56	< 0.1	2015
Otud6b	0117268.2	OTU domain containing 6B	103	Sites in UTR	1	0	1	0	3	1	2	0	2	mmu-miR-21a-5p	0.0102	0.0723	0.4221	-0.19	-0.37	0.11	2007, '09, '11, '15
Pitx2	0029657.10	paired-like homeodomain transcription factor 2	47	Sites in UTR	1	0	1	0	0	0	0	0	0	mmu-miR-21a-5p	0.0100	0.0694	0.3551	-0.34	-0.34	0.32	2005, '07, '09, '11, '15
Smad7	0026999.4	SMAD family member 7	871	Sites in UTR	1	1	0	0	0	0	0	0	0	mmu-miR-21a-5p	0.0099	0.0690	0.3699	-0.45	-0.45	0.64	2005, '07, '09, '11, '15
Rmnd5a	0002292.9	required for meiotic nuclear division 5 homolog A (S. cerevisiae)	291	Sites in UTR	1	1	0	0	0	0	0	0	1	mmu-miR-21c	0.0097	0.0687	0.4019	-0.34	-0.34	0.63	2007, '09, '11, '15
Scml2	0112345.2	sex comb on midleg-like 2 (Drosophila)	44	Sites in UTR	1	1	0	0	1	0	1	0	1	mmu-miR-21a-5p	0.0099	0.0686	0.3445	-0.18	-0.44	0.50	2005, '07, '09, '11, '15
Cntfr	0102961.4	ciliary neurotrophic factor receptor	58	Sites in UTR	1	0	1	0	0	0	0	0	0	mmu-miR-21a-5p	0.0098	0.0684	0.3519	-0.26	-0.26	0.20	2005, '07, '09, '11, '15
Xkr6	0119973.2	X Kell blood group precursor related family member 6 homolog	5	Sites in UTR	1	0	1	0	0	0	0	0	0	mmu-miR-21a-5p	0.0098	0.0683	0.3660	-0.25	-0.25	0.31	2005, '07, '09, '11, '15

It was discovered that 303 mRNA transcripts (target genes) had conserved binding sites for miR-21-5p. Among these, the transcripts contain 322 conserved sites and 106 poorly conserved sites. miR-21-5p can interact with multiple target genes as it only requires partial complementarity with the 3' UTR of target mRNA sequences to exert its regulatory effects. This unique characteristic of miRNAs enables miR-21-5p to regulate a broad spectrum of genes simultaneously, thereby influencing various signalling pathways and cellular functions [59].

The binding of miR-21 to its target genes involves both seed pairing and 3’ supplemental pairing [60]. Watson–Crick base-pairing principles govern seed pairing, where uracil (U) pairs with adenosine (A) and cytosine (C) pairs with guanine (G) [40]. The 'seed region’, encompassing nucleotides 2–8 at the 5’-end of the miRNA facilitates the canonical miRNA–target interactions, where they will bind to the miRNA responsive elements (MREs), the complementary sites that typically located in the 3’UTRs

of target mRNAs, leading to translational inhibition or repression. However, MREs can also be found within the protein coding sequence or 5'UTRs [61]. Among the canonical binding sites, 8mer-A1 sites which are characterised by complementarity at positions 2–8 and the presence of a t1A, demonstrate the highest regulatory efficiency. These sites are followed in decreasing regulatory strength by 7mer sites (complementarity at positions 2–8), 7mer-A1 sites (complementarity at positions 2–7 and a t1A), 6mer sites (complementarity at positions 2–7) and 6mer offset sites (complementarity at positions 3–8), as illustrated in Figure 4 [62].

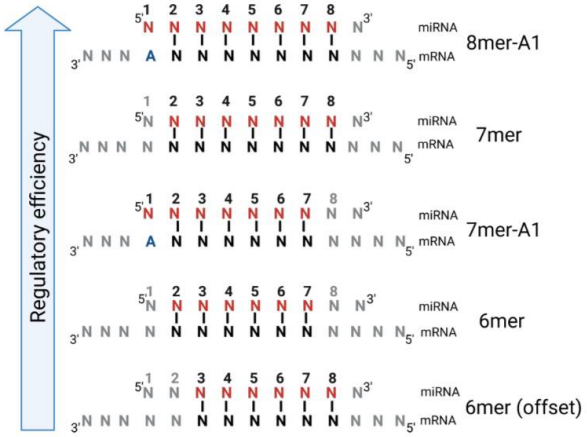

Fig. 4. Types of miRNA-mRNA interaction contributing to different regulatory efficiencies [62]

As the seed region of miR-21 has been reported to have low GC content, seed pairing with target mRNAs is relatively weak and 3' supplemental pairing is required [60]. The 3' supplemental pairing plays a crucial role in determining the specificity and efficiency of miRNA target recognition. During this process, additional nucleotides, specifically positions 13–16, become accessible after the initial seed pairing between the miRNA seed region and its target mRNA. This interaction induces a conformational change in the AGO protein, further stabilising the miRNA-mRNA complex and enhancing target regulation [63].

Alternative polyadenylation (APA), a process that occurs during the maturation of pre-mRNA, allows most mammalian genes to utilise multiple polyadenylation sites [64]. This mechanism generates distinct 3' termini on mRNAs, enabling a single gene to produce multiple transcript variants with diverse 3' UTR compositions. Such variations in 3' UTR isoforms can include or exclude miRNA binding sites, leading to changes in transcript abundance, stability and translation efficiency. Poly(A)-position profiling by sequencing (3P-seq) is a technique capable of globally identifying novel APA events by generating reads directly adjacent to the poly(A) tail, which facilitates precise quantification of 3' UTR isoforms [65]. In other words, a higher number of 3P-seq tags provides strong experimental evidence for miRNA-mRNA interactions, enhancing confidence in the predicted regulatory relationships.

Out of the 303 predicted target genes, SMAD7 was selected for further validation via RT-PCR analysis. This selection was primarily based on the high-confidence interaction between miR-21-5p and SMAD7, supported by a high number of 3P-seq tags (871) and the presence of an 8mer seed match, a site type considered the most reliable for miRNA target prediction [66]. Figure 5 illustrates the predicted binding interaction, showing the 8mer site located at positions 1165–1172 within the SMAD7 3' UTR. In addition, SMAD7 is a disease-relevant gene, acting as a negative regulator of the TGF-β signalling pathway. Its downregulation has been associated with pathological processes such

as uncontrolled cell proliferation, fibrosis and chronic inflammation [67]. This highlights its importance as a target for further investigation.

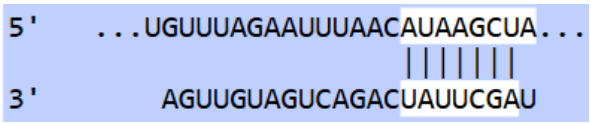

Fig. 5. Predicted consequential pairing between SMAD7 (top) and miR-21-5p (bottom) generated using TargetScanMouse 8.0

3.5 RT-PCR Products of SMAD7 and GAPDH Analysed by Agarose Gel

The RT-PCR amplification was successful, yielding a single distinct band at approximately 100 bp across most of the lanes as shown in Figure 6. No non-specific amplification products of varying sizes were detected but higher degree of smearing was observed in the lanes corresponding to the treated group. This observation may reflect partial cDNA template degradation resulting from compromised RNA integrity [68]. Furthermore, the band intensity for the housekeeping gene, GAPDH appeared inconsistent across samples. This inconsistency may be attributed to RNA degradation, which can eliminate critical primer binding regions required for efficient reverse transcription [69]. Such degradation can lead to biased or reduced cDNA synthesis, potentially causing some transcripts to be underrepresented or undetectable in downstream analysis. Consequently, it is crucial to maintain RNA integrity by avoiding repeated freeze–thaw cycles and ensuring the use of RNase-free reagents and equipment throughout the extraction and PCR procedures. On top of that, variation in GAPDH band intensity may also result from pipetting errors, as improperly calibrated pipettes can introduce significant systematic bias. Therefore, regular pipette calibration is crucial to ensure accuracy and reproducibility [70].

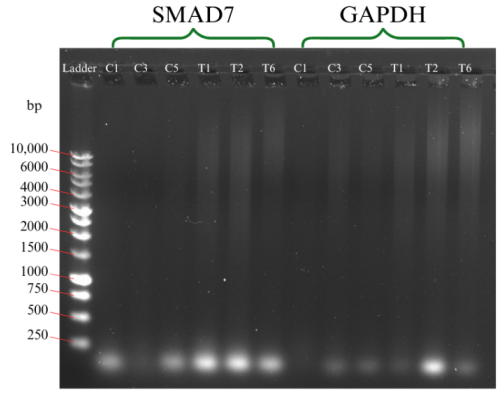

Fig. 6. Gel image of PCR products. Lane 1: 1 kb DNA ladder; Lanes 2–4: SMAD7 (control); 5–7: SMAD7 (treatment); 8–10: GAPDH (control); 11–13: GAPDH (treatment).

3.6 Relative Gene Expression Analysis of miR-21-5p and Its Target Gene Using ImageJ

The band intensities corresponding to miR-21-5p and the reference gene U6 were quantified using ImageJ software. In order to determine the relative expression of miR-21-5p, normalisation was

carried out by calculating the ratio of the target gene (miR-21-5p) integrated density to that of the reference gene (U6). The normalised band intensity for each sample is presented in Table 4, reflecting the expression levels of miR-21-5p. The mean relative expression for each group based on triplicate samples was subsequently calculated.

Table 4
Normalised band intensity of miR-21-5p in the control (C) and treatment (T) group respectively

Sample	Integrated density		Normalised intensity	Mean ± standard error
	miR-21-5p	U6		
C1	5041.731	7614.853	0.66	0.55±0.059
C3	5508.439	11889.924	0.46	
C5	6109.095	11651.711	0.52	
T1	2787.811	13543.439	0.21	0.21±0.009
T2	3315.104	14534.439	0.23	
T6	2642.861	13102.803	0.20	

A reduction in miR-21-5p expression was observed, decreasing from 0.55 ± 0.059 in the control group to 0.21 ± 0.008 in the treatment group. As shown in Figure 7, this reduction was statistically significant, as indicated by a p-value of 0.028, suggesting that Moringa treatment significantly downregulated miR-21-5p expression.

This observation aligns with earlier research reporting the downregulation of miR-21-5p expression following treatment with naturally derived compounds. Moringa treatment significantly lowered miR-21-5p levels in high fat diet (HFD)-induced C57BL/6J mice, following an eight weeks supplementation at a dosage of 290 mg/kg [71]. Similarly, quercetin treatment at a concentration of 15 mg/mL effectively attenuated TGF-β-induced miR-21-5p upregulation in HK-2 cells after 72 hours of exposure [72]. Furthermore, a significant reduction in miR-21 expression in thioacetamide-induced albino Wistar rats treated with 20 mg/kg of gallic acid and ferulic acid for six weeks [73].

This modulation of miRNA expression following Moringa treatment is likely attributed to its rich phytochemical composition, particularly its high flavonoid content [74,75]. Among these, quercetin stands out as the predominant compound in Moringa extracts, with the highest reported concentration being 16.64 mg/g of dry weight [76]. Quercetin exhibits strong anti-inflammatory and antioxidant properties, which contribute to the suppression of inflammatory cytokines such as IL-6 via inhibition of the IKK/NF-κB signalling pathway [77]. Specifically, quercetin interferes with IκB kinase (IKK) activity, thereby preventing phosphorylation and subsequent proteasomal degradation of IκBα. This stabilisation of IκBα inhibits the translocation of NF-κB to the nucleus, leading to decreased transcription of NF-κB-regulated genes, including IL-6. Reduced IL-6 levels subsequently diminish STAT3 phosphorylation, as activation of the JAK/STAT3 pathway is attenuated, impairing STAT3 dimerisation and nuclear translocation [78]. Since STAT3 is a key transcription factor that initiates miR-21 transcription by binding to promoter elements located between −4528 and −3340 base pairs upstream of the transcription start site, its inhibition consequently leads to decreased expression of miR-21-5p [79].

The normalised band intensity of SMAD7 for each sample was calculated by dividing its integrated density by that of the reference gene, GAPDH. The mean relative expression for each group based on triplicate samples was subsequently calculated and presented as in Table 5.

Table 5
Normalised band intensity of SMAD7 in the control (C) and treatment (T) group respectively

Sample	Integrated density		Normalised intensity	Mean ± standard error
	SMAD7	GAPDH		
C1	13645.957	3354.669	4.07	2.61±0.979
C3	4253.205	5646.075	0.75	
C5	13630.472	4534.347	3.01	
T1	21368.371	3667.79	5.83	3.29±1.374
T2	21872.371	19679.714	1.11	
T6	17190.421	5846.51	2.94	

An increase in SMAD7 mRNA expression was observed, rising from 2.61 ± 0.979 in the control group to 3.29 ± 1.373 in the treatment group. However, as shown in Figure 8, this increase was not statistically significant (p = 0.7083), suggesting that Moringa treatment did not significantly affect SMAD7 expression levels.

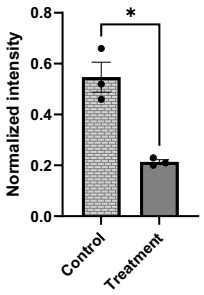

Fig. 7. Relative expression of miR-21-5p in control and treatment groups (p < 0.05); Data were presented as mean ± SEM, n = 3 mice/group

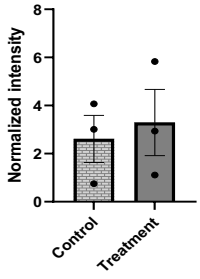

Fig. 8. Relative expression of SMAD7 in control and treatment groups; Data were presented as mean ± SEM, n = 3 mice/group

This finding is consistent with a previous study that demonstrated increased SMAD7 mRNA expression in miR-21 knockout mice using RT-PCR [80]. Similarly, treatment with 50 mg/kg of quercetin resulted in decreased miR-21 expression and elevated SMAD7 protein levels in cadmium chloride-treated rats [81]. These support the role of miR-21-5p as a negative regulator of SMAD7.

SMAD7 has been validated as a direct target of miR-21-5p in a previous study using dual-luciferase reporter assays, qRT-PCR, and Western blot analysis [82]. An 8-nucleotide miR-21-5p binding site was identified within the 3′ UTR of SMAD7. Transfection of miR-21-5p mimics into NIH3T3 fibroblasts led to reduced luciferase activity in reporter constructs containing the wild-type SMAD7 3′ UTR, along with decreased SMAD7 mRNA and protein levels.

The observed increase in SMAD7 mRNA expression may be attributed to a reduced occurrence of miR-21-5p-induced mRNA degradation via deadenylation mechanisms as a result of decreased miR-21-5p levels [83]. In this process, GW182, a key component of the miRISC recruits the CCR4–NOT deadenylase complex to facilitate the removal of the mRNA's poly(A) tail. Following deadenylation, the mRNA undergoes decapping by the DCP2 enzyme, after which it is degraded by the major cytoplasmic 5′ to 3′ exonuclease XRN1. On top of that, target mRNA translation is essential for miRNA-

mediated mRNA destabilisation, as reduced miRNA levels significantly increased mRNA expression of protein-coding genes, whereas long noncoding RNAs (lncRNAs), which are less actively translated were minimally affected by miRNA loss [84].

Furthermore, a previous study proposed that animal miRNAs primarily regulate the expression of functional target genes at the protein level [85]. Therefore, additional analysis such as Western blotting could be employed to assess changes in SMAD7 protein levels, providing further validation of whether the observed changes in mRNA expression are reflected at the translational level.

4. Conclusions

In conclusion, all objectives of this study were successfully achieved. Oral administration of 300 mg/kg Moringa extract for 12 consecutive days significantly reduced miR-21-5p expression in BALB/c mice ($p < 0.05$). TargetScan predicted 303 potential target genes, among which SMAD7 was selected for RT-PCR analysis due to its strong predicted interaction with miR-21-5p and its relevance to disease pathways. The downregulation of miR-21-5p was associated with an upregulation of SMAD7 mRNA expression, supporting the possibility that SMAD7 is a direct target of miR-21-5p. For future studies, techniques such as Western blotting could be employed to assess SMAD7 protein levels. Additionally, qRT-PCR is recommended for future miRNA and target gene expression analysis due to its high sensitivity, specificity and efficiency.

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