Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 6s, https://carcinogenesis.com/

Zebrafish Model to Study the Effects of Thymoquinone on Circadian Rhythms via Clock

Shreya Madhubala B K , S.Sangeetha*, Taniya Mary Martin, Meenakshi Kishore Kumar

[1]Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science, Saveetha University, Chennai - 600077

Email: 152401064.sdc@saveetha.com

Mobile no: 9042912606

[2]Assistant Professor Department of anatomy, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science,

Saveetha University, Chennai - 600077

Email: Sangeethas.sdc@saveetha.com

[3]Junior Research Fellow Department of anatomy, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science, Saveetha University, Chennai - 600077

[4] Assistant Professor Department of anatomy, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science,

Saveetha University, Chennai - 600077

*Corresponding author

Mrs.S.Sangeetha

Assistant Professor Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Science, Saveetha University, Chennai - 600077 No. 162, Poonamallee High Road, Velappanchavadi, Chennai - 600077

Email: Sangeethas.sdc@saveetha.com

Mobile no: +91 9944953239

ABSTRACT

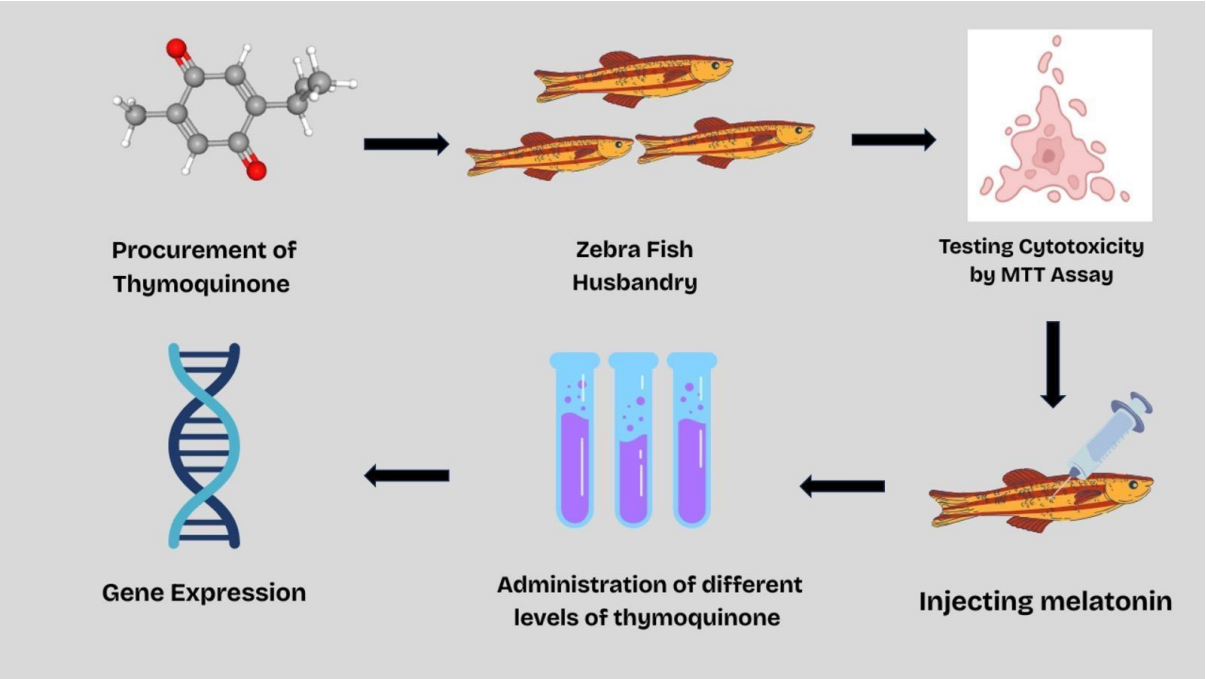
Background: All living organisms depend on circadian rhythms which are managed by clock1a, bmal1, per2, cry1 and nr1d1 genes. The disruption of this causes various illnesses including chronic diseases over time. Thymoquinone which exists in Nigella sativa produces antioxidant effects together with anti-inflammatory and anti-cancer activities. Its effects on regulating biological clocks has not yet been explored. The research investigates Thymoquinone effects on zebrafish circadian gene expression and evaluates its neuroblastoma cell toxicity. This study investigates how Thymoquinone alters zebrafish circadian gene expression while measuring human neuroblastoma cell toxicity through MTT assay.

Materials and Methods: The study administered Thymoquinone to zebrafish using various concentration levels. After the RNA extraction expression levels of clock1a, bmal1, per2, cry1 and nr1d1 were measured using RT-qPCR. Expression changes were determined through 2^−ΔΔCt calculations. The data was adjusted based on the expression of housekeeping genes. The MTT assay tested neuroblastoma cells treated with different Thymoquinone concentrations at 1,10,25,50,100 µg/mL. Spectrophotometry at 570 nm was used to measure cell viability.

Results: In zebrafish, Thymoquinone affected the expression of circadian genes by upregulation of bmal1 through nr1d1 and downregulation of per2 and cry1. The thymoquinone demonstrated dose related killing action on neuroblastoma cells and kept the cell viability above 85% at 10 µg/mL concentration, while higher doses (25 and 50µg/mL) showed significant degree of cytotoxicity suggesting its anticancer effects.

Conclusion: Our data suggest that thymoquinone alters zebrafish circadian gene expression and exerts selective killing in neuroblastoma cells. A concentration of 27.67 µg/mL was found to be most effective. Thymoquinone serves as both a

chronomodulatory agent and an anticancer agent which establishes its potential for future development of circadian-based cancer therapies.



GRAPHICAL ABSTRACT.

Keywords: *Circadian rhythm, Thymoquinone, Zebrafish, CLOCK1a, BMAL1, PER2, CRY1, NR1D1, Neuroblastoma, MTT assay, gene expression.*

How to Cite: Shreya Madhubala B K , S.Sangeetha*, Taniya Mary 1Martin, Meenakshi Kishore Kumar., (2025) Zebrafish Model to Study the Effects of Thymoquinone on Circadian Rhythms via Clock, *Journal of Carcinogenesis*, *Vol.24, No.6s*, 187-197.

1. INTRODUCTION

Circadian Rhythm is a natural process that helps us maintain a 24-hour sleep wake cycle. This way it regulates the behavioural, biochemical and physiological conditions of the body. Regulation of this circadian is dependent on the period of light and darkness(1). Through this the internal clock is synchronized with the day night cycle. Circadian rhythm can influence the body in many ways ranging from hormone release, body temperature to feeding behaviour. Various disorders can be caused by imbalance in the circadian clock(2). Organisms possess an internal biological clock named the circadian clock which maintains rhythms. This mainly supports the organism in adapting to daily light and dark changes. The Earth's rotation represents the primary factor which produces this variation. Through this there is an equilibrium among different body systems. Scientists have discovered that disturbances to this clock system lead to many health conditions including metabolic syndrome, obesity, depression, neurodegenerative diseases and also cancer(3), (4). The biological clock functions through a molecular process that uses feedback systems. Through this it controls specific clock genes in transcription and translation (5) .The clock genes and the proteins generated from them, interact with a sleep-wake cycle that repeats every 24 hours(6). The system features two positive factors called CLOCK-1a and BMAL1 which function together as transcription factors (7). The regulation of negative components PER2 and CRY1 depends on these factors. Increase in PER and CRY proteins causes inhibition of CLOCK: BMAL1 activity. This creates a feedback loop that maintains rhythmic gene expression. The process is also regulated by NR1D1 which functions as a suppressor to control BMAL1 expression. The circadian clock in vertebrates requires five essential genes-clock 1a, bmal1, per2, cry1 and nr1d1 to operate correctly (8).

Zebrafish are one of the ideal organisms to function as a model system for this study as they have a high degree of genetic similarity to humans (9). They are being used to carry out research on various branches of science including genetics, physiology, ecology, biotechnology and many more (10), (11) . The other advantage is their behavioural patterns which are very easy to observe. In mammals, the Suprachiasmatic nucleus (SCN) of the brain is where the central pacemaker lies.

Zebrafish on the other hand have multiple circadian clocks throughout their body, including in the brain, liver, heart and eyes. By this, gene expression studies can be carried out at multiple tissues(12) .The transparent nature of Zebrafish embryos and larvae, external development and their quick response to light-dark cycle make them suitable for circadian research that requires real time tracking and molecular examination (13). Many plants like *Curcuma longa, Withania somnifera, Ocimum sanctum, Azadirachta indica, Ficus benghalensis* are used in modern medicine and drugs for their therapeutic purposes (14), (15) . The research focused on the effects of Thymoquinone on the circadian clock system of Zebrafish (16) . Thymoquinone is a natural compound obtained from *Nigella sativa* (black seed). This molecule is well known for its antioxidative, anti-inflammatory, and anticancer activities like many other natural extracts (17) , (18), (19), . Studies reveal Thymoquinone also protects the body against oxidative stress. It also regulates inflammation as well as programmed cell death. Although there is a lot of research on the therapeutic benefit of Thymoquinone, its effect on circadian rhythm is not well understood (20).

The research investigated the effects of Thymoquinone on core clock machinery in zebrafish through analysis of clock1a, bmal1, per2, cry1 and nr1d1 expression levels. The selection of genes occurred because these elements form the basis of circadian rhythm regulation and sustenance. Our research proposed that Thymoquinone may affect gene expression levels through two pathways: direct action on redox sensitive transcription factors and indirect effects on cellular oxidative status which impacts circadian timing.

Thymoquinone comes from Nigella sativa. It was used as the natural compound to investigate its ability to affect the circadian clock through regulatory gene targets in zebrafish. Zebrafish within the age range of 6 to 8 weeks were used as our experimental model. It is because their circadian system responds to light signals and can be thoroughly studied. The zebrafish received different concentrations of Thymoquinone in controlled light dark environments. DMSO treated zebrafish functioned as vehicle controls. The MTT assay was performed for cytotoxicity testing to determine Thymoquinone concentrations which maintained cell viability above 85-90% (21) . This was done to ensure non-toxicity of selected doses. The activation and function of multiple genes required doses that were free of cytotoxicity. Total Isolation of the RNA from the zebrafish was done. From that by cDNA synthesis was carried out. The subsequent step involved quantitative RT-PCR(real time PCR) to measure gene expression. The measurement of expression levels used β-actin as the normalization reference because it functions as a housekeeping gene. The relative expression levels were calculated through the $2^{-\Delta\Delta Ct}$ method. The entire experimental process included three independent repetitions to establish data reproducibility and statistical reliability. Through this method we examined how Thymoquinone influences biological rhythms in a vertebrate animal.

There is the clock1a gene that encodes a transcription factor. This transcription factor combines to form a heterodimer with bmal1 to start the transcription of target genes including per2 and cry1. These two genes , per2 and cry1 develop into proteins in the cytoplasm (22).Then the two proteins translocate into the nucleus to inhibit the transcription mediated by CLOCK: BMAL1 and complete the feedback loop. NR1D1 adds another level of regulation by suppressing the transcription of bmal1 to stabilize the oscillatory cycle of the clock (23) . These were the genes we had chosen in order to get a clear picture as to how Thymoquinone might affect the circadian regulation from transcriptional activation, to suppression and fine tuning. If any of these were either significantly up- or down-regulated, it would mean that Thymoquinone can reset or modulate circadian timing in zebrafish. The study of redox modulation with respect to circadian rhythm is important as the CLOCK: BMAL1 complex is sensitive to cellular redox conditions (24) . The antioxidant action of Thymoquinone may modulate the activity of CLOCK: BMAL1 by acting on the NAD+/NADH ratio. This controls the transcriptional feedback loops. Studies show that oxidative stress has a destructive influence on circadian rhythms across models. Thymoquinone could prevent this by maintaining the redox balance (25). Zebrafish heartbeat rhythms were also recorded. The treated zebrafish went through light-dark cycles while their heart beat patterns were observed. This data helps us determine whether changes in gene expression also reflect in the circadian pattern. Therefore this study functions as an emerging research topic, that explains how natural compounds including Thymoquinone alter circadian rhythms. Zebrafish functions as an ideal model because of its high investigatory potential. This study functions as a starting point in understanding the relation between natural therapeutic compounds and circadian biology. Through more studies, many more natural treatments could also be developed for disorders related to circadian rhythm.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Thymoquinone was purchased from a certified supplier. DMSO (Dimethyl Sulfoxide) was used to dissolve thymoquinone. This was done to prepare the stock solution. The working concentrations were freshly diluted using system water to attain the final test concentrations of 1μg/mL, 10 μg/mL, and 25 μg/mL. The DMSO concentration was controlled to stay under 0.1% during the final preparation. Pharmacological rhythm modulation was achieved through the usage of Melatonin together with OSMI-1 and ethanol. The standard protocols were followed by using all molecular biology grade reagents for RNA extraction, cDNA synthesis, and RT-qPCR which included TRIzol and reverse transcription kits as well as SYBR

Green master mix.

2.2 Zebrafish Husbandry and Ethical Compliance

Ethical guidelines were properly followed in all the procedures involving all animal procedures. The procedures were also approved by Institutional Animal Ethical Committee of Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), under the approval number BRULAC/SDCH/SIMATS/IAEC/03-2024/12. For this study, adult zebrafish (AB strain) aged 6 to 8 months, were obtained from a certified local supplier. After purchase of the fish, they were adapted to laboratory tanks under standardized and stable conditions. A light-dark cycle of 14 hours of light and 10 hours of darkness was maintained. This was done to support the fish's natural rhythm. Water in the tank was held constant at 28 ± 1°C temperature. Continuous aeration and filtration was also done. Zebrafish were given a nutritionally balanced diet twice daily. The tank conditions were monitored regularly to ensure health of the fish throughout the study.

2.3 Induction of Circadian Rhythm Disruption

To induce circadian rhythm disruption, adult zebrafish were subjected to two pharmacological treatments. One group received intraperitoneal injections of melatonin (50 µg/g body weight) at 8:00 am daily for three consecutive days using a Hamilton syringe. A separate set of fish received 0.1% of DMSO, which was administered via intracranial injection at 8:00 pm daily for three days to disturb central circadian regulation. Fish were monitored for abnormal swimming behaviour post injection, and any individuals with irregular motion were excluded from the study.

2.4 Neuroblastoma cell line and cell culture conditions

Human neuroblastoma cells were purchased from National Centre for Cell Science (NCCS), Pune, India. All materials and procedures were executed under sterile conditions. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, USA). The medium contained 10% fetal bovine serum together with 1% penicillin-streptomycin solution. The cultures were incubated at 37°C with 5% CO2. The incubator maintained the proper humidity levels. The cells were subcultured at 80-90% confluence using 0.25% trypsin EDTA which detached adherent cells. All experiments used the same batch of cells which guaranteed both consistency and reproducibility in experimental outcomes.

2.5 MTT assay in Neuroblastoma cells

The cytotoxic effect of Thymoquinone was measured through MTT assay in human neuroblastoma cell cultures before implementing any in vivo experiments. Human neuroblastoma cells received Thymoquinone treatment at five different concentrations (1,5,10,25, and 50µg/mL) for twenty-four hours in 96 well plates. After treatment, MTT reagent (0.5 mg/mL) was mixed. Following that, incubation was done for 4 hours. The Formazan crystals that formed during the reaction dissolved in DMSO. The absorbance level at 570 nm was read by using a microplate reader. The experiment measured cell viability by comparing it to cells that received no treatment. Concentrations that maintained ≥ 85% viability were considered safe for in vivo use. Based on these results, 1,10, and 25 µg/mL were selected for zebrafish treatment.

2.6 Thymoquinone Treatment Design

Following the circadian rhythm disruption the zebrafish were divided randomly into five different experimental groups. The negative control group consisted of fish that remained in regular light-dark cycles without any treatment. The positive control group consisted of rhythm disrupted fish which did not receive any Thymoquinone treatment. Thymoquinone was administered to zebrafish at three different concentrations including 1 µg/mL 10 µg/mL and 25 µg/mL which made up the treatment groups III, IV, and V. The zebrafish received Thymoquinone treatment by immersion for a period of 24 to 48 hours under controlled environmental conditions. The three concentrations were selected from MTT assay results which demonstrated that all three doses maintained over 85% cell viability, indicating their non-cytotoxic nature and suitability for downstream gene expression.

2.7 Contextual Fear conditioning and Memory Testing

To evaluate the potential influence of Thymoquinone on learning and memory regulated in part by circadian rhythms a contextual fear conditioning test was performed. After 1 hour acclimatization to the testing room, each fish was placed in a black acrylic two compartment chamber (dark and light compartments) separated by a barrier with a 3 cm diameter opening. The fish was allowed to stabilize in the dark compartment for 3 minutes, after which the door to the light side was opened. Upon entering the light side, an aversive stimulus was applied (tapping the tank) to induce fear. The latency to cross and total crossing time was recorded. This was repeated three times. Two hours later the memory test was performed using the same setup but without stimulus. Longer latency times indicated retention of the learned fear response.

2.8 Gene expression Analysis

Following treatment, zebrafish were euthanized and tissues from the whole body were collected for gene expression analysis. Total RNA was extracted using TRIzol reagent and quantified spectrophotometrically. First strand cDNA

synthesis was performed using 1µg of total RNA. SYBR Green based detection was used to carry out Real time PCR. This was done to quantify the expression of five main circadian genes: clock1a, bmal1, per2, cry1 and nr1d1 (Table 1). The housekeeping gene β-atin was used for normalization. Amplification was conducted using standard cycling conditions. The 2^−ΔΔCt method was used to calculate the gene expression levels. Triplicate analysis was performed for every sample.

Table 1: Primers used for gene expression

Gene	Former Primer (5' →3')	Reverse Primer 5'→ 3'
clock1a	ATGCCTCAGCTTTGTCCAGT	AGCAGGGAGGAAAGGTTGAG
bmal1	TGACTTCGACTTCGGTTTGG	TGTGTTGGGTTGTTGCTGAT
per2	CTGCTGCTGTCCATCTACCT	AGAGGACAGGGAAGAGGAAG
cry1	TCGGAGAGAGCGTTACCAAG	TCTGAGGTCATCGCAGAGGT
nr1d1	GGTGCTGATGCTGTTGTAGC	GTAGGGCTTGGTCAGGAGGT
β-actin	TGCCCCTCGTGCTGTTTT	TCTGTCCCATGCCAACCAT

2.8 Statistical Analysis

The data from the experiments were all presented in the form of mean ± standard error of the mean. To access the group wise variations, the tests that were performed are one way ANOVA followed by Turkey's post hoc test. For statistical significance a value of $p < 0.05$ was considered. GraphPad Prism software was used for data visualization and analysis

3. RESULTS

3.1 Scanning Electron Microscopy (SEM) analysis of Thymoquinone

The surface morphology of Thymoquinone was characterized using SEM to evaluate its physical structure and particulate nature. The SEM image revealed that Thymoquinone appeared as irregularly shaped crystalline particles with a moderately rough surface texture, which may facilitate its disruption observed. It was consistent with previously reported micron-scale ranges, indicating suitable properties for biological interaction and absorption in aquatic models like zebrafish. These morphological features support its physicochemical stability and contribute to the effective bioavailability of Thymoquinone during short term immersion exposure in the present study (Figure 1).

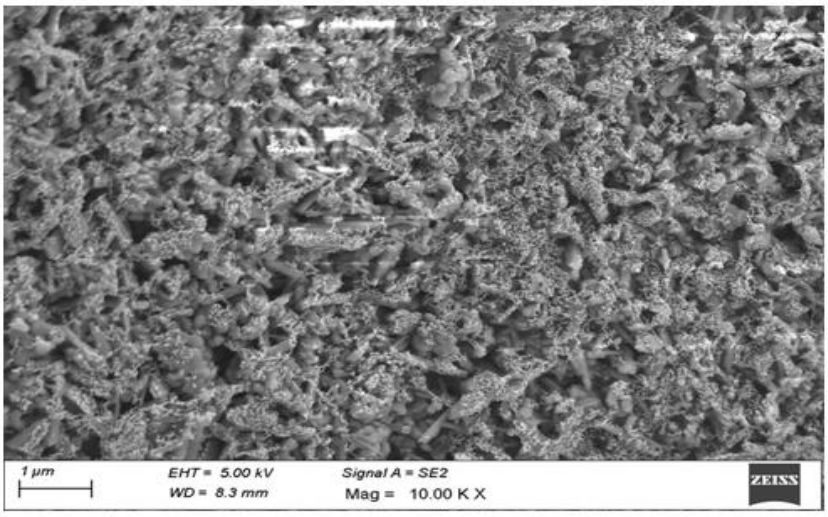


Figure 1: Scanning Electron Microscopy image of Thymoquinone

3.2 Cytotoxicity evaluation of Thymoquinone in neuroblastoma cell lines

The MTT assay served to measure Thymoquinone cytotoxicity against human neuroblastoma cells. The cells experienced lowered viability rates after thymoquinone treatment which increased from 1 to 50 µg/mL concentrations. Cells maintained strong viability at lower thymoquinone doses of 1, 5, and 10 µg/mL but showed slight decreases at 25 µg/mL. The cell viability dropped significantly at the 50 µg/mL concentration. Cell viability remained above 85% during treatment at concentrations below 25 µg/mL which indicates these doses lack toxicity and show compatibility with cells. The in vivo zebrafish experiments for circadian gene modulation used 1 µg/mL, 10 µg/mL, and 25 µg/mL concentrations based on these findings. (Figure 2).

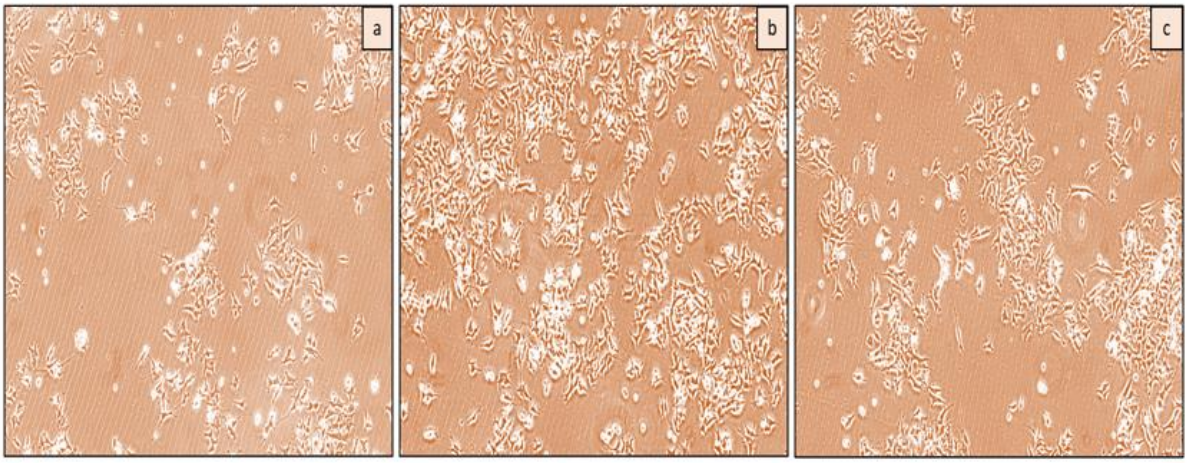


Figure 2: Microscopic images showing the morphological features of neuroblastoma cells under different treatment conditions a) positive control group b) cells treated with the lowest concentration of Thymoquinone (1µg/mL) and c) cells treated with highest concentration of Thymoquinone (25 µg/mL). Changes in cell density and morphology are indicative of concentration dependent cytotoxic effects.

3.3 MTT Assay: Comparative Cytotoxicity of Thymoquinone and Doxorubicin

The MTT assay was used to measure Thymoquinone's cytotoxic effects against Doxorubicin on neuroblastoma cells. The neuroblastoma cells were treated with Thymoquinone at five different concentrations which included 1, 10, 25, 50 and 100 µg/mL**.** Later the cytotoxicity percentages were measured after 24 hours. The cytotoxic effect of Doxorubicin showed strong dose dependency through which cell survival rates dropped with rising concentrations. The test results showed an average cytotoxicity at 6.64% for 1 µg/mL while the 10 µg/mL concentration produced 12.86% and the 25 µg/mL concentration resulted in 28.03% and the 50 µg/mL concentration reached 73% and the 100 µg/mL concentration obtained 93.8%. The rise in Thymoquinone's cytotoxicity revealed a gradual progression over the entire testing span. At 1 µg/mL Thymoquinone concentration the average cytotoxicity reached 2.71% which increased to 7.04% at 10 µg/mL and 18.12% at 25 µg/mL. At 50 µg/mL, cytotoxicity was at 45.61%, which increased to 87.20% at 100 µg/mL. Compared to Thymoquinone, Doxorubicin showed much greater cytotoxicity at every concentration tested. Despite its higher doses displaying cytotoxicity, Thymoquinone was still relatively biocompatible, as it maintained over 85% cell viability at concentrations below 25 µg/mL. We chose the 1 µg/mL and 10 µg/mL doses for further in vivo zebrafish studies because of their safety profile (Figure 3).

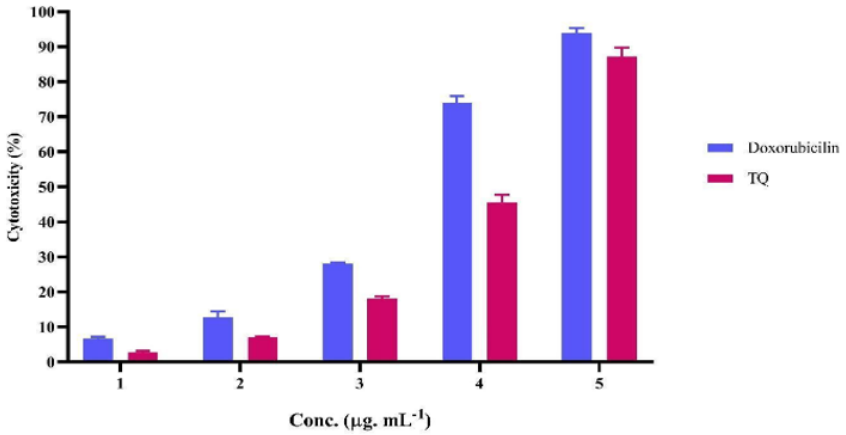


Figure 3: Comparative cytotoxicity of Thymoquinone and Doxorubicin in neuroblastoma cells assessed by MTT assay.

3.4 Effect of Thymoquinone on Circadian rhythm in Zebrafish

To evaluate the effects of Thymoquinone on the regulation of circadian rhythms, heart rate was monitored as a physiological marker in zebrafish after rhythm disruption. Within the positive control group's (rhythm disrupted, untreated) results, heart rate was significantly diminished, portraying disrupted circadian regulation. On the contrary, the negative control group (untreated, kept in a light-dark cycle) demonstrated a stable and coherent heart rate rhythm, portraying preserved circadian function. The zebrafish given Thymoquinone at the concentration of 1µg/mL showed only a moderate increase in heart rate as compared to the positive control group suggesting partial loss of restoration at this concentration. Nonetheless, at the higher doses of 10 µg/mL and 25 µg/mL of Thymoquinone, there was marked recovery in heat rate, reaching values close to negative control. These findings confirmed the dose-dependent restoration of circadian rhythm by Thymoquinone, illustrating its proposed use as a chronomodulatory zebrafish agent (Figure 4).

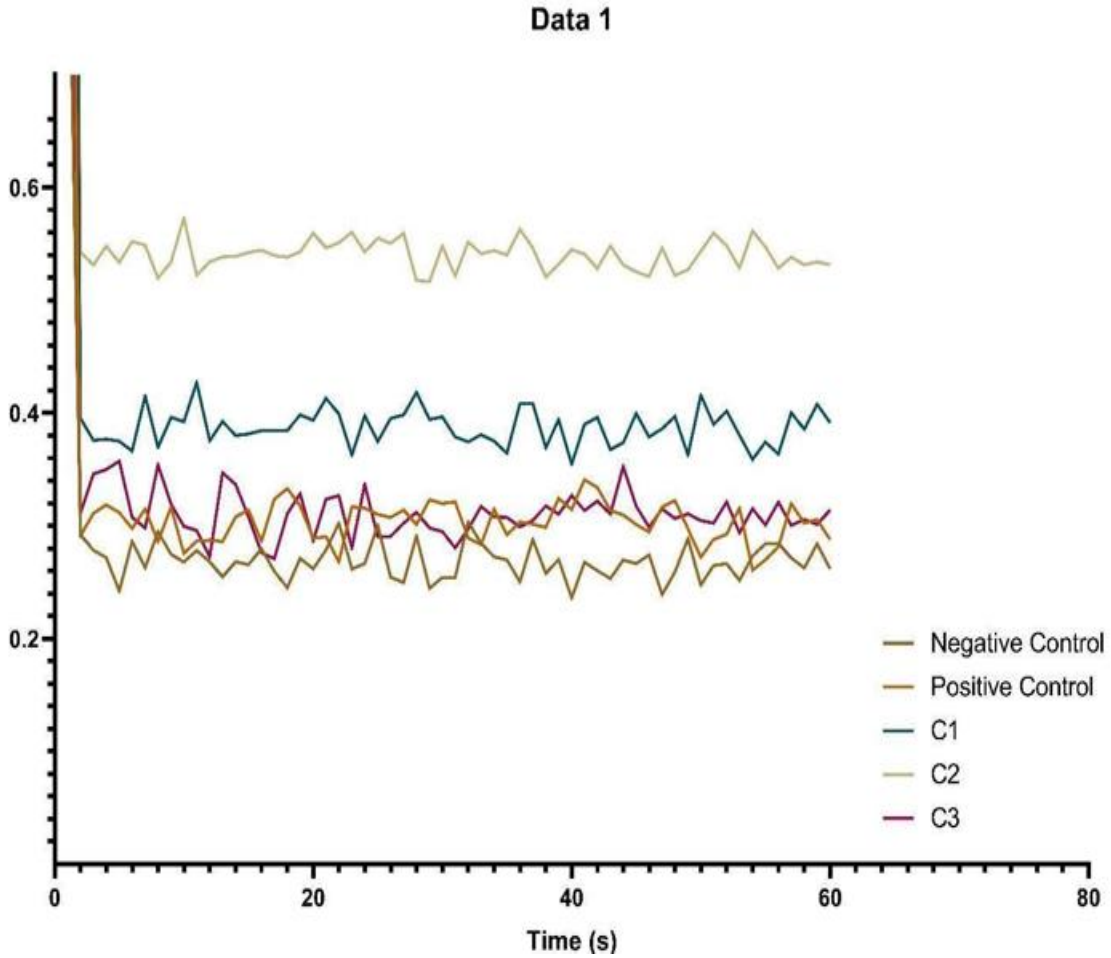


Figure 4: Effect of Thymoquinone on cardiac rhythm in zebrafish following circadian rhythm disruption. Heart rate was measured across five groups: Negative control, positive control and Thymoquinone treated groups at 1,10,25 µg/mL. Thymoquinone treatment at 10 and 25 µg/mL significantly restored cardiac rhythmicity compared to the positive control.

3.5 Effects of Thymoquinone on Circadian gene expression in Zebrafish

To establish the effects of Thymoquinone on zebrafish circadian regulation on a molecular level, the expression of the major clock genes were measured using RT-qPCR. In the positive control group, all five circadian genes were significantly downregulated relative to the negative control group, confirming the loss of the circadian rhythm within the system and molecular clock. Treatment of Thymoquinone TQ, at a concentration of 1 µg/mL resulted in a slight increase in clock1a and bmal1 expression, with negligible changes to per2, cry1 and nr1d1, indicative of some form of partial recovery. At 10 µg/L, marked increases in clock1a and bmal1 expression and moderate recovery of per2 and cry1 were observed suggesting strong positive influence on circadian gene activation. The highest dose of 25 µg/mL TQ, resulted in strong upregulation of all 5 genes with expression levels approaching, or in some cases exceeding those of the negative control. This shows Thymoquinone restores the expression of the genes in a dose dependent manner, with an increase in concentration, suggesting its potential use as a chronotherapeutic zebrafish modulator (Figure 5).

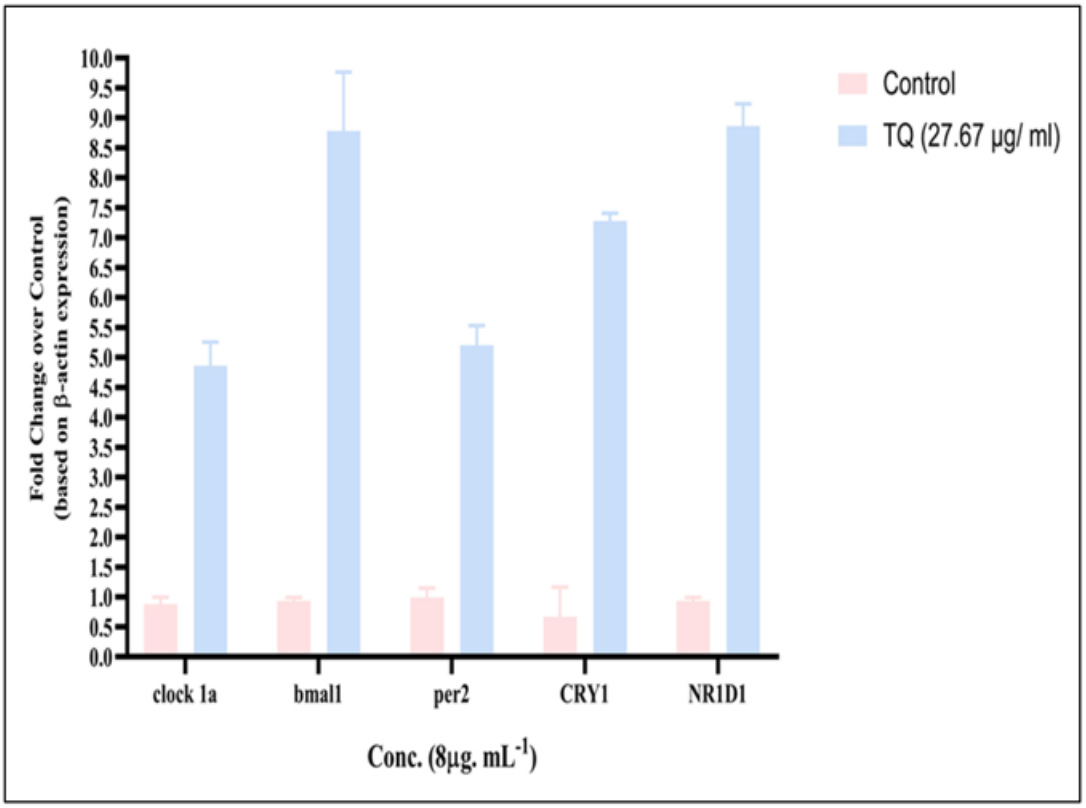


Figure 5: Relative mRNA expression levels of core circadian genes in zebrafish following Thymoquinone treatment.

4. DISCUSSION

Organisms use circadian rhythms to adapt themselves to environmental changes which include light and temperature patterns throughout the day. The main clock genes clock 1a, bmal1, per2 cry1, and nr1d1 regulate the intrinsic molecular clock . It is mainly carried out through feedback loops. Multiple physiological and pathological factors can disrupt circadian rhythms. This can cause metabolic disorders, hormonal disorders, neurodegenerative and cardiac disorders and also cancer (26). Therefore it has become very important to focus on discovering natural compounds that can both regulate and fix circadian function. Thymoquinone is an active constituent present in Nigella sativa which has been identified to have anti-inflammatory properties alongside its anticancer and antioxidant effects. However, the effect of Thymoquinone on circadian rhythm at the genetic level is not fully understood yet (27) .

The study explored whether Thymoquinone exhibits chronomodulatory effects on circadian rhythm. Zebrafish were used as model organisms because they have high genetic similarity to humans and transparent developmental stages and distributed clock gene organization. The study used adult zebrafish at 3-6 weeks of age. Circadian rhythm disruption through melatonin and OSMI-1 injections was done. Then Thymoquinone at different doses of 1, 10 and 25 µg/mL was administered. Measurement of heart rate was done to identify the physiological effect of Thymoquinone. RT-qPCR testing of five essential circadian genes was also done. It helped in identifying whether there was reestablishment of proper circadian rhythm. The positive control group showed a significant decrease in heart rate compared to the negative control group. This proves that Thymoquinone has the potential to restore circadian rhythm. The heart rate showed only a minimal improvement at 1 µg/mL. But significant restoration occurred at 10 µg/mL and 25 µg/mL doses which indicates Thymoquinone may help in reestablishing physiological circadian outputs. Thymoquinone demonstrates a dose-dependent influence on circadian regulation which probably happens through the molecular control of clock genes according to these findings. RT-qPCR measurement of CLOCK1a, bmal1, per2, cry1 and nr1d1was done to analyze the molecular effects (28). The rhythm-disrupted group showed a significant reduction in the expression of all five genes which also matched the physiological changes. The gene expression levels in Thymoquinone treated groups increased based upon the dosage administered. 10 µg/mL of Thymoquinone enabled partial expression of clock1a, bmal1 and nr1d1 whereas the 25µg/mL dosage nearly restored gene expression to levels matching the negative control. The molecular oscillator contains these genes as its fundamental components. The transcriptional activator complex is formed when CLOCK1a combines with BMAL1. The negative feedback inhibition occurs through PER2 and CRY1 while NR1D1 acts as a bmal1 repressor which provides precise control (29).

The reason which explains upregulation of these genes by Thymoquinone can be its antioxidant properties. which are known to influence circadian regulation. The redox state of the cell can influence circadian proteins such as CLOCK and BMALI. Oxidative stress, which is an imbalance between the reactive oxidative species, can lead to disruption of circadian oscillations (30). Thymoquinone helps restore redox balance. It functions as a powerful ROS scavenger to eliminate reactive oxygen species from the environment. The transcriptional feedback loops controlling circadian gene expression gain stability through this process. Additionally, Thymoquinone establishes interactions with signalling pathways which include NF-κB, Nrf2 and P13K/Akt. These are related to the circadian regulator. This could also explain the observed gene expression modulation.

The MTT assay served to determine the cell toxicity of Thymoquinone on human neuroblastoma cells. This assay confirmed that Thymoquinone was non-cytotoxic at concentrations up to 25 μg/mL, maintaining more than 85% cell viability (31), (32). This is important to ensure that the results seen in Zebrafish are due to positive effects of Thymoquinone and not because of its toxic effects. This also ensures biocompatibility. The standard chemotherapeutic agent is Doxorubicin. When compared to Doxorubicin, Thymoquinone had less cytotoxicity although Thymoquinone did display dose dependent cytotoxicity. This shows that Thymoquinone has lesser side effects than synthetic drugs. The effect of Thymoquinone on both gene expression as well as heart rate shows that it has a significant effect on circadian homeostasis. Improvement and reestablishment of heart rate by Thymoquinone, shows that imbalance in circadian rhythm can also affect cardiovascular functioning as per the previous studies (33) . The cardiovascular system together with major systemic physiological controls potentially may be impacted by circadian gene expression.

The molecular CLOCK and metabolic functions particularly lipid and glucose metabolism require NR1D1 which showed increased expression in thymoquinone-treated groups. The raised NR1D1 expression indicates that thymoquinone might regulate metabolism through circadian mechanisms. However further research is needed to validate this theory in metabolic disease investigations. The research generates multiple important consequences for understanding the studied phenomena. First, the research establishes initial proof that Thymoquinone works as a chronomodulatory agent which could fix or maintain circadian rhythm disturbances at both genetic and physiological stages. Second, it supports the utilization of zebrafish as a model for chronobiology research, especially for screening natural compounds with potential therapeutic value. Third, it introduces a safe concentration range for in vivo use of Thymoquinone in aquatic models, supported by in vitro toxicity screening. However, the study also has some limitations. Heart rate stands as a practical physiological indicator for circadian output yet this model did not evaluate circadian behaviours including locomotor activity or melatonin secretion or light/dark cycle entrainment. To gain a complete understanding of Thymoquinone's chronobiological effects future research should incorporate these endpoints. The gene expression analysis occurred only at one specific moment which failed to demonstrate the natural rhythm of CLOCK gene transcription. Time course analysing circadian gene expression across multiple zeitgeber times would help confirm whether Thymoquinone can truly re-entrain the CLOCK or simply boost expression levels. Thus, this study demonstrates that Thymoquinone can significantly modulate circadian gene expression and restore rhythm associated physiological functions in zebrafish following pharmacological disruption. The dose-dependent upregulation of clock1a, bmal1, per2, cry1, and NR1D1, along with the recovery of heart rate, suggests that Thymoquinone may serve as a natural chronotherapeutic agent. The favourable compatibility with cells makes it a promising candidate for future use in treatments of circadian-related conditions. The results establish a solid foundation for upcoming experimental research to explore how Thymoquinone affects circadian rhythm control and its general impact on wellness and illness.

5. CONCLUSION

This study demonstrates that Thymoquinone effectively modulates the circadian system in zebrafish by restoring both physiological rhythms and molecular clock gene expression following pharmacological disruption. Treatment with Thymoquinone at varying levels or concentrations of 10 μg/mL and 25 μg/mL caused significant improvements in heart rate and the upregulation of core circadian genes including clock1a, bmal1, per2, cry1 and NR1D1. It was also found that at a concentration of 27.67 μg/mL, the treatment is most effective. These findings indicate a dose dependent chronomodulatory effect, highlighting Thymoquinone's potential as a natural agent for reestablishing circadian balance. The analysis of cytotoxicity in neuroblastoma cells demonstrated its safety within tested doses to support its safe use in biological systems. The future research of Thymoquinone shows promise as a circadian therapeutic target especially for rhythm-based disorders.

6. ACKNOWLEDGMENT

We extend our sincere gratitude to Saveetha dental College and hospitals for their constant support and successful completion of this work.

7. CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest in this study.

REFERENCES

[1] Foster RG. Sleep, circadian rhythms and health. Interface Focus. 2020 Jun 6;10(3):20190098.
[2] Ramar K, Malhotra RK, Carden KA, Martin JL, Abbasi-Feinberg F, Aurora RN, et al. Sleep is essential to health: an American Academy of Sleep Medicine position statement. J Clin Sleep Med. 2021 Oct;17(10):2115–9.
[3] Roenneberg T, Foster RG, Klerman EB. The circadian system, sleep, and the health/disease balance: a conceptual review. J Sleep Res. 2022 Aug;31(4):e13621.
[4] Chan CMH, Siau CS, Wong JE, Wee LH, Jamil NA, Hoe VCW. Prevalence of insufficient sleep and its associated factors among working adults in Malaysia. Nat Sci Sleep. 2021 Jul 13;13:1109–16.
[5] Cox KH, Takahashi JS. Circadian clock genes and the transcriptional architecture of the clock mechanism. J Mol Endocrinol. 2019 Nov;63(4):R93–102.
[6] Bolsius YG, Zurbriggen MD, Kim JK, Kas MJ, Meerlo P, Aton SJ, et al. The role of clock genes in sleep, stress and memory. Biochem Pharmacol. 2021 Sep;191(114493):114493.
[7] Trott AJ, Menet JS. Regulation of circadian clock transcriptional output by CLOCK:BMAL1. PLoS Genet. 2018 Jan;14(1):e1007156.
[8] Hastings M. The brain, circadian rhythms, and clock genes. BMJ. 1998;317(7174):1704–7.
[9] Adhish M, Manjubala I. Effectiveness of zebrafish models in understanding human diseases-A review of models. Heliyon. 2023 Mar;9(3):e14557.
[10] Chokkattu JJ, Mary DJ, Shanmugam R, Neeharika S. Embryonic toxicology evaluation of ginger- and clove-mediated titanium oxide nanoparticles-based dental varnish with zebrafish. J Contemp Dent Pract. 2022 Nov 1;23(11):1157–62.
[11] Garapati B, Malaiappan S, Rajeshkumar S, Murthykumar K. Cytotoxicity of lycopene-mediated silver nanoparticles in the embryonic development of zebrafish-An animal study. J Biochem Mol Toxicol. 2022 Oct;36(10):e23173.
[12] Dooley K, Zon LI. Zebrafish: a model system for the study of human disease. Curr Opin Genet Dev. 2000 Jun;10(3):252–6.
[13] Goldsmith JR, Jobin C. Think small: zebrafish as a model system of human pathology. J Biomed Biotechnol. 2012 Jun 3;2012:817341.
[14] Deepika BA, Ramamurthy J, Jayakumar ND, Rajesh Kumar S. Comparative clinical data for gingivitis treatment using gels from (Tulsi) and chlorhexidine (CHX). Bioinformation. 2021 Dec 31;17(12):1091–8.
[15] Tahoora, Rajeshkumar S, Ezhilarasan D, Lakshmi T. Preparation of Stevia and Ficus benghalensis hexane extract and its based mouthwash. J Complement Med Res. 2022;13(3):1.
[16] Krylov VV, Izvekov EI, Pavlova VV, Pankova NA, Osipova EA. Circadian rhythms in zebrafish (Danio rerio) behaviour and the sources of their variability. Biol Rev Camb Philos Soc. 2021 Jun;96(3):785–97.
[17] Thymoquinone and its pharmacological perspective: A review. Pharmacological Research - Modern Chinese Medicine. 2021 Dec 1;1:100020.
[18] Monica K, Rajeshkumar S, Ramasubramanian A, Ramani P, Sukumaran G. Anti-inflammatory and antimicrobial effects of herbal formulation using karpooravalli, mint, and cinnamon on wound pathogens. J Adv Pharm Technol Res. 2022 Dec;13(Suppl 2):S369–73.
[19] Ghasemian M, Owlia S, Owlia MB. Review of Anti-Inflammatory Herbal Medicines. Adv Pharmacol Sci. 2016 May 10;2016:9130979.
[20] Khader M, Eckl PM. Thymoquinone: an emerging natural drug with a wide range of medical applications. Iran J Basic Med Sci. 2014 Dec;17(12):950–7.
[21] Tolosa L, Donato MT, Gómez-Lechón MJ. General cytotoxicity assessment by means of the MTT assay. Methods Mol Biol. 2015;1250:333–48.
[22] Langmesser S, Tallone T, Bordon A, Rusconi S, Albrecht U. Interaction of circadian clock proteins PER2 and CRY with BMAL1 and CLOCK. BMC Mol Biol. 2008 Apr 22;9:41.
[23] Cahill GM. Clock mechanisms in zebrafish. Cell Tissue Res. 2002 Jul;309(1):27–34.
[24] Huang N, Chelliah Y, Shan Y, Taylor CA, Yoo SH, Partch C, et al. Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex. Science. 2012 Jul 13;337(6091):189–94.
[25] Taysi S, Algburi FS, Mohammed ZR, Ali OA, Taysi ME. Thymoquinone: A Review on its Pharmacological Importance, and its Association with Oxidative Stress, COVID-19, and Radiotherapy. Mini Rev Med Chem.

2022;22(14):1847–75.
[26] Schrader LA, Ronnekleiv-Kelly SM, Hogenesch JB, Bradfield CA, Malecki KM. Circadian disruption, clock genes, and metabolic health. J Clin Invest [Internet]. 2024 Jul 15;134(14). Available from: http://dx.doi.org/10.1172/JCI170998
[27] Das S S, Kannan, George S, Ps BC, Maliakel B, Ittiyavirah S, et al. Thymoquinone-rich black cumin oil improves sleep quality, alleviates anxiety/stress on healthy subjects with sleep disturbances– A pilot polysomnography study. J Herb Med. 2022 Mar;32(100507):100507.
[28] Kim MY, Jung S, Kim J, Lee HJ, Jeong S, Sim SJ, et al. Highly sensitive and multiplexed one-step RT-qPCR for profiling genes involved in the circadian rhythm using microparticles. Sci Rep. 2021 Mar 19;11(1):6463.
[29] Duong HA, Robles MS, Knutti D, Weitz CJ. A molecular mechanism for circadian clock negative feedback. Science. 2011 Jun 17;332(6036):1436–9.
[30] Zhang HY, Li KY, Wang YL, Wei CJ, Gao YX, Ren-Zhou, et al. ROS regulates circadian rhythms by modulating Ezh2 interactions with clock proteins. Redox Biol. 2025 Apr;81:103526.
[31] Shoieb AM, Elgayyar M, Dudrick PS, Bell JL, Tithof PK. In vitro inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. International Journal of Oncology. 2003 Jan 1;22(1):107–13.
[32] Ibrahim WN, Muizzuddin Bin Mohd Rosli L, Doolaanea AA. Formulation, cellular uptake and cytotoxicity of thymoquinone-loaded PLGA nanoparticles in malignant melanoma cancer cells. Int J Nanomedicine. 2020 Oct 20;15:8059–74.
[33] Ojha S, Azimullah S, Mohanraj R, Sharma C, Yasin J, Arya DS, et al. Thymoquinone protects against myocardial ischemic injury by mitigating oxidative stress and inflammation. Evid Based Complement Alternat Med. 2015 May 25;2015:143629.