ORIGINAL ARTICLE

Therapeutic Effect of Thymoquinone on MAPK Signalling Pathway in K562 Chronic Myeloid Leukaemia Cells

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ABSTRACT

INTRODUCTION: Chronic Myeloid Leukaemia (CML) is a hematopoietic malignancy caused by the BCR-ABL1 fusion oncoprotein, originating from Philadelphia chromosome translocation that enhances leukemic cell survival and therapeutic resistance. Aberrant MAPK pathway activation promotes proliferation and inhibits apoptosis, contributing to CML progression and resistance to tyrosine kinase inhibitors like imatinib with prolonged use. Thymoquinone (TQ), a bioactive molecule, has attracted considerable interest Zfor its anticancer characteristics that are worth investigating. **MATERIALS AND METHODS**: K562 CML cells were divided into an untreated group and a TQ-treated group and observed for 24 and 48 hours. Different TQ concentrations were administered to the TQ-treated group. Dose and time dependent effects on cell growth were assessed to evaluate cytotoxicity and determine the IC50 value in both groups. RNA was extracted from K562 CML cells based on the IC50 value and proceeded with RT-qPCR analysis on 7 genes involved, assigned as *Raf1, B-Raf, ERK1, ERK2, K-Ras, H-Ras,* and *N-Ras* genes, while the *beta-actin* gene was used as a housekeeping gene. Protein was extracted for the determination of protein and phosphorylation levels of Raf, MEK1/2, and ERK1/2, and assessed using the Jess Simple Western protocol. The Wilcoxon Signed-Rank test was performed using IBM SPSS, with $p<0.05$ considered statistically significant. **RESULTS**: TQ treatment significantly reduced the expression of all genes analysed in K562 cells. It also decreased protein and phosphorylation levels of Raf, MEK1/2, and ERK1/2. **CONCLUSION**: These findings suggest that TQ effectively inhibits MAPK signalling in K562 CML cells, highlighting its potential as a future treatment for CML.

Keywords
leukaemia, signalling pathways, thymoquinone, K562 CML, MAPK

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INTRODUCTION

Chronic myeloid leukaemia (CML), commonly referred to as CML, is a type of myeloproliferative neoplasm that arises from the stem cells of the haematopoietic system.[1] The onset of CML is driven by the BCR-ABL chimeric oncogene, which arises from a translocation between the breakpoint cluster region (BCR) gene on chromosome 22 and the Abelson murine leukaemia (ABL) gene on chromosome 9.[1] This translocation results in the formation of a fusion oncogene.[2,3] The constitutive activation of the BCR-ABL kinase plays a central role in CML pathogenesis by overactivating several downstream signalling pathways, including the MAPK pathway, ultimately causing uncontrolled proliferation of myeloid cells.[4,5]

Patients diagnosed with CML have shown a favourable response to tyrosine kinase inhibitors (TKIs) that target BCR-ABL, including imatinib (IM). Conversely, the overwhelming majority of individuals with CML do not achieve remission and may acquire resistance to TKIs

during prolonged treatment.[6,7] Consequently, diverse therapeutic strategies are necessary to address aberrant signalling pathways, which are crucial for the progression of chronic myelogenous leukaemia.[8]

The MAPK signalling pathway involves key protein kinases, including MEK, ERK, Raf and Ras which belong to multigene families. The Ras family consists of K-Ras, H-Ras, and N-Ras, while the Raf family includes Raf1, A-Raf, and B-Raf. MEK has five members (MEK1, MEK2, MEK3, MEK4, and MEK5), and ERK comprises four gene members (p38 MAPK, ERK1/2, ERK5 and JNK1/2/3). Among the ERK family, only ERK1/2 serve as the downstream target of MEK1/2 kinase activity.[9,10]

The MAPK signalling pathway plays a crucial role in regulating cell growth, apoptosis, and differentiation.[9,11] Its hyperactivation is a key contributor to the development of various cancers, including CML.[5,12] Consequently, targeting and inhibiting this pathway represents a vital therapeutic approach for managing CML.[13]

Natural phytochemicals have demonstrated potential epigenetic activity and may offer supportive benefits in leukaemia management, though further clinical validation is required to establish their efficacy and safety.[14] Among these, thymoquinone (TQ) a monoterpene quinone and the principal bioactive compound of Nigella sativa seeds has garnered attention for its broad pharmacological profile.[15,16] Biochemically, TQ exhibits antioxidant, anti-inflammatory, and anticancer properties. It exerts anticancer effects by modulating multiple molecular targets, including NF-κB, STAT3, PI3K/Akt, and MAPK pathways, and by inducing apoptosis, cell cycle arrest, and autophagy in various cancer cell lines.[17,18,19] In haematological malignancies, TQ has been shown to suppress proliferation and promote apoptosis in leukaemia cells by downregulating anti-apoptotic proteins (e.g., Bcl-2) and upregulating pro-apoptotic markers (e.g., Bax, caspases).[20] Although recent studies have explored its anticancer effects, the specific anti-leukaemia properties of TQ and the underlying mechanisms remain insufficiently understood. This study aimed to examine the effects of TQ in K562 CML cells, focusing on its impact on the expression of genes such as *K-Ras, H-Ras, N-Ras, Raf1, B-Raf, ERK1,* and *ERK2.*

MATERIAL AND METHODS

Cell Culture of K562 CML cell line

The BCR-ABL-positive K562 CML cell line, acquired from ATCC (American Type Culture Collection), was cultivated in a growth medium. This medium consisted of Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Kyoto, Japan), supplemented with 10% foetal bovine serum (FBS) (Tico Europe, Netherland) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, United State America). Cells were incubated in T-25 and T-75 culture flasks at 37°C with 5% CO2 in a humidified environment. The culture medium was refreshed every 3-4 days to ensure adequate cell nourishment. For subsequent experiments, cells were subcultured and allowed to grow until they reached approximately 80% of their maximum capacity. To ensure reproducibility and account for biological variability, all experiments were conducted using three independent biological replicates derived from separate culture batches.

TQ Treatment of K562 CML cell line

TQ was purchased from Sigma Aldrich (Sigma Aldrich, USA). Stock solution was prepared by adding dimethyl sulfoxide (DMSO) in TQ as 39 mM and stored at -80°c for not more than a month. Appropriate working solutions were prepared by diluting the stock solution with complete RPMI-1640 culture medium. Final concentration of DMSO in culture media was less than 1%. A series of TQ concentrations (5, 10, 15, 20, 25 μM) were prepared from a stock solution diluted with RPMI-1640 culture medium and incubated with BCR-ABL-positive K562 CML for 24 and 48 hours for IC50 determination.

RNA Extraction of Treated and Untreated K562 CML cell line

Total RNA of cultured cells was extracted from both untreated cells and TQ-treated cells (16μm for 48 hours) by using miniprep kit from Zymo Quick-RNA (Zymo Research Coorporation, Murphy Avenue, Irvine, USA)

according to the protocol from manufacturer. Extracted RNA was evaluated the purity and concentration by using Nanodrop photometer (Thermo Fisher Scientific, USA).

Quantitative Reverse Transcription PCR (RT-qPCR)

cDNA was synthesized from 100 ng of total RNA using GoScript™ Reverse Transcriptase. Quantitative PCR (qPCR) was then performed using GoTaq® qPCR Master Mix and gene-specific primers. PCR reactions were carried out in a 20µL volume and included 2µL of cDNA template, 2µL primers (1µL forward, 1µL reverse), 5.8µL of RNase free water, 0.2µL of CXR dye and 10µL of qPCR MasterMix. Cycling settings consisted of an initial denaturation step (95℃, 2 minutes) followed by 40 cycles of denaturation (95℃, 15 seconds) and lastly annealing and extension (60℃, 1 minute). Data analysis was performed using StepOne Software v2.3 (Applied Biosystems, USA). β-actin serving as the housekeeping gene. All samples were measured in triplicate manner. Primer sequences were designed and verified using database in NCBI (Table I).

Table I: Sequence of primers employed in RT-qPCR for gene expression studies

Genes name	Primer sequence (5' – 3')	References
K-RAS	Forward: CTAGAACAGTAGACACAAAACAGG Reverse: CGAACTAATGTATAGAAGGCATC	(18)
H-RAS	Forward: TACGGCATCCCCTACATCGAGAC Reverse: CACCAACGTGTAGAAGGCATCCTC	(18)
N-RAS	Forward: GAGTTACGGGATTCCATTCATTGAAAC Reverse: TGGCGTATTTCTCTTACCAGTGTGTAAAA	(18)
B-Raf	Forward: AGAAAGCACTGATGATGAGAGG Reverse: TGGCGTATTTCTCTTACCAGTGTGTAAAA	(19)
RAF1	Forward: TATTGGGAAATAGAAGCCAGTGAAGTGA Reverse: AACATCTCCGTGCCATTTACCCTTATA	(18)
ERK1	Forward: CGCTTCCGCCATGAGAATGTC Reverse: CAGGTCAGTCTCCATCAGGTCCTG	(18)
ERK2	Forward: CGTGTTGCAGATCCAGACCATGAT Reverse: TGGACTTGGTGTAGCCCTTGGAA	(18)
β-actin	Forward: GAGCGCGGCTACAGCTT Reverse: TCCTTAATGTCACGCACGATTT	(20)

Protein Extraction

Total Protein was extracted from both untreated cells and TQ-treated (16µm for 48 hours) by using Macherey-Nagel NucleoSpin® RNA/Protein extraction kit (Macherey-Nagel, Germany) accordance to the guidelines from company. In this study, both treated and untreated K562 leukaemia cells were collected in 15mL Falcon tubes and centrifuged at 10,000 x g for 5 minutes. Following centrifugation, cell lysis was initiated by adding 350µL of Buffer RP1 supplemented with 3.5µL of β-mercaptoethanol to the cell pellet. The mixture was thoroughly vortexed to ensure proper mixing. The lysate was then transferred into a NucleoSpin® Filter within a collection tube and centrifuged at 11,000 x g for 1 minute. After centrifugation, the NucleoSpin® Filter was discarded, and 350µL of 70% ethanol was added to the flow-through. This mixture was then transferred into a NucleoSpin® RNA/Protein Column in a new collection tube and centrifuged again at 11,000 x g for 30 seconds. Next, the flow-through was moved to a fresh collection tube, where one volume of Protein Precipitation (PP) solution was added. For about 10 minutes, the sample was incubated at room temperature, allowing for adequate protein precipitation. After incubation, the sample was centrifuged at 11,000 x g for 5 minutes, and the supernatant was completely removed. To wash the pellet, 500µL of 50% ethanol was added, which was later centrifuged at 11,000 x g for about 1 minute. Following centrifugation, the supernatant was again discarded. The pellet was air-dried for 10 minutes at room temperature with the lid open to ensure complete drying. Subsequently, 50µL of PSB-TCEP was added to the pellet, and the sample was incubated at 96°C for 3 minutes. Finally, the sample underwent a final centrifugation for 1 minute, allowing for the recovery of the supernatant, which was then prepared for further analysis. This method provides a reliable process for extracting RNA and proteins from K562 leukaemia cells, with the use of specific buffers and centrifugation steps ensuring purity and efficiency in sample preparation.

Jess Simple Western Analysis for Protein Analysis

Protein analysis was conducted using the Jess Simple Western system (Biomed Global, Wilayah Persekutuan Kuala Lumpur, Malaysia), following the protocol from manufacturer. Preparation of cell lysate that achieves final concentration of 1mg/mL start by diluting 0.1X sample buffer and heating to 95°C for five minutes. Fluorescent 5X mastermix were then mixed with the prepared lysate at 4:1 ratio. The plate was then filled with a total of 3µL of each sample. The analysis utilized a cartridge with a size range of 12-230 kDa. Primary antibodies, including anti-Raf 1, anti-pMEK1/2, anti-ERK1/2, and anti-pERK1/2 (R&D Systems, Minneapolis, MN, USA), were diluted at a 1:10 ratio in antibody buffer to probe the target proteins. HRP-conjugated secondary antibodies were applied afterward. In each sample, primary and secondary antibodies were added with total volume of

both antibodies 10 µL. To remove bubbles, the plate was centrifuged at 1000x g for five minutes. The plate and capillaries were then placed into Jess analyser. In this analyser, process of automated protein separation, blocking, incubation of antibody, and signal detection occurs. Data analysis was conducted using the manufacturer -supplied Compass software. Protein normalization was achieved using a reagent that binds biomolecules to amine groups, eliminating variability due to inconsistent housekeeping protein expression. No control was required for the Jess system during this experiment.

SPSS Statistical Analysis

Wilcoxon Signed Ranked statistical test was performed to analyse Ras/Raf/MEK/ERK gene expression analysis and protein phosphorylation analysis in both TQ-treated and untreated K562 leukaemia cells. Data were presented as medians with interquartile ranges (IQRs), based on three independent biological replicates (n=3). Statistical analysis was conducted by using International Business Machines® (IBM) Statistical Package for Social Sciences® (SPSS) software, version 21 (IBM, USA) in which p-value <0.05 was considered significant.

RESULTS

TQ inhibits K562 CML cell proliferation

K562 leukaemia cells were treated with varying concentrations of TQ (5,10,15,20,25 µM) with incubation period for 24 and 48 hours in order to identify cytotoxicity of TQ on the cells. Based on the result, TQ concentration that inhibited 50% of cell viability (IC50) were 21.5µM after treatment for 24 hours and 16 µM after incubation for 48hours (Figure 1).

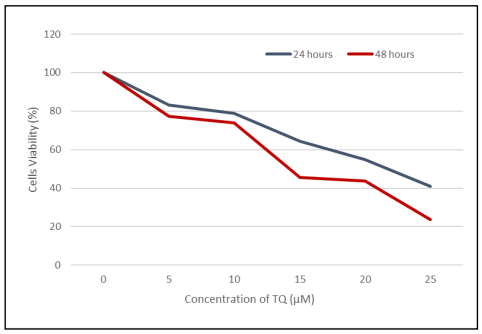

Figure 1: The suppression of K562 cell proliferation by thymoquinone in a dose and time dependent manner. Various concentrations of TQ (5 µM, 10 µM, 15 µM, 20 µM, 25 µM) were tested to evaluate its cytotoxic effects after 24 and 48 hours of treatment. The calculated IC50 values were 21.5 µM at 24 hours and 16 µM at 48 hours, respectively.

TQ reduces the expression of genes involved in MAPK Signalling pathway in K562 CML cells

Gene expression that involved in MAPK signalling pathway in both treated and untreated K562 CML cells was assessed with RT-qPCR. The results demonstrated a significant decrease in the expression of *Raf1, B-Raf, ERK1, ERK2, K-Ras, H-Ras,* and *N-Ras* genes in TQ-treated cells ($p<0.05$) compared to the untreated cells (**Figure 2, Table II, Table III**).

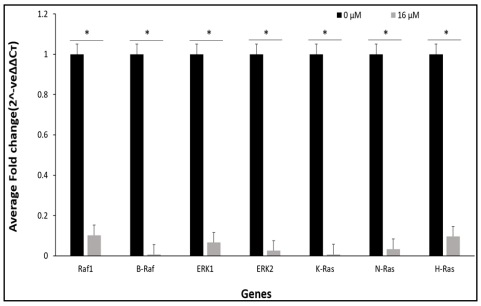

Figure 2: Gene expression analysis of genes participating in MAPK Signalling Pathway in K562 Leukaemia Cells. Analysis was done by using RT-qPCR. The graph shows the downregulation of all genes after 48 hours of incubation with 16 µM TQ in K562 leukaemia cells. Wilcoxon signed-rank test was used to determine the statistical significance. Data are presented as medians with interquartile ranges (error bars), based on three independent measurements (n=3) conducted on separate days to ensure reproducibility. *Denotes statistical significance when p is < 0.05.

Table II: Expression level of Untreated and Treated K562 Leukaemia Cells

Gene	Sample Type	ΔCr	ΔΔCr	Fold change(2^-veΔΔCr)
Raf1	Untreated	-4.04921659	0	1
	TQ Treated	-0.76298714	3.286229451	0.10250531
B-Raf	Untreated	-7.84446971	0	1
	TQ Treated	2.701745987	10.54621569	0.000668764
ERK1	Untreated	-3.92287572	0	1
	TQ Treated	0.004199982	3.927075704	0.065740411
ERK2	Untreated	-5.27196955	0	1
	TQ Treated	0.004199982	5.276169535	0.025805644
K-Ras	Untreated	-7.36014951	0	1
	TQ Treated	-0.25081126	7.109338252	0.00724229
N-Ras	Untreated	-3.96586545	0	1
	TQ Treated	0.937428157	4.90329361	0.033416545
H-Ras	Untreated	-3.18306732	0	1
	TQ Treated	0.190394592	3.373461914	0.096490993

Table III: Median Gene Expression Data for Untreated and Treated K562 Leukaemia Cells

Gene	n	Z-statistic[a]	Medians (IqR)		p-value [a]
			Untreated K562 cells	TQ-treated K562 cells	
Raf 1	3	-2.27	25.48 (0.28)	22.87 (0.34)	0.023
B-RAF	3	-2.27	21.96 (0.58)	26.22 (0.22)	0.023
ERK1	3	-2.22	22.25 (0.22)	23.20 (0.21)	0.026
ERK2	3	-2.22	24.33 (0.07)	23.46 (0.21)	0.026
K-RAS	3	-2.22	22.25 (0.22)	23.20 (0.21)	0.026
N-RAS	3	-2.22	26.58 (0.44)	23.70 (0.17)	0.026
H-RAS	3	-2.27	25.68 (0.09)	24.40 (0.18)	0.023

[a]Wilcoxon signed-rank test was employed in evaluate differences of statistical significance in gene expression between treated K562 leukaemia and untreated K562 leukaemia cells.

TQ inhibits MAPK Signalling pathway in K562 CML cells

Phosphorylation of protein that involved in MAPK signalling pathway in both untreated and TQ-treated K562 CML cells were evaluated by using Jess Simple Western Analysis. Results demonstrated reduction of the protein level of *Raf-1, p-Raf-1, MEK1/2, p-MEK1/2, ERK1/2* and *p-ERK1/2* in K562 cells after being treated

with TQ ($p<0.05$) as opposed to untreated K562 CML cells (**Figure 3 and Table II**).

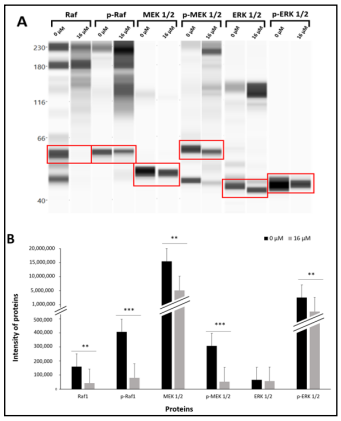

Figure 3: The effect of thymoquinone on the activation of MAPK signalling in K562 leukaemia cells. The cells were treated for 48 hours in incubator with 16µM TQ. Protein levels were quantified using Jess Simple Western protocol. Figure A illustrates the results of this analysis on target proteins before and upon treatment with TQ. Figure B presents the bar graph demonstrating a significant reduction in the protein levels of Raf-1, p-Raf-1, MEK1/2, p-MEK1/2, ERK1/2, and p-ERK1/2 in K562 cells upon TQ treatment. Statistical significance was determined using the Wilcoxon signed-rank test. Data are presented as medians with interquartile ranges (error bars) and are based on three independent measurements (n=3).
**Denotes statistical significance at $p<0.01$, while ***Denotes statistical significance at $p<0.001$ compared with untreated control cells.

Table IV: Medians of The Protein Expression levels within K562 Leukaemia Cells.

Proteins	n	M.Ws (kDa)	Protein Expression Levels medians (IqR)		p-value [a]
			Untreated K562 CML	TQ-treated K562 CML	
Raf 1	3	60	150,600 (31,413)	42,470 (29,423)	0.004
p-Raf 1	3	60	3,760,087 (450,379)	75,592 (11,561)	< 0.001
MEK 1/2	3	50	14,086,331 (614,461)	5,445,683 (153,350)	0.006
p-MEK 1/2	3	59	288,079 (67,531)	44,117 (12,621)	< 0.001
ERK1/2	3	45	61,216 (47,923)	53,680 (30,745)	0.037
p-ERK1/2	3	46	2,591,558 (202,440)	1,493,945 (112,560)	0.004

[a]Wilcoxon signed-rank test was employed in evaluate differences of statistical significance in protein intensities between treated and untreated K562 leukaemia cells.

DISCUSSION

CML is a type of myeloproliferative neoplasm marked by excessive granulocyte proliferation and the presence of the Philadelphia chromosome translocation, t(9;22)(q34;q11.2).[1,8] CML is characterized by the presence of the BCR-ABL1 fusion gene. The aberrant activation of MAPK signalling plays a critical role in the development of CML.[4] BCR-ABL induces the hyperactivation of numerous pro-oncogenic molecules involved in cellular signalling pathways including Ras, Raf, MEK, and ERK. Consequently, cell proliferation is augmented, while apoptosis and cell differentiation are markedly suppressed.[4,25]

TKIs have emerged as the most effective first-line therapy for CML.[26] TKIs are effective medications for treating patients with CML. Nonetheless, not all patients achieve long-term disease-free survival with TKIs, as some individuals exhibit intolerance to the medication.[27,28] Consequently, addressing abnormal signalling pathways critical to the progression of CML necessitates alternative therapeutic strategies.

Previous studies have shown that Thymoquinone (TQ) exhibits anti-cancer properties in various malignancies both in vitro and in vivo.[28] However, the specific mechanisms underlying TQ's anti-leukemic effects remain unclear. This study aimed to investigate the anti-leukemic effects of TQ in CML by examining its impact on the MAPK signalling pathway. This involved analysing the phosphorylation status and protein levels of key molecules in this pathway, as well as assessing the expression of genes involved in this signalling cascade.

MAPK signalling is known for its involvement to play crucial role in complex cellular programs such as differentiation, transformation, proliferation, development and apoptosis. The pathophysiology of CML is significantly influenced by the aberrant activation of the MAPK pathway.[12] Given the crucial role of genes within the MAPK signalling pathway in CML, this study aimed to elucidate the mechanisms underlying the anti-proliferative and apoptotic effects of TQ by assessing its ability to regulate the expression of *K-Ras, H-Ras, N-Ras, Raf1, B-Raf, ERK1,* and *ERK2* genes in K562 CML cells. The findings from this investigation revealed a significant decrease in the mRNA levels of *K-Ras, H-Ras, N-Ras, Raf1, B-Raf, ERK1,* and *ERK2* in K562 cells after treatment with TQ (Figure 3.2). These findings align with a previous study that demonstrated significant suppression of *BCR-ABL, JAK2, STAT3, STAT5A*, and *STAT5B* genes in K562 CML cells following TQ treatment.[29] The findings of the current study also agree with previously reported findings in which revealed TQ significantly inhibited *STAT3, STAT5a, STAT5b* and *JAK2* gene in HL60 cells after treatment with TQ.[30]

The constitutive activation of the MAPK pathway is significant in the progression of haematological malignancies, including CML. The BCR-ABL oncoprotein exhibits abnormal tyrosine kinase activity, which enhances the function of signal transduction pathways, such as MAPK signaling.[4,5,31] In turn, the suppression of activated proteins implicated in MAPK signalling is one possible therapeutic strategy for CML

treatment. This research evaluated the influence of TQ on the activation of the MAPK signalling pathway in K562 cells. The results indicated that TQ treatment caused a substantial decrease in the phosphorylation and protein levels of Raf, MEK1/2, and ERK1/2 in K562 CML cells (Figure 3.3 and Table 3.2).

These findings align with earlier research that demonstrated TQ-mediated suppression of STAT5, JAK2, Akt, STAT3, and PI3K phosphorylation and protein levels in K562 CML cells, HL60 AML cells and MV4-11.[16,31,33,34] While the reduction in total ERK1/2 protein levels was relatively modest, this is consistent with the established understanding that ERK1/2 activity is predominantly regulated through phosphorylation rather than total protein abundance. According to previous studies, ERK1/2 activation occurs via dual phosphorylation on threonine and tyrosine residues (Thr202/Tyr204 for ERK1 and Thr185/Tyr187 for ERK2), which induces conformational changes essential for downstream signalling functions such as proliferation, differentiation, and apoptosis.[34] Therefore, the observed decrease in phosphorylated ERK1/2 (p-ERK1/2) in this study is of greater functional relevance, indicating suppression of ERK activation rather than degradation of the protein itself. This distinction underscores the importance of evaluating both total and phosphorylated forms of signalling proteins when assessing pathway modulation.

Another study on other natural compound which is Asperuloside shows a significant reduction in the protein levels of RAF, RAS, MEK, p-MEK, p-ERK and ERK in K562 CML cells as opposed to the control group.[5] Similarly, a previous study using the phytochemical compound Hinokiflavone also demonstrated inhibition of ERK phosphorylation within the MAPK/NF-κB pathway in K562 cells, supporting the broader potential of phytochemicals in modulating this signalling axis.[35] In the present study, TQ at 16μM reduced cell viability by approximately 50% in K562 cells. While this concentration was effective in vitro, further investigation is required to determine its selectivity and safety profile in vivo, particularly regarding potential cytotoxicity toward non-malignant cells. Although TQ was dissolved in DMSO in this study, the final concentration was maintained below 1%, which is generally considered non-toxic and unlikely to interfere with MAPK signalling. Supporting this, a recent study reported that DMSO concentrations ranging from 0.25% to 1.5% did not result in cellular toxicity and produced results comparable to DMSO-free controls.[36] Nonetheless, the absence of a DMSO-only control in the present study limits definitive attribution of MAPK modulation solely to TQ. Future studies should incorporate vehicle controls to fully exclude solvent-related influences.

Although TQ's anticancer effects have been previously reported, its direct impact on MAPK gene and protein expression in CML remains incompletely explored. This study addresses this gap by demonstrating that TQ significantly downregulates key MAPK pathway genes and proteins in K562 cells, suggesting a mechanistic role in modulating oncogenic signalling. By providing evidence of TQ's ability to suppress both transcriptional and post-translational activation of MAPK components, this research offers new insights into its potential as a targeted therapeutic agent for CML. However, the study is limited by its use of a single cell line (K562), the absence of apoptosis assays to confirm downstream functional effects, and lack of in vivo validation. Future investigations should include multiple CML cell lines, assess apoptotic markers, and explore the efficacy and safety of TQ in animal models. Additionally, evaluating TQ in combination with TKIs may reveal synergistic effects and provide a basis for integrative therapeutic strategies.

CONCLUSION

The findings of this study demonstrate that TQ exhibits promising anti-leukemic activity against K562 CML cells. TQ treatment significantly inhibited cell proliferation, as evidenced by reduced cell viability. Gene expression analysis revealed that TQ downregulated key components of the MAPK signalling pathway, including *K-Ras, H-Ras, N-Ras, Raf1, B-Raf, ERK1*, and *ERK2*, indicating its potential to suppress oncogenic transcriptional activity. Furthermore, protein analysis using the Jess assay confirmed that TQ effectively reduced the phosphorylation

and protein levels of Raf, MEK1/2, and ERK1/2, suggesting inhibition of MAPK pathway hyperactivation a hallmark of CML pathogenesis. Collectively, these results support the hypothesis that TQ exerts its anti-proliferative effects through modulation of MAPK signalling. Given its ability to target critical molecular mechanisms involved in CML progression, TQ may serve as a potential therapeutic candidate, particularly for patients who exhibit resistance or intolerance to conventional tyrosine kinase inhibitors. However, given the limitations of the current study including the use of a single cell line, a single phytochemical agent, and short-term exposure, further investigations are warranted. Future studies should incorporate additional CML models, extended treatment durations, and in vivo validation to comprehensively assess the therapeutic potential and mechanistic specificity of TQ in targeting MAPK signalling in leukaemia. Further in vivo studies and clinical validation are necessary to establish its safety, selectivity, and translational applicability.

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