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Combined Therapeutic Potential of Thymoquinone and Theaflavin Through P13k Pathway Suppression

Keerat Mann[1], Sangeetha S[2], Taniya Mary Martin[3], Meenakshi Sundaram Kishore Kumar[4]
[1,2,3,4]Department of Anatomy, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai-600077, Tamil Nadu, India.
Corresponding Author: Sangeetha S, E-mail: sangeethas.sdc@saveetha.com

Abstract

Background: *The synergistic potential of natural phytochemicals in modulating key cancer related pathways offers a promising approach for therapeutic development. This study investigates the combined anti-cancer, anti-inflammatory, and antioxidant effects of Thymoquinone and theaflavin on KB cells.*

Methods: *Thymoquinone and theaflavin were evaluated individually and in combination (1:1,2:1,1:2) across a concentration range of 1 to 200 µg/mL. Cytotoxicity was assessed by MTT assay, anti-inflammatory activity was measured using Bovine serum albumin method, and antioxidant potential was determined via the DPPH radical scavenging assay. Gene expression analysis of P13K, Akt, mTOR, NF-kB, IL-6, Bcl-2 and BAX was performed using real-time PCR.*

Results: *Combination treatments, especially at a 1:1 ratio, demonstrated significantly enhanced cytotoxicity, superior inhibition of protein denaturation, and greater DPPH radical scavenging compared to individual compounds. Gene expression analysis revealed strong downregulation of the P13K/Akt/mTOR and NF-kB/IL-6 pathways and a shift toward apoptosis characterised by decreased Bcl-2 and increased BAX expression.*

Conclusion: *The synergistic interaction between Thymoquinone and theaflavin effectively modulates survival, inflammatory, and apoptotic pathways, offering a promising natural combination strategy for anticancer and anti-inflammatory therapies. Further preclinical investigations are warranted to validate their clinical applicability.*

Keywords: *Thymoquinone, Theaflavin, KB Cells, P13K/Akt/mTOR, NF-kB, Bcl-2, BAX, antioxidant, synergy*

1 INTRODUCTION

Over the past few years, therapeutic strategies have undergone a notable transformation, with increasing emphasis placed on targeted molecular interventions and the exploration of naturally derived bioactive compounds (1). Among these, thymoquinone and theaflavin have garnered substantial attention for their impressive pharmacological properties and potential utility in modulating key signalling pathways implicated in cancer, inflammation, and metabolic disorders (2). In particular, the potential synergism between these two naturally sourced molecules has garnered scientific interest for its possible role in modulating the Phosphoinositide 3-kinase (PI3K) signalling pathway—a key axis that governs cell growth, metabolism, and survival.

Thymoquinone (TQ), the main active constituent of Nigella sativa (black cumin) seeds, has long been celebrated in traditional medicinal systems for its therapeutic versatility. Modern pharmacological studies have validated many of these traditional claims, demonstrating that thymoquinone exhibits a wide spectrum of biological activities including antioxidant, anti-inflammatory, immunomodulatory, and antitumor effects (3). Structurally classified as a monoterpenoid, TQ has the ability to traverse cell membranes and interact with a multitude of molecular targets, thereby influencing gene expression and apoptotic pathways (4,5).

In contrast, theaflavins represent a class of polyphenolic compounds that form during the enzymatic oxidation of catechins in black tea (Camellia sinensis) during fermentation. Of these, Theaflavin-3,3'-digallate (TF3) stands out for its pronounced anticancer, anti-inflammatory, and antimicrobial efficacy. Due to their polyphenolic structure, theaflavins are excellent scavengers of reactive oxygen species (ROS), and they have been shown to modulate several intracellular signalling cascades including MAPK, NF-κB, and PI3K/AKT pathways (6–8).

The PI3K/AKT signalling network is among the most commonly dysregulated pathways across a spectrum of human cancers. It orchestrates essential cellular processes such as proliferation, energy metabolism, angiogenesis, and mobility. Dysregulation or hyperactivation of this pathway—either through genetic mutations, loss of PTEN (Phosphatase and Tensin Homolog deleted on chromosome 10) function, or external oncogenic signals—is implicated in a wide array of malignancies, including breast, prostate, liver,

and colorectal cancers (9). Consequently, this pathway has emerged as a prime target for pharmacological inhibition (10).

Individual studies have demonstrated that both thymoquinone and theaflavin exhibit inhibitory effects on the PI3K/AKT pathway. Thymoquinone, for instance, has been shown to downregulate PI3K activity and decrease AKT phosphorylation, triggering apoptosis in several cancer models such as breast, colon, and pancreatic cancers.(11) Similarly, TF3 inhibits PI3K/AKT signalling by decreasing phosphorylated AKT levels and boosting the expression of pro-apoptotic proteins like Bax and p53.(12) Nevertheless, monotherapy approaches often encounter challenges such as resistance development, limited efficacy, and toxicity at escalated doses. Hence, combinational regimens involving multiple agents that can work synergistically have gained traction for their enhanced effectiveness and reduced side effects. This synergy not only helps to target multiple nodes within the signaling network but also reduce the required dose of each agent, thereby decreasing the likelihood of adverse effects.

In combination, thymoquinone and theaflavin are posited to offer amplified synergistic inhibition of the PI3K pathway compared to their individual actions. Early in vitro and computational docking studies indicate that the two compounds may interact with different regions of the PI3K enzyme, contributing complementary effects on pathway inhibition. This also reduces the required dose of each agent (13). In addition, their combined antioxidant properties can attenuate oxidative stress which often acts as a co-activator of the PI3K/AKT signaling cascade.(14,15). The dual targeting approach could lead to more profound suppression of tumour cell growth, angiogenesis, and metastasis.

Another crucial element of their therapeutic profile lies in their shared anti-inflammatory potential. Persistent inflammation is a recognized facilitator of cancer progression, frequently acting through signalling routes like PI3K/AKT and NF-κB. TQ, for instance, has demonstrated the capacity to inhibit kinase activation driven by inflammatory cytokines. The synergistic anti-inflammatory effects of both compounds may therefore further potentiate the inhibition of PI3K signalling, reinforcing their disease-modulating capacity. Additionally, their physicochemical properties support co-administration: while TQ is lipophilic and readily enters cells, theaflavin's hydrophilic nature allows for complementary uptake mechanisms. Utilizing ethanol-based extraction techniques can enhance their solubility and ensure stability, thereby improving bioavailability and ensuring efficient delivery to target tissues (16).

Accordingly, this study is designed to assess the combined effects of ethanol-extracted thymoquinone and theaflavin on PI3K signalling in vitro, focusing on changes in PI3K/AKT gene and protein expression, apoptotic markers, and indicators of oxidative stress. The results may offer critical evidence supporting the rational design of combination therapies using natural compounds to achieve enhanced efficacy and safety. Given the frequent dysregulation of the PI3K pathway across disease states, uncovering how thymoquinone and theaflavin function in tandem could contribute to the broader understanding of plant-based molecular pharmacology and encourage clinical exploration of these agents (17). As drug resistance and patient preference shift the focus toward natural treatment alternatives, this dual-compound approach presents a promising frontier in the evolution of molecularly targeted therapeutics.

2 MATERIALS AND METHODS

2.1 Chemicals and Reagents

The chemicals used in this study included Thymoquinone (≥98%) and Theaflavin (≥95%) were procured from Sigma-Aldrich (St. Louis, MO, USA), Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), both obtained from Sigma Chemical Pvt Ltd, USA. From Gibco (Canada), Cell culture reagents such as Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics solution, RPMI 1640 medium and phosphate buffered saline (PBS) were purchased and the Real-Time PCR kit were procured from Takara (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

2.2 Preparation of Herbal Compounds

Based on the solubility profiles of Thymoquinone and theaflavin, sterile DMSO was used as a solvent and stock solutions of both of them, having concentration 10mg/mL, were prepared. The solutions were passed through a 0.22 µm syringe filter for sterilization and until further use were stored at temperature of 4°C while ensuring simultaneous protection from light. Serial working dilutions of each compound were freshly prepared for the cytotoxicity assay in complete DMEM culture medium to achieve final concentrations of 1,5,10, 25,50,75,100, and 200µg/mL. Each concentration was tested individually as well as in combination at fixed ratios. Drug combinations, at fixed molar ratios of 1:1, 2:1 and 1:2, were prepared of Thymoquinone and theaflavin and evaluated at the same total concentrations (1-200 µg/mL).

According to the intended total concentration and the corresponding ratio, the required volume of each compound was calculated. (For example, to prepare a 200 µg/mL of theaflavin: 1:1 ratio: 100 µg/mL of thymoquinone + 100 µg/mL of theaflavin; 2:1 ratio: 133.3 µg/mL of thymoquinone + 66.7 µg/mL of theaflavin; 1:2 ratio: 66.7 µg/mL of thymoquinone + 133.3 µg/mL of theaflavin). Each combination was mixed thoroughly in complete media following which they were filtered through a sterile 0.22 µm filter prior to cell treatment.

2.3 Cell Culture

From NCCS (Pune, India), Mouse fibroblast KB cell lines were obtained and cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, and maintained in a humidified incubator at 37°C and 5% CO_2. Cells were passaged using 0.25% trypsin-EDTA upon reaching 80–90% confluency.

2.4 Biocompatibility: MTT Assay

At a density of 5× 10^3 cells per well, KB cells were seeded in 96 well plates. These cells were allowed to adhere for 24 hours and after they were treated with varying concentrations of thymoquinone (1-200 µM), theaflavin (1-200 µM), and their combinations at fixed ratios of 1:1, 1:2 and 2:1 based on IC20, IC50 and IC70 values. The negative control consisted of 0.1% DMSO, while the blank included media without cells. The plates were incubated for 48 hours after the treatment at a temperature of 37°C. Subsequently, 10µL of MTT solution (5 mg/ml in PBS) was added to each well and incubated for an additional 4 hours after which the supernatant was carefully removed, and the resulting formazan crystals were dissolved in 100 µL of DMSO. After this with the help of a microplate reader the absorbance of each well was measured at 570 nm. The percentage of cell viability was determined using the formula: (A 570 of treated cells/ A570 of control cells) x 100, where A represents the absorbance. In the graphical representations, the Y-axis depicts the percentage of cell viability, whereas the X-axis represents the respective sample concentrations. For comprehensive comparisons of cell viability across different treatment conditions both cell control and sample control were included for each assay.

2.5 Anti-inflammatory Assay: Protein Denaturation Method

Bovine Serum Albumin (BSA) was prepared for the anti-inflammatory assay, based on the protein denaturation method, at a concentration of 1% in phosphate-buffered saline (PBS) with a pH of 6.4. The test samples including thymoquinone, theaflavin and their combinations were diluted to final concentrations of 1,5,10,25,50,75,100 and 200µg/mL. Ascorbic acid was used as a positive control. In the assay procedure, 0.5 mL of the prepared BSA solution was mixed with 0.5 mL of the respective test sample followed by incubation of the mixture at 37°C for 20 minutes to allow interaction. Following incubation, the samples were heated for 5 minutes to induce protein denaturation at a temperature of 70°C. The samples were allowed to cool to room temperature after heating and the absorbance was measured at 660 nm using a spectrophotometer.

2.6 Antioxidant Assay: DPPH Radical Scavenging

For the antioxidant assay using the DPPH radical scavenging method, a 0.1 mM DPPH solution was prepared in methanol, following which the test samples were diluted to concentrations of 1,5,10,25,50,75,100 and 200µg/mL. Diclofenac was used as a positive control. In the experimental procedure, 1 mL of DPPH solution was mixed with 1 mL of each test sample, and for 30 minutes the mixtures were incubated in the dark. Using a UV-Visible spectrometer, the absorbance was measured at 517 nm after incubation.

2.7 Gene expression analysis

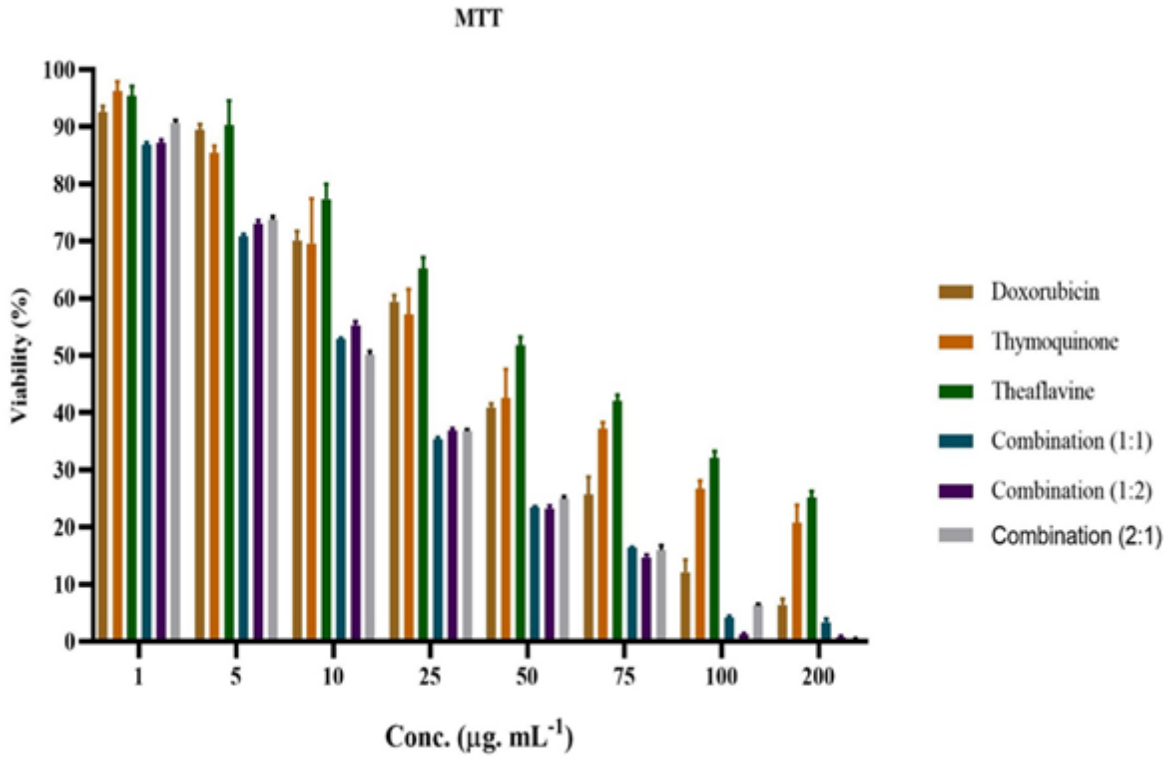
KB cells were treated with IC_{50} concentrations for 24 hours, after which total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at −80°C until further analysis. For cDNA synthesis, 2µg of total RNA was used in a 10µL reaction volume using superscript II reverse transcriptase (Invitrogen), following the manufacturer's instructions. Real-time PCR analysis was conducted in a total volume of 20µl which comprised 1µl of cDNA, 10µl of 2× Qpcr Master Mix (Takara, USA), and 9µl of doubled distilled water (ddH_20). CFX96 Touch Real Time PCR Detection System (Bio-Rad, USA) was used for the reactions under the following thermal cycling conditions: initial denaturation at 95°C, 15 seconds at 60°C, and 20 seconds at 72°C. This was followed by a melting curve analysis consisting of 5 seconds at 95°C, 60 seconds at 60°C, and a gradual increase in temperature for continuous melting. Melting curves were recorded for all samples to verify amplification specificity, for the sake of ensuring quality control, with each primer pair's product validated by melt curve analysis (Table 1). Comparative Ct method (ΔΔCt) was used to perform data analysis and fold changes were calculated according to the

2^(-ΔΔCt) formula as described by Schmittgen and Livak (2008), using CFX Manager version 2.1 software (Bio-Rad, USA).

Table 1: Primers used for gene expression

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
PI3K	GACCTGTGCGTCCTCAGTTT	CTGTGTGGTGGGCTCAATAA
Akt	TGGACTTCCGAGCGACGTGG	CTGCGGGGCCATCTGGAAGG
mTOR	CTGAAGATGATGCTGACCAAGGA	TGGTCCGTTCCAGGATTGTT
NF-κB	AACTGGAAACGACCTGGAAGCA	TTGGTGGGTGCGTCTTAGTG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
BCL-2	GAGTTCGGTGGGGTCATGTGTGTG	GGTGCCGGTTCAGGTACTCAGTC
BAX	TTTGCTTCAGGGTTTCATCC	GATCAGCTCGGGCACTTTAG

3 RESULTS



The cytotoxicity of Theaflavin, Thymoquinone and their combinations (1:1, 1:2 and 2:1 ratios) on KB cells was evaluated using the MTT assay, a standard method for assessing cellular metabolic activity and viability. KB cells were treated with varying concentrations (1,5,10,25 50, 75,100 and 200 µg/mL) of Theaflavin, Thymoquinone and their combinations for 24 hours. The reduction of MTT to insoluble formazan crystals by mitochondrial dehydrogenases indicated the presence of viable, metabolically active cells.

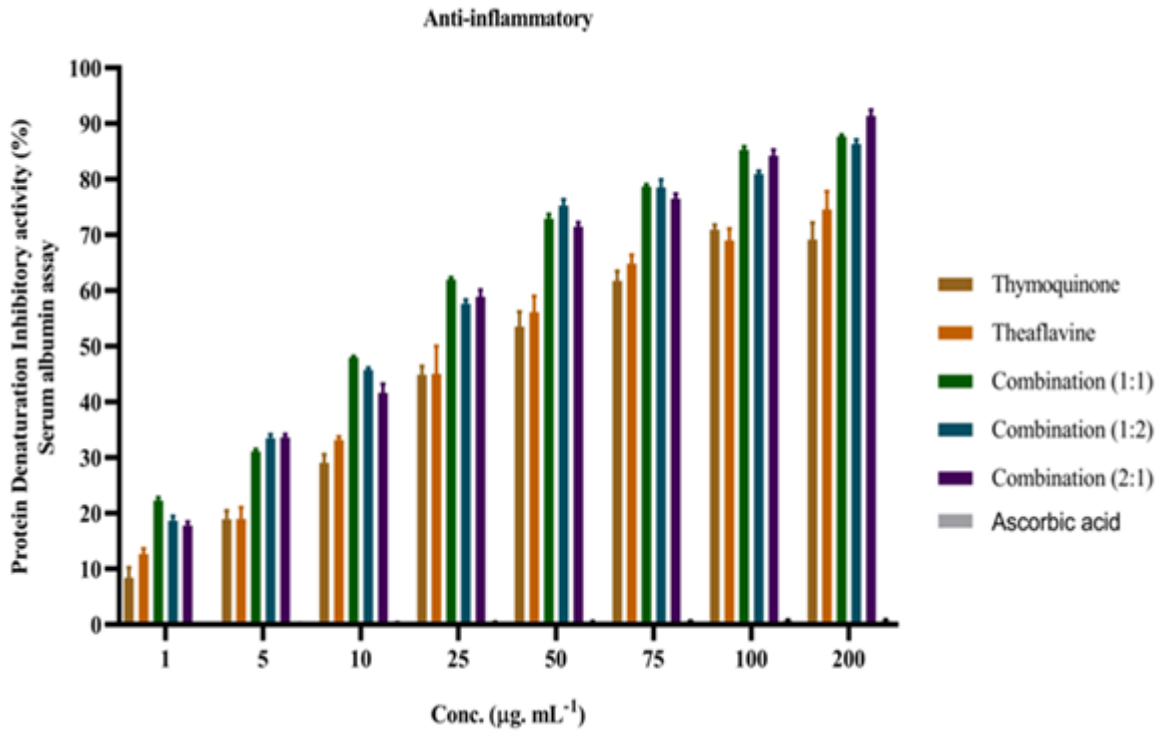
Figure 1: The cytotoxic effect of Thymoquinone, theaflavin and its combination in 1:1, 1:2 and 2:1 ratio on KB cell lines

Statistical analysis using two-way ANOVA revealed significant contributions from the interaction (2.47%), column factor (12.78%), and row factor (84.75%) to the total variation, each achieving high statistical significance ($p < 0.001$). Based on this findings, lower concentrations of 5 and 10 µg/mL were selected for subsequent experiments, as they maintained over 80% cell viability while demonstrating measurable biological effects without inducing cytotoxicity. These results underline the importance of optimizing the concentration range for therapeutic applications of Theaflavin and Thymoquinone combinations and set the foundation for further functional and mechanistic studies (Figure 1).

The anticancer effect of thymoquinone and theaflavin was evaluated in combination at different ratios (1:1, 2:1, and 1:2) after 24 hours of treatment in KB cell lines. Various concentrations ranging from 1 to 200 µg/mL were tested to assess the dose dependent cytotoxicity. The combination treatments demonstrated enhanced cytotoxic effects compared to individual compounds, suggesting a potential synergistic interaction between thymoquinone and theaflavin. Cell viability was determined using the MTT assay, and the results indicated that certain combination ratios achieved greater inhibition of KB cell proliferation than either agent alone (Figure 2).

Figure 2: The anticancer effect of the Thymoquinone and Theaflavin in combination ratios at 24 h in KB cell lines at various concentrations.
The image shows (a) control, (b) Thymoquinone, (c) Theaflavin, (d) combination 1:1, (e) combination 1:2, (f) combination 2:1 in the highest concentration 200 µg/mL
The results showed a clear concentration dependent decrease in cell viability across all treatment groups. Higher concentrations (≥75 µg/mL) of Theaflavin, Thymoquinone and their combinations significantly reduced KB cell viability compared to lower concentrations (≤10 µg/mL). Among the combinations the 1:2 (Theaflavin: Thymoquinone) ratio demonstrated slightly enhanced cytotoxicity compared to the 1:1 and 2:1 ratio, suggesting a synergistic effect favouring Thymoquinone dominant formulations.

3.2 Anti-Inflammatory Activity by Protein Denaturation Assay



The anti-inflammatory activity of theaflavin, thymoquinone and their combinations was evaluated using the BSA protein denaturation method, with ascorbic acid serving as the positive control. The results demonstrated a concentration- dependent increase in inhibition of protein denaturation, reflecting enhanced anti-inflammatory potential at higher doses. At the lowest concentration tested (1 µg/mL), the positive control (ascorbic acid) exhibited moderate inhibition (mean 14.16%), whereas thymoquinone and theaflavin showed lower inhibition values (8.39% and 12.69%, respectively). However, the 1:1 combination showed a significantly higher inhibition (22.28%), indicating early synergistic activity. At 5 µg/mL, inhibition increased to 24.52% for positive control. Thymoquinone and theaflavin showed inhibition of 18.26% and 18.99%, respectively, while the combinations enhanced activity, with 1:1, 1:2, and 2:1 mixture showing 31.08%, 33.51% and 33.34% inhibition, respectively. By 10 µg/mL, the trend became more apparent. Ascorbic acid reached 35.19%, with thymoquinone at 29.07%, and theaflavin at 33.18%. The combinations further improved inhibition, with 1:1 reaching 47.95%, 1:2 at 45.75% and 2:1 at 41.66%. At 25 µg/mL, the positive control showed 49.33% inhibition. Thymoquinone and theaflavin achieved 44.91% and 45.91% and 45.06%, respectively. The combinations maintained superior efficacy with the 1:1 mix at 61.98%, 1:2 at 57.67%, and 2:1 at 59.56%. At 50 µg/mL, inhibition

levels further increased across all groups. Ascorbic acid averaged 59.35%, thymoquinone and theaflavin reached 53.54% and 56.14%, respectively. Notably, the 1:1, 1:2, and 2:1 combination showed 72.89%, 75.29%, and 71.48% inhibition, highlighting strong anti-inflammatory synergy. At 75 µg/mL, ascorbic acid demonstrated 75.31% inhibition. Theaflavin showed improved activity (64.82 %) compared to thymoquinone (61.75%). The combinations again showed superior results, with 1:1 at 78.69%, 1:2 at 78.53%, and 2:1 at 76.52%. At 100 µg/mL, peak inhibitory effects were observed for most samples. The positive control recorded 90.98%, while thymoquinone and theaflavin achieved 70.95% and 68.97% respectively. The combination treatments exhibited robust inhibition: 85.24% (1:1), 80.96% (1:2), and 84.26% (2:1). Finally, at the highest concentration of 200 µg/mL, the positive control reached 96.83% inhibition. Thymoquinone averaged 69.18%, theaflavin 74.56%, while the combinations continued to exhibit the strongest anti-inflammatory activities, with 87.67% (1:1), 86.34% (1:2), and 91.38% (2:1), suggesting near-parity with ascorbic acid. Overall, the combination treatments consistently outperformed the individual compounds, and the data clearly suggest a synergistic interaction, particularly evident in the 1:1 and 2:1 ratio. These findings support the therapeutic potential of combining theaflavin and thymoquinone in anti-inflammatory formulations (Figure 3).

Figure 3: Inhibition of BSA protein denaturation by thymoquinone, theaflavin and their combinations. Higher percentage inhibition reflects stronger anti-inflammatory activity.

3.3 Antioxidant Activity by DPPH Assay

The antioxidant capacity of theaflavin, thymoquinone and their combinations was evaluated using the DPPH radical scavenging assay, with diclofenac serving as the positive control. The results demonstrated a concentration- dependent increase in radical scavenging activity across all samples. At the lowest concentration of 1 µg/mL, diclofenac showed moderate activity with an average inhibition of approximately 21.8%, while thymoquinone and theaflavin showed slightly lower scavenging activities (17.1% and 19.5%, respectively) However, the combination groups (1:1, 1:2, and 2:1) exhibited higher scavenging around 22-23%, indicating an early synergistic effect. At 5 µg/mL, the inhibition increased, with diclofenac reaching around 27.6% and the combination groups showing approximately 38-40 % scavenging, outperforming individual compounds. At 10 µg/mL, diclofenac exhibited about 40.4% inhibition, while combinations surpassed 50%, highlighting strong antioxidant potential when theaflavin and thymoquinone were used together. A notable enhancement was observed at 25 µg/mL, where combinations reached up to 67% scavenging, compared to 46.6% by diclofenac alone. At higher concentrations of 50 and 75 µg/mL, combination groups consistently maintained superior radical scavenging activities (>78% and >84%, respectively), compared to individual compounds and even matching or exceeding the positive control. At 100 µg/mL, diclofenac demonstrated around 89% inhibition, whereas combinations achieved approximately 90%, confirming their robust antioxidant potential. Finally, at 200 µg/mL, diclofenac showed maximum scavenging of about 96%, while combination groups remained highly effective at 96-98%, thereby demonstrating a strong, dose dependant, and synergistic antioxidant effect of theaflavin and thymoquinone when combined (Figure 4).

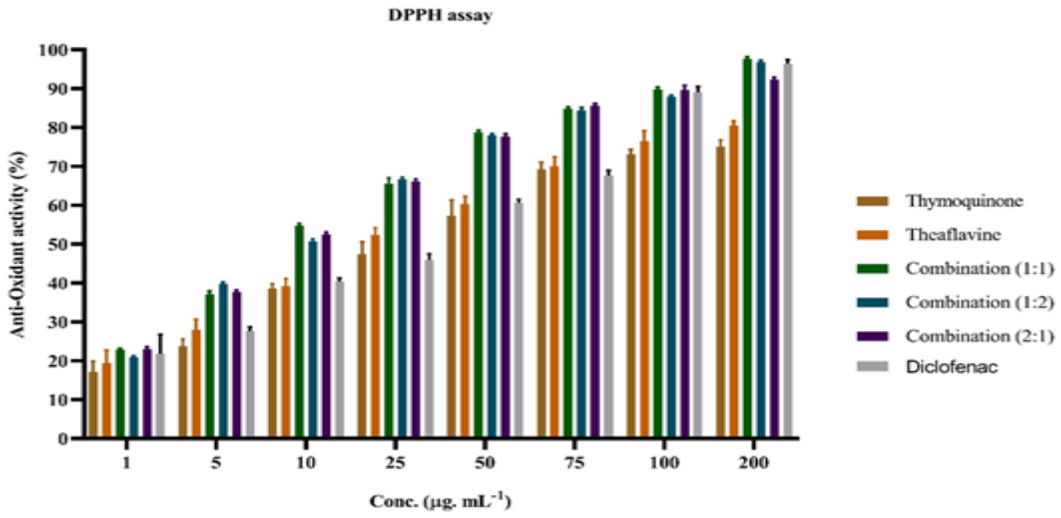


Figure 4: The antioxidant capacity of thymoquinone, theaflavin, and their combinations was assessed by the DPPH radical scavenging assay. Higher percentage scavenging indicates stronger antioxidant potential.

3.4 Gene Expression modulation by Thymoquinone, theaflavin and their combinations in KB cells

The gene expression analysis revealed that treatment with Thymoquinone, theaflavin, and their combinations significantly modulated key signalling molecules in KB cells. A marked downregulation of P13/Akt/mTOR expression was observed, indicating suppression of the P13K/Akt/mTOR survival pathway (Table 2). Similarly, the inflammatory markers NF-kB and IL-6 were significantly reduced in cells treated with the combination therapies compared to individual treatments, suggesting an anti-inflammatory effect. Regarding apoptosis related genes, Bcl-2 expression was downregulated while BAX expression was notably upregulated in combination-treated groups, the 1:1 combination of thymoquinone and theaflavin produced the most pronounced effects on inhibiting cell survival pathways and promoting apoptotic signalling. These findings collectively demonstrate the potent synergistic activity of thymoquinone and theaflavin in modulating critical molecular pathways involved in cancer progression and inflammation (Figure 5).

Table 2: Gene expression modulation by thymoquinone, theaflavin, and their combinations in KB cells

Gene	Thymoquinone (TQ)	Theaflavin (TF)	TQ + TF (1:1)	TQ + TF (2:1)	TQ + TF (1:2)
PI3K	Downregulated	Downregulated	Strongly downregulated	Downregulated	Moderately downregulated
Akt	Downregulated	Downregulated	Strongly downregulated	Downregulated	Moderately downregulated
mTOR	Downregulated	Moderately downregulated	Strongly downregulated	Downregulated	Moderately downregulated
NF-kB	Downregulated	Downregulated	Strongly downregulated	Downregulated	Moderately downregulated
IL-6	Downregulated	Downregulated	Strongly downregulated	Downregulated	Moderately downregulated
BCL-2	Downregulated	Moderately downregulated	Strongly downregulated	Downregulated	Moderately downregulated
BAX	Upregulated	Upregulated	Strongly upregulated	Upregulated	Moderately upregulated

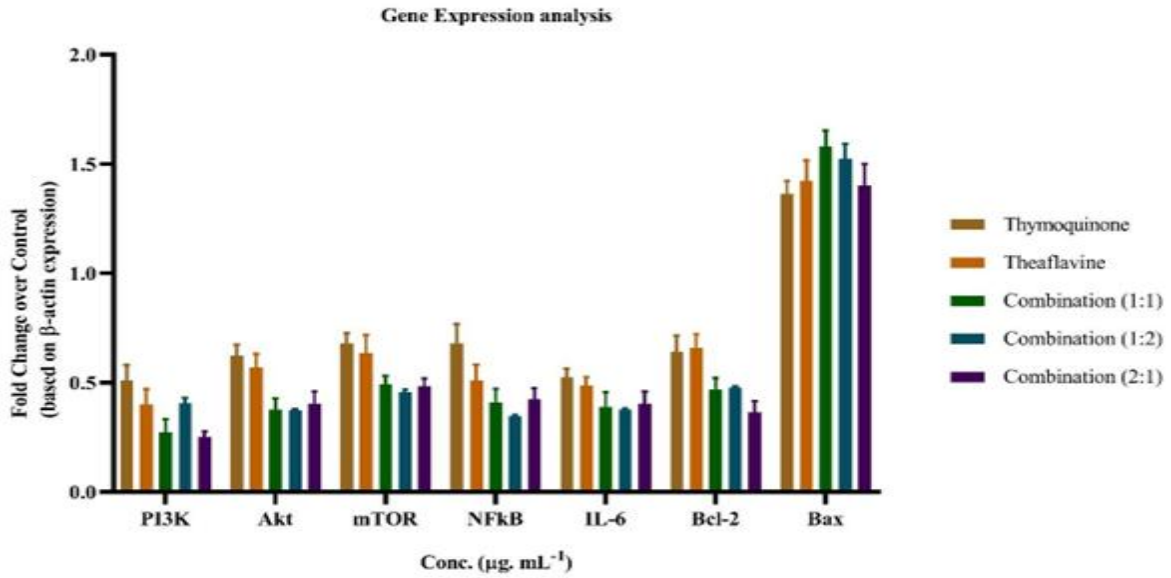


Figure 5: Relative mRNA levels of P13K, Akt, mTOR, NF-kB, IL-6, Bcl-2 and BAX were assessed by real time PCR in KB cells treated with Thymoquinone, Theaflavin and their combinations.

4 DISCUSSION

This investigation focused on assessing the anticancer, anti-inflammatory, and antioxidant capabilities of thymoquinone and theaflavin—both as standalone agents and in combination—on kb cells.(18) the experimental outcomes demonstrated that while each compound exhibited considerable biological activity individually, their co-administration led to synergistic enhancements across multiple biological domains, including cytotoxic effects, inflammation suppression, oxidative stress alleviation, and modulation of molecular signaling pathways.

During cytotoxicity testing, both thymoquinone and theaflavin reduced the viability of kb cells in a dose-dependent fashion. Notably, when combined—especially in a 1:1 ratio—the cytotoxic effect was markedly more significant than that of either compound alone at equivalent concentrations. These findings point towards a synergistic mechanism of action that promotes cell death through more than just additive effects. Such enhanced interactions are well-documented in phytochemical research, where multi-compound therapies are known to affect diverse molecular targets concurrently, thereby intensifying anticancer responses.(19,20) as kb fibroblasts are widely used to model cell proliferation, the pronounced inhibition observed supports the role of this combination as a potent antiproliferative approach.

In evaluating anti-inflammatory activity using the bsa protein denaturation assay, additional insights were gained. Inflammation plays a pivotal role in cancer development, and protein denaturation serves as a surrogate marker of inflammatory insult. Both agents demonstrated significant capacity to prevent protein denaturation, and their combined application outperformed individual treatments at matching doses. This suggests improved protein stabilization under inflammatory conditions, leading to reduced inflammatory responses. Thymoquinone's known inhibition of pro-inflammatory cytokines and theaflavin's suppression of nuclear transcription pathways likely work together to achieve this enhanced effect.(21,22)

Antioxidant potential was examined using the dpph free radical scavenging assay. Both thymoquinone and theaflavin exhibited strong antioxidant responses independently; however, when used together, their radical scavenging capacity was significantly improved. Since oxidative stress is a key driver of carcinogenesis through mechanisms such as dna damage and inflammatory signaling activation, this synergistic antioxidant activity suggests improved cellular defence. The outcome is consistent with previous literature indicating that combining antioxidants may broaden the range and depth of ros neutralization, enhancing cellular protection (23).

To better understand the molecular underpinnings of these biological effects, gene expression analysis was performed on regulatory genes associated with cell proliferation, inflammation, and programmed cell death. The pi3k/akt/mtor signaling cascade—known for regulating growth, metabolism, and survival—was notably downregulated by both compounds individually. However, the combination, particularly in the 1:1 ratio, led to a more substantial decrease in the expression of pi3k, akt, and mtor genes. Since overactivation of this pathway is a hallmark in numerous cancers, its strong inhibition through combination therapy presents a promising therapeutic angle.

Alongside this, inflammatory markers such as nf-κb and il-6 showed significant reductions in expression levels upon treatment with the compound mixtures. Nf-κb, a central regulator of inflammation and cancer cell survival, is often aberrantly activated in tumour cells, promoting proliferation and resistance to cell death. The concurrent suppression of nf-κb and il-6 by thymoquinone and theaflavin indicates a robust anti-inflammatory effect that could help dismantle the tumour-supportive inflammatory microenvironment (18).

Apoptosis-related genes were also assessed to explore the effect of treatments on programmed cell death pathways. Expression levels of bcl-2, an anti-apoptotic gene, were significantly lowered, while the pro-apoptotic gene bax was upregulated. The combination treatments shifted the balance further toward apoptosis than either compound alone, reinforcing the idea that co-treatment promotes cell death through intrinsic apoptotic pathways. Regulating the bcl-2/bax ratio is essential in tipping cells towards apoptosis—an outcome that is highly desirable in cancer therapy.(18)

Among the tested ratios of thymoquinone and theaflavin, the 1:1 combination consistently demonstrated superior efficacy across all experimental parameters. This suggests that a balanced proportion allows optimal interaction at the molecular level, potentially enhancing uptake, bioavailability, and multi-target pathway modulation. Although 2:1 and 1:2 ratios also yielded beneficial effects, they were marginally less potent, reinforcing the importance of dosing balance in maximizing therapeutic synergy.

These findings align with a growing body of evidence advocating for phytochemical combinations over monotherapies. Such regimens can provide therapeutic benefits at lower concentrations of each agent, potentially minimizing side effects and toxicity. Moreover, multi-target approaches can help circumvent cellular redundancy and resistance mechanisms often observed with single-compound treatments.

Despite these encouraging results, certain limitations must be noted. While the kb model offers preliminary insight, comprehensive studies involving cancer cell lines, animal models, and detailed mechanistic investigations are essential to validate these findings. Moreover, pharmacokinetic assessments are necessary to understand how thymoquinone and theaflavin interact metabolically in vivo, as their bioavailability and systemic behaviour will significantly influence their efficacy.

In conclusion, this study highlights that the combination of thymoquinone and theaflavin exhibits improved anticancer, anti-inflammatory, and antioxidant properties when compared to their individual effects on kb cells. The coordinated suppression of the pi3k/akt/mtor and nf-κb/il-6 pathways, along with enhanced induction of apoptosis via bcl-2 and bax regulation, underscores the therapeutic potential of this natural compound duo. These promising results warrant further preclinical and clinical exploration, with the potential to incorporate thymoquinone and theaflavin as adjuncts or alternatives in the management of cancer.

5 CONCLUSION

This study highlights the significant therapeutic potential of Thymoquinone and theaflavin, both individually and in combination, in modulating key biological processes in KB cells. The combination treatments, particularly at a 1:1 ratio, exhibited enhanced anticancer, anti-inflammatory, and antioxidant activities compared to single agent therapies. Mechanistically, the synergistic inhibition of the P13K/Akt/mTOR and NF-kB/IL-6 pathways, along with the induction of apoptosis through downregulation of Bcl-2 and upregulation of BAX, underscores their ability to target multiple signalling pathways simultaneously. These results support the concept that phytochemical combinations can offer superior biological efficacy with potentially lower toxicity. Although further studies in cancer-specific models and in-vivo systems are needed, the findings provide a strong foundation for considering Thymoquinone and theaflavin as promising candidates for future anticancer strategies.

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