International Journal of Medical Toxicology & Forensic Medicine
2026, Volume 16, E52823

Research Paper

Concentration-Dependent Modulation of CASP9 Expression by Metformin and Glycyrrhiza glabra in LNCaP Prostate Cancer Cells

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Citation Al-Azabi HM, Al-Oubaidy SA, Bash AA. Concentration-Dependent Modulation of CASP9 Expression by Metformin and Glycyrrhiza glabra in LNCaP Prostate Cancer Cells. International Journal of Medical Toxicology and Forensic Medicine. 2026; 16:E52823.

https://doi.org/10.22037/ijmtfm.v16.52823

Article info:
Received: 17 July, 2026
Accepted: 12 August, 2026
Published: 01 November, 2026

Keywords:
Glycyrrhiza, Metformin, Prostatic neoplasms, Caspase 9, Apoptosis

ABSTRACT

Background: Resistance to mitochondrial apoptosis contributes to prostate cancer progression and therapy failure. Metformin and *Glycyrrhiza glabra* exhibit anticancer activity; however, their individual and combined effects on caspase-9 (CASP9) transcription remain unclear. To evaluate the concentration-dependent effects of metformin and *G. glabra* extract, alone and in combination, on CASP9 expression in LNCaP human prostate cancer cells.

Methods: LNCaP cells were treated for 24 h with either *G. glabra* extract (62.5 or 2000 µg/mL), metformin (125 or 4000 µg/mL), or a combination of 4000 µg/mL metformin with either concentration of the extract. Each experimental condition was performed as two independent biological replicates (n=2), and each biological replicate was assayed in technical duplicate. CASP9 mRNA was quantified by reverse transcription quantitative PCR, normalized to GAPDH, and analyzed using the $2^{-}\Delta\Delta Ct$ method. Group differences were assessed using one-way ANOVA, followed by Tukey's post hoc test.

Results: High-dose metformin increased CASP9 expression 8.09-fold compared to the control ($p=0.0032$), whereas high-dose *G. glabra* produced a 2.15-fold increase ($p=0.0052$); neither low-dose treatment had a significant effect. The addition of low-dose *G. glabra* to high-dose metformin resulted in a 3.66-fold expression, which was significantly lower than that observed with metformin monotherapy ($p<0.0001$). The high-dose combination reduced the expression to 0.39-fold of the control ($p<0.0001$).

Conclusion: Co-treatment resulted in concentration-dependent interference rather than enhancement, highlighting the importance of optimizing the dose ratio. Functional apoptosis assays and clinically relevant exposures are required because CASP9 mRNA modulation alone does not induce apoptotic activity.

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Introduction

Despite advances in early detection, localized therapy, and improved survival, prostate cancer (PC) remains the most commonly diagnosed solid malignancy among men worldwide and the second leading cause of cancer-related mortality globally [1]. Androgen deprivation therapy continues to be the mainstay of treatment for advanced disease, but in many cases, it ultimately leads to the development of castration-resistant prostate cancer (CRPC) and carries a critical need for novel therapeutic modalities that target different cell survival pathways. Dysregulation of the apoptosis machinery, particularly the intrinsic mitochondrial pathway, is a hallmark of PC progression and therapy resistance [2].

Caspase-9 (CASP9) is an upstream death caspase that acts in the intrinsic apoptotic cascade. In the apoptosome, caspase-9 autoactivates upon release of cytochrome c from compromised mitochondria, which in turn cleaves and activates effector caspases-3 and -7 [3]. Caspase-9 is downregulated or functionally inhibited in several malignancies, including PC, promoting cell survival and chemoresistance [4]. Thus, a rational strategy to overcome apoptosis resistance in PC therapeutics is through the pharmacological restoration or augmentation of caspase-9 expression.

Metformin is one of the most widely used first-line pharmacotherapeutics for type 2 diabetes mellitus (T2D) and has received much interest for its anti-neoplastic properties. Multivariate analysis from these studies revealed that metformin was associated with a reduced incidence of PC and improved outcomes compared with other hypoglycemic agents [5]. Metformin has multiple mechanisms of action that are implicated in its anti-cancer functions, including activation of AMP-activated protein kinase (AMPK), inhibition of mTOR signaling, disruption of mitochondrial complex I function, and modulation of cell cycle regulators [6]. Notably, metformin has been reported to alter apoptosis induction in PC cell lines by activating the intrinsic mitochondrial pathway; however, its direct effects on caspase-9 transcriptional regulation are not fully characterized [7].

Botanical compounds are emerging as useful adjuncts or alternatives to standard chemotherapy. *Glycyrrhiza glabra* (licorice root) is a perennial herb native to the Mediterranean and Western Asian regions. The bioactive triterpenoid saponins responsible for the pharmacological effects of licorice root include glycyrrhizin (glycyrrhizic acid), a highly soluble glycoside, and glycyrrhetic acid, as well as flavonoids. These compounds exhibit a wide range of pharmacological effects, including anti-inflammatory, antiviral, and anticancer activities [8]. Glycyrrhizin induced cell cycle arrest and apoptosis in PC models associated with the generation of reactive oxygen species (ROS), mitochondrial membrane potential collapse, and cytochrome c However, the regulation of apoptotic initiators such as caspase-9 by *Glycyrrhiza* constituents remains elusive [9].

The integration of metformin and phytotherapeutic agents offers opportunities but also challenges. Both agents promote apoptotic signaling, but their joint action may induce additive effects, pharmacological antagonism, or synergistic enhancement [10]. By comprehending these interactions, rational combination protocols can be designed to optimize therapeutic benefits.

We hypothesized that metformin and *Glycyrrhiza glabra* would both increase caspase-9 gene expression in LNCaP PC cells, resulting in subsequent apoptotic competence. Finally, we postulated that the use of both agents together would result in dose-dependent potentiation or suppression of this effect, where low-dose *Glycyrrhiza* extract enhanced metformin-mediated caspase-9 expression and high-dose *Glycyrrhiza* extract diminished this response via compensatory survival signaling or antagonistic transcriptional activity.

Materials and Methods

Cell Culture and Treatment Protocol

LNCaP cells, a human prostate adenocarcinoma cell line, were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated in six-well plates at a density of 1×10^5 cells/well and allowed to adhere for 24 h before treatment.

The experimental groups were as follows: (1) untreated control; (2) *G. glabra* extract, 62.5 µg/mL; (3) *G. glabra* extract, 2000 µg/mL; (4) metformin, 125 µg/mL; (5) metformin, 4000 µg/mL; (6) metformin, 4000 µg/mL + *G. glabra* extract, 62.5 µg/mL; and (7) metformin, 4000 µg/mL + *G. glabra* extract, 2000 µg/mL. Treatments were applied for 24 h. Before seeding, the density of the cell suspension was adjusted so that an equal number of cells was plated in every well, according to previously established counting conditions.

Definition of replicates

In the present study, a *biological replicate* denotes an independent culture experiment performed on a separate passage of LNCaP cells, seeded, treated, harvested, and processed for RNA extraction and reverse transcription on a separate occasion. A *technical replicate* denotes repeated amplification of the same complementary DNA (cDNA) preparation within the same quantitative PCR run. Two independent biological replicates were generated for each of the seven experimental conditions (n = 2 biological replicates per group; 14 independent cultures in total), and each biological replicate was amplified in technical duplicate. All summary statistics, error bars, and inferential tests reported in this manuscript were computed from the biological replicates; technical duplicates were averaged before analysis and were used solely to monitor pipetting and amplification precision.

RNA Extraction and Purification

Total RNA was extracted from the cells using the FavorPrep™ Blood/Cultured Cell Total RNA Kit (Favorgen, Korea) as recommended by the manufacturer, with modifications for adherent cell lines. In brief, cells were harvested and centrifuged at 18,000 × g for 2 min at 4 °C. Cell pellets were resuspended in a lysozyme reaction solution (100 µL) and incubated for 10 min at 37 °C with the addition of FARB Buffer (350 µL) and β-mercaptoethanol (3.5 µL). Samples were vortexed with 250 mg acid-washed glass beads (500–700 µm) for 5 min to achieve mechanical disruption. Supernatants were placed on FARB mini-columns after centrifugation for 2 min at 18,000 × g. Genomic DNA was removed by on-column DNase I treatment (60 µL RNase-free DNase I solution, 0.25 U/µL) for 15 min at room temperature. The columns were then washed sequentially with Wash Buffer 1 and Wash Buffer 2, ending each wash with a 3 min centrifugation to dry the column membrane. RNA was eluted in 30–50 µL RNase-free water and stored at −70 °C until processing. RNA quantification and purity were determined using spectrophotometry (A260/A280 ratio > 1.8).

cDNA Synthesis

Complementary DNA was synthesized using the AddScript cDNA Synthesis Kit (Addbio, Korea). Each 20 µL reaction contained 10 µL of 2× reaction buffer, 2 µL of 10 mM dNTP mixture, 2 µL of the respective primer (oligo-dT_{20} or random hexamer), 1 µL of AddScript reverse transcriptase enzyme solution, 1 µL of nuclease-free water, and 4 µL of RNA template (equivalent to 1 µg of total RNA), in accordance with the low-quantity, high-efficiency protocol supplied by the manufacturer. The thermal cycling protocol comprised 10 min of primer annealing at 25°C, followed by reverse transcription for 60 min at 50°C.

Quantitative Real-Time PCR (qPCR)

Gene expression was analyzed using the GoTaq® qPCR System (Promega, USA) and the Mx3005P Stratagene Real-Time PCR system (Agilent Technologies, USA). For the amplification of caspase-9 (CASP9), the forward and reverse primer sequences were 5′-GTTGAGGACCTTCGACCAGCT-3′ and 5′-CAACGTACCAGGAGCCACTCTT-3′, respectively, generating a 129 bp amplicon (OriGene Technologies). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene (forward 5′-GGAGTCAACGGATTTGGT-3′ and reverse 5′-GTGATGGGATTTCCATTGAT-3′; product size, 206 bp).

Each 20 µL qPCR reaction contained 10 µL GoTaq® qPCR Master Mix (2×), 2 µL forward primer (10 µM), 2 µL reverse primer (10 µM), 4 µL nuclease-free water, and 2 µL cDNA template. Thermal cycling conditions comprised hot-start activation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s and combined annealing/extension with fluorescence data acquisition at 60°C for 30 s. Amplification of a single product was confirmed by melting-curve (dissociation) analysis performed immediately after cycling, over a continuous ramp from 60°C to 95°C. All samples were run in technical duplicates, and no-template controls were included for each primer pair.

Data Analysis

Quantitative analysis was conducted using the comparative cycle threshold (2^(−ΔΔCt)) method [11]. Ct values were obtained automatically using the Mx3005P software. ΔCt was obtained by subtracting the Ct value of the reference gene (GAPDH) from that of the target gene (CASP9). ΔΔCt was obtained by subtracting the mean ΔCt of the untreated control from the ΔCt of each treated sample. Fold-change values are expressed as $2^{-\Delta\Delta Ct}$.

The untreated control is normalized to a fold change of exactly 1.00 by definition of the 2^(−ΔΔCt) method; consequently, its dispersion is zero, and no error bar is displayed for the control group in Figures 1 and 2. A single, common untreated control group was used as the reference for both monotherapy and combination comparisons; the two separate control bars presented in the original version of Figure 1 have therefore been consolidated into one control bar.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, USA). Descriptive statistics were used to assess data distribution. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare group means, and statistical significance was set at $p < 0.05$. All results are expressed as the mean fold change ± standard deviation (SD) of two independent biological replicates (n = 2 per group). The standard deviation is the only measure of dispersion used anywhere in this manuscript, in the figures, and in Table 1; the standard error of the mean (SEM) is not reported, and error bars in Figures 1 and 2 therefore represent SD. Because each group comprises only two independent biological replicates, the SD is presented as a descriptive indicator of the agreement between the two replicate cultures rather than as a robust estimate of population variance, and the corresponding p-values and coefficients of determination should be interpreted as exploratory. To maximize transparency, the individual fold-change value for each biological replicate is plotted as an open circle superimposed on the corresponding bar in Figures 1 and 2 and is also tabulated in Table 1. Degrees of freedom, F-statistics, R^2 values, and Tukey-adjusted p-values are reported in full for each ANOVA model.

Results

Baseline Gene Expression and Technical Validation

GAPDH was detected in all treatment groups (Ct values 18.29 to 33.69). The mean Ct value for caspase-9 in the untreated control samples indicated a detectable basal expression level (28.58±0.10). Reaction specificity was confirmed by single-phase exponential amplification with consistent threshold crossings across replicates in the amplification plots and by single-peak melting curves for both CASP9 and GAPDH, whereas no-template controls yielded no amplification (Table 1).

Effect of Glycyrrhiza glabra on Caspase-9 Expression

Glycyrrhiza glabra treatment modified caspase-9 expression in a concentration-dependent manner ($F(2,3) = 56.90$, $p = 0.0041$, $R^2 = 0.9743$). Low-dose *Glycyrrhiza* (62.5 µg/mL) produced a mean fold change of 1.04 ± 0.19 (individual biological replicates: 1.17 and 0.91) which did not differ significantly from the untreated control (adjusted $p = 0.9442$). In contrast, high-dose *Glycyrrhiza* (2000 µg/mL) increased caspase-9 expression to 2.15 ± 0.09-fold relative to the control (individual biological replicates: 2.22 and 2.09) ($p = 0.0052$). The difference between the low- and high-dose *Glycyrrhiza* treatments was also significant ($p = 0.0058$), consistent with a concentration threshold for transcriptional activation (Figure 1, Table 1).

Effect of Metformin on Caspase-9 Expression

Metformin modulated caspase-9 expression with a significant concentration-dependent effect ($F(2,3) = 79.37$, $p = 0.0025$, $R^2 = 0.9815$). Low-dose metformin (125 µg/mL) produced a marginal increase in CASP9 mRNA of 1.23 ± 0.04-fold (individual biological replicates: 1.26 and 1.20) that did not reach statistical significance relative to the control ($p = 0.9344$). In contrast, high-dose metformin (4000 µg/mL) induced a substantially stronger upregulation of caspase-9 (8.09±0.78-fold versus control; individual biological

Table 1. Individual biological replicate values of CASP9 mRNA expression in LNCaP cells.

Treatment group	BR1	BR2	Mean	SD	CV (%)	Adjusted p vs. control
Untreated control	1.00	1.00	1.00	0.00	0.0	Reference
G. glabra 62.5 µg/mL	1.17	0.91	1.04	0.19	18.3	0.9442 (ns)
G. glabra 2000 µg/mL	2.22	2.09	2.15	0.09	4.2	0.0052
Metformin 125 µg/mL	1.26	1.20	1.23	0.04	3.3	0.9344 (ns)
Metformin 4000 µg/mL	8.65	7.54	8.09	0.78	9.6	0.0032
Metformin 4000 + *G. glabra* 62.5 µg/mL	3.89	3.44	3.66	0.32	8.7	0.0002
Metformin 4000 + *G. glabra* 2000 µg/mL	0.40	0.37	0.39	0.02	5.2	< 0.0001

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Relative expression is given as 2^(−ΔΔCt) fold change after 24 h of treatment, normalized to GAPDH and to the untreated control. Each biological replicate (BR1, BR2) represents an independent culture experiment processed separately from RNA extraction onwards, and each was amplified in technical duplicate. Values are mean ± standard deviation (SD) of n = 2 biological replicates; the standard error of the mean is not reported. CV, coefficient of variation. Adjusted p-values are derived from one-way ANOVA with Tukey's multiple comparison test versus the untreated control; ns, not significant.

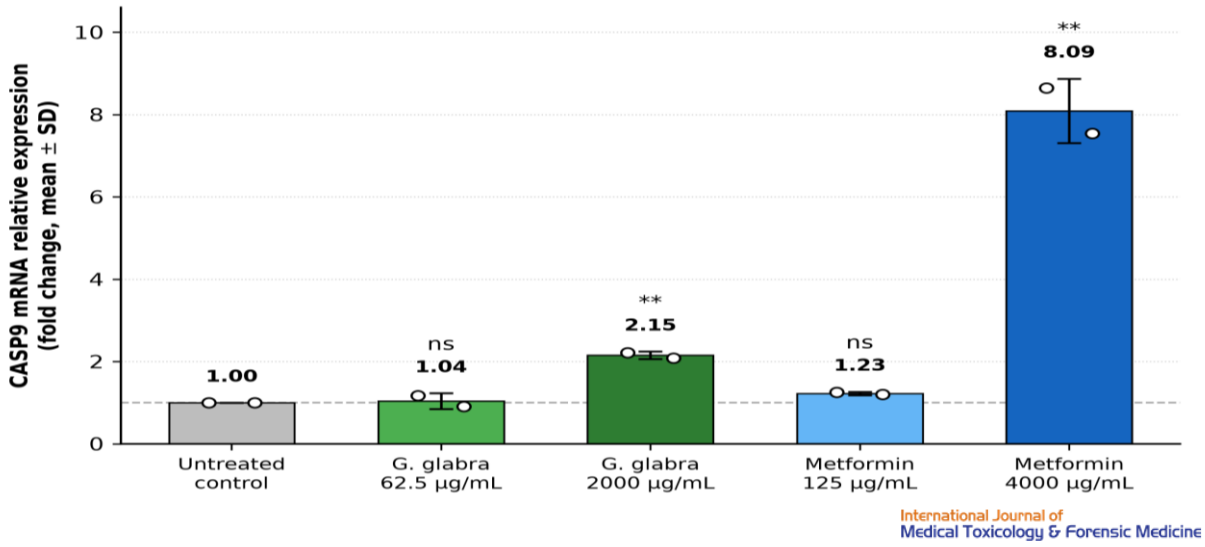


Figure 1. Effect of *Glycyrrhiza glabra* extract and metformin monotherapy on CASP9 mRNA expression in LNCaP prostate cancer cells.

Cells were treated for 24 h. Bars represent the mean relative expression (2^(−ΔΔCt) fold change, normalized to GAPDH and to the untreated control) of n = 2 independent biological replicates per treatment group; each biological replicate was an independent culture experiment amplified in technical duplicate, and technical duplicates were averaged before analysis. Error bars represent the standard deviation (SD) of the two biological replicates, not the standard error of the mean. The untreated control is fixed at 1.00 by definition of the 2^(−ΔΔCt) method and therefore has no dispersion or error bar; the dashed horizontal line marks this control level. Groups were compared using one-way ANOVA with Tukey's multiple comparison test: **p < 0.01 versus untreated control; ns, not significant (G. glabra 62.5 µg/mL, adjusted p = 0.9442; G. glabra 2000 µg/mL, adjusted p=0.0052; metformin 125 µg/mL, adjusted p = 0.9344; metformin 4000 µg/mL, adjusted p = 0.0032). Because n = 2, the SD is descriptive of the agreement between the two independent cultures and the associated inference is exploratory.

replicates: 8.65 and 7.54; p = 0.0032). The difference between low- and high-dose metformin was statistically significant (p=0.0036), indicating a steep concentration–response relationship (Figure 1, Table 1).

Combination Treatment

When metformin 4000 µg/mL was combined with *Glycyrrhiza glabra*, the direction of the response depended on the concentration of the botanical extract ($F(4,5) = 197.2$, $p < 0.0001$, $R^2 = 0.9937$). Caspase-9 expression reached 3.66 ± 0.32-fold (individual biological replicates: 3.89 and 3.44) in the combination of metformin 4000 µg/mL with low-dose *Glycyrrhiza* (62.5 µg/mL). Although this value was significantly higher than that of the untreated control (p = 0.0002), it was significantly lower than that obtained with metformin monotherapy (adjusted $p < 0.0001$ versus metformin 4000 µg/mL alone).

In contrast, the combination of metformin (4000 µg/mL) with high-dose *Glycyrrhiza* (2000 µg/mL) reduced caspase-9 expression to 0.39 ± 0.02-fold of the untreated control (individual biological replicates: 0.40 and 0.37), representing significant downregulation below the basal level ($p < 0.0001$). The difference between the two combination regimens was also highly significant (adjusted $p < 0.0001$) (Figure 2, Table 1).

Discussion

The present study provides preliminary evidence that CASP9 transcription in LNCaP PC cells is influenced by both the treatment concentration and co-exposure to metformin and *Glycyrrhiza glabra*. High-dose metformin (4000 µg/mL) produced the largest response, with an 8.09-fold increase in CASP9 expression relative to the untreated control, whereas high-dose *G. glabra* extract (2000 µg/mL) generated a smaller 2.15-fold increase. Neither low-dose metformin nor low-dose *G. glabra* significantly altered the expression. Importantly, co-treatment did not reproduce the response observed with metformin alone: the combination containing low-dose *G. glabra* remained above control (3.66-fold) but was markedly lower than high-dose metformin monotherapy, whereas the high-dose combination reduced CASP9 expression below the control value (0.39-fold). Collectively, these findings indicate a nonlinear, concentration-dependent transcriptional response. However, they did not establish that apoptosis occurred or that the interaction was pharmacodynamically antagonistic because only one apoptosis-related transcript was measured. As now displayed explicitly in Figures 1 and 2 and tabulated in Table 1, the two independent biological replicates within each group were concordant in both magnitude and direction (coefficient of variation 3.3–18.3%), which supports the internal consistency of the measurements while not compensating for the small number of independent experiments.

The increase in CASP9 expression following high-dose metformin treatment is mechanistically plausible

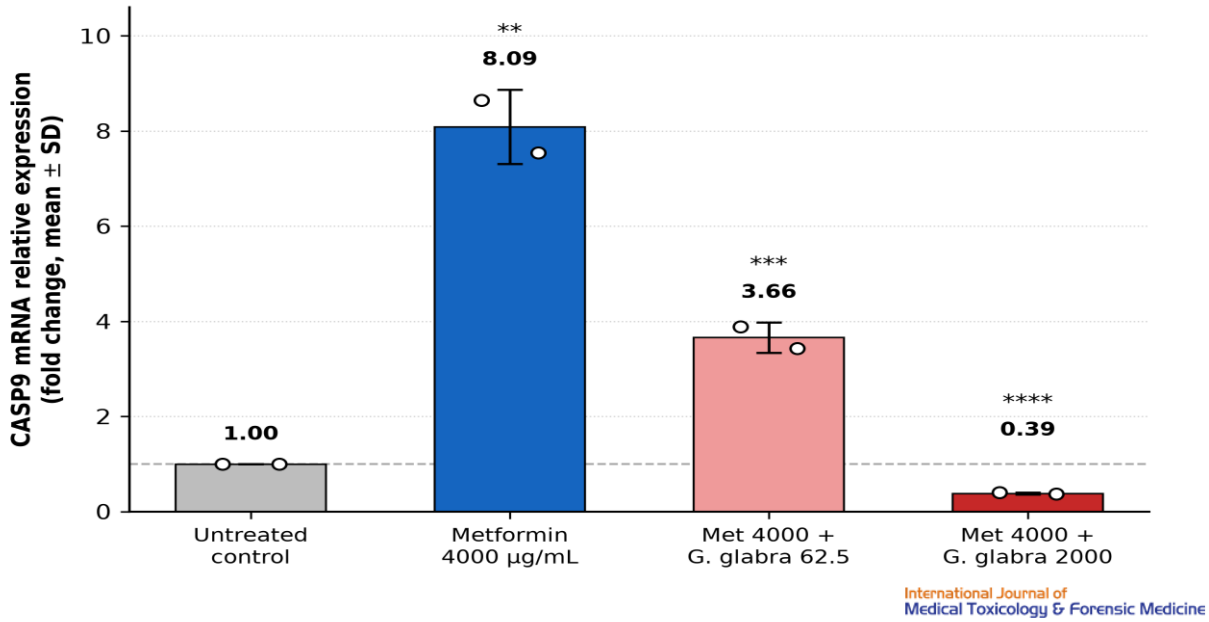


Figure 2. Effect of metformin combined with *Glycyrrhiza glabra* extract on CASP9 mRNA expression in LNCaP prostate cancer cells.

Cells were treated for 24 h with metformin (4000 µg/mL) alone or in combination with low-dose (62.5 µg/mL) or high-dose (2000 µg/mL) *G. glabra* extract. Bars represent the mean relative expression (2^(−ΔΔCt) fold change, normalized to GAPDH and to the untreated control) of n = 2 independent biological replicates per treatment group, each amplified in technical duplicate. Error bars represent the standard deviation (SD) of the two biological replicates; the standard error of the mean is not shown. The untreated control and the metformin 4000 µg/mL monotherapy group are the same biological replicates presented in Figure 1 and are reproduced here for direct comparison; they are not additional experiments. The untreated control is fixed at 1.00 by definition of the 2^(−ΔΔCt) method and has no dispersion; the dashed horizontal line marks this control level. Groups were compared using one-way ANOVA with Tukey's multiple comparison test: **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus untreated control. The difference between the two combination regimens was also significant (adjusted $p < 0.0001$). Because n = 2, the SD is descriptive and the associated inference is exploratory.

in the context of metformin-induced mitochondrial stress. Metformin can inhibit mitochondrial complex I, reduce ATP production, alter the AMP:ATP ratio, and activate AMPK, with downstream effects on mTOR signalling and cellular biosynthesis [11, 12]. Recent discussions on metformin in PC similarly emphasize that its effects on mitochondrial respiration, reactive oxygen species, and apoptotic signalling are context-dependent rather than uniform [13]. Zhao et al. reported a metformin half-maximal inhibitory concentration of approximately 3.61 mM after 48 h in LNCaP cells and showed that metformin combined with paclitaxel increased apoptosis, caspase-3/7 activity, and oxidative stress while reducing mitochondrial membrane potential and intracellular ATP [14]. These observations support the capacity of metformin to engage mitochondrial death pathways; however, they differ from the present study in terms of exposure time, concentration, co-treatment, and measured endpoints. Furthermore, metformin-associated cell death in androgen-sensitive PC cells may also involve PARP-1- and apoptosis-inducing factor-dependent pathways, with limited caspase-3 cleavage under certain conditions [15]. Therefore, the current CASP9 response should be interpreted as evidence of transcriptional modulation within an apoptosis-related pathway rather than as confirmation of apoptotic execution.

The concentration used for the strongest metformin response also limits the translational interpretation. Depending on whether the reported mass concentration refers to metformin hydrochloride or the free base, 4000 µg/mL is approximately 24–31 mM. This is substantially higher than the systemic exposure in humans. Phillips et al. found that metformin concentrations achieved in plasma and resected tissues from patients receiving approved diabetes doses were lower than the 5–50 mM concentrations frequently used in cell culture studies, with plasma values ranging from below detection to approximately 47.84 µM [16]. Consequently, the eight-fold increase observed here may reflect a high-intensity in vitro metabolic stress response that cannot be assumed to occur at clinically achievable exposure. Future experiments should specify the metformin salt form and test a concentration range that includes micromolar and low millimolar exposures.

The response to *G. glabra* should also be considered in relation to the chemical complexity of licorice preparations. The 2.15-fold increase produced by the high-dose extract is compatible with previous evidence that licorice constituents can affect prostate cancer cell survival, but the published mechanisms are

heterogeneous. Thirugnanam et al. found that purified glycyrrhizin reduced the viability of LNCaP and DU-145 cells and increased Annexin-V positivity and DNA damage; nevertheless, caspase-3 and caspase-8 activities were not detected, leading the authors to propose a caspase-independent mechanism [17]. In contrast, Yo et al. reported that whole licorice preparations and licochalcone A induced both apoptosis and autophagy in LNCaP cells, accompanied by reduced Bcl-2 expression and inhibition of mTOR signalling [18]. Hawthorne and Gallagher also showed that glycyrrhetinic acid reduced proliferation and prostate-specific antigen secretion in androgen-dependent LNCaP cells, while having little effect on androgen-independent PC-3 and DU145 cells [19]. These differences demonstrate that biological activity depends on the constituent examined, extraction method, concentration, exposure period, and cellular context. As the extract used in the present study was not chemically standardized, the observed CASP9 response cannot be attributed specifically to glycyrrhizin, glycyrrhetinic acid, licochalcone A, or any other individual component.

The combination results were the most distinctive finding. The addition of 62.5 µg/mL *G. glabra* to high-dose metformin attenuated the metformin-associated increase in CASP9 expression, although the expression remained above that of the untreated control. However, increasing the concentration to 2000 µg/mL reversed the direction of the transcriptional response. The prior observation that 18β-glycyrrhetinic acid transcriptionally suppresses the androgen receptor in LNCaP cells offers one candidate explanation for a divergent, concentration-dependent transcriptional outcome in this androgen-sensitive model [9]. This pattern may reflect concentration-dependent competition between stress, survival, redox, and androgen-receptor-related pathways, or may arise from changes in cell number, RNA integrity, reference gene behavior, or the relative abundance of active and inactive botanical constituents. These explanations remain hypotheses because the relevant pathways and extract compositions have not been measured. Therefore, the present data should be described as attenuation and reversal of the metformin-associated CASP9 transcriptional response, rather than definitive antagonism. The formal classification of a combination as synergistic, additive, or antagonistic requires an adequately sampled dose–response matrix and a prespecified reference model. The Chou–Talalay method, for example, defines synergy, additivity, and antagonism through a combination index derived from dose–effect relationships [20], whereas Bliss independence, Loewe additivity, and highest single-agent models use different assumptions and may yield different conclusions [21]. The application of at least one appropriate model is necessary before making a pharmacodynamic interaction claim. Because dosage ratio determined not only the magnitude but also the direction of the transcriptional response, dose-ratio optimization should be regarded as a prerequisite for any future polypharmacy protocol combining a biguanide with a licorice preparation.

Several limitations require particular emphasis. First, CASP9 mRNA abundance is not equivalent to caspase-9 activation. Caspase-9 is regulated through apoptosome assembly and proteolytic activation, and apoptotic execution requires downstream biochemical and cellular events [3]. Therefore, the findings require confirmation by measuring cleaved caspase-9 and cleaved caspase-3/7 or caspase activity, together with Annexin-V/propidium iodide staining, cytochrome-c release, mitochondrial membrane potential, and cell viability. Second, the reported GAPDH Ct range of 18.29–33.69 spans approximately 15 cycles and does not, without formal validation, support the assertion that the reference gene was stable across treatments. In accordance with the MIQE guidelines, subsequent studies should validate multiple reference genes under experimental conditions, report amplification efficiency and assay specificity, and provide complete thermal cycling and quality control information [22]. Additional limitations include the use of a single androgen-sensitive cell line, one exposure time, an uncharacterized botanical extract, a restricted set of concentration pairs, and the absence of normal prostate epithelial cells as a selectivity comparator.

Therefore, future studies should repeat the experiment using a larger number of independent biological replicates, a time course, and a full factorial concentration matrix. Chemical profiling of the *G. glabra* extract using HPLC or LC–MS, with quantification of the major constituents, would improve reproducibility and permit constituent-specific testing. Mechanistic experiments should integrate transcript, protein, and functional end points and examine AMPK–mTOR, mitochondrial stress, oxidative balance, Bcl-2-family proteins, androgen-receptor signalling, and autophagy. Testing in additional androgen-sensitive and androgen-independent prostate cancer models, alongside non-malignant prostate epithelial cells, would determine whether the response is cell-line specific and whether it offers any therapeutic selectivity. The combination should be evaluated in animal models only after reproducible interaction modelling and functional confirmation.

Conclusion

In summary, high-dose metformin and high-dose *G. glabra* individually increased CASP9 mRNA levels in

LNCaP cells, whereas co-treatment attenuated or reversed the metformin-associated transcriptional response in a concentration-dependent manner. The results identified a potentially important interaction at the level of CASP9 gene regulation; however, they remain preliminary because apoptosis, pharmacodynamic antagonism, and clinical relevance were not directly established, and because each condition was represented by only two independent biological replicates. A chemically standardized extract, clinically informative metformin exposures, rigorous qPCR validation, an increased number of independent biological replicates, and multiple functional apoptosis assays will be required to determine the biological and therapeutic significance of this observation.

Ethical Approval

The Central Biological Ethics Committee of the College of Medicine, Babylon, approved the experimental procedures (4-52 19/4/2026).

Acknowledgment

None.

Funding

No external funding was received for this research.

Conflicts of Interest

The authors report there are no competing interests to declare.

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