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A NATURAL COUMESTAN WEDELOLACTONE TARGETS INFLAMMATORY CYTOKINES TO INITIATE APOPTOSIS IN HUMAN CERVICAL CANCER CELLS

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ABSTRACT: Objective: Wedelolactone is a naturally (WDL) occurring coumestan of phytoestrogen category that possess anticancer and anti-inflammatory property. In this study, the anti-inflammatory effect of WDL on human cervical cancer cells (HeLa) and initiation of apoptosis mechanism were investigated. **Methods:** MTT assay and Trypan blue assay were demonstrated to detect the inhibitory effects of WDL on cell proliferation. The mRNA expression of IL-1β, TNFα, IL-6, TGFβ, and NFκB were detected by RT PCR. Western blotting was performed to detect the expression of apoptosis-associated proteins; cleaved caspase-3, cleaved PARP, Bcl-2, and Bax. **Results:** WDL significantly inhibited the growth and proliferation of HeLa cells in a concentration dependent manner with the IC_{50} of 10µM. mRNA expression of inflammatory cytokines and NFκB was significantly reduced in WDL treated groups. Moreover, WDL promoted the apoptosis of HeLa cells dose-dependently by down-regulating Bcl-2 and up-regulating cleaved caspase-3, cleaved PARP, and Bax. **Conclusion:** Collectively, WDL exerted anti-inflammatory effect through the suppression of NF-kB activation, IL-1β, TNFα, IL-6 and promoted apoptosis in HeLa cells. Thus, use of WDL, especially through its anti-inflammatory effect, may have future applications in treating human cancers by sensitizing cancer cells to conventional cancer therapies.

INTRODUCTION: Cervical cancers are the most common malignancies among the gynecological oncology. They pose an exclusive threat to treat and cause morbidity. Especially inflammation exerted by HPV (Human papillomavirus) leads to cervicitis that can progress into cervical cancer [1]. Unregulated chronic inflammation seems to play a compelling role that leads to the pathogenesis of various malignant tumors such as cervical, ovarian, hepatic and esophageal cancers [2]. Inflammation plays essentially an important role in governing cervix pathology and viral infection, such as HPV sensitivity establishing tumor environment [3].

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Biochemical, epidemiological, immunological and genetic studies have proved the link between inflammation and malignancy development. Infiltrated immune cells along with its cytokines, chemokines, and growth factors at inflammatory sites lead to cervical cancer. Dysregulated cytokine secretion increases excessive cell growth, transformation into malignancy and its survival [4]. Once the tumor initiation stage is established it creates excessive inflammation, which in turn tends to facilitate tumor progression [5].

NF-κB, the key switch mediates crosstalk between inflammation and cancer at various levels. Constitutive activation of NFκB piles up proinflammatory cytokines and establish pro-tumorigenic environment, enhancing the expression of antiapoptotic genes [6]. This contributes to the cell survival mechanism by combating the physiological stress that induced the inflammatory response.

NFκB persuades the cytokines that regulate the immune response such as TNFα, IL-1β, IL-6 and TGFβ [7]. These inflammatory signals, in turn, induce phosphorylation and proteosomal degradation of IKB by IKKB. This paves way for NFκB nuclear translocation where it activates the genes for transcription. These genes include some of the cytokines IL-1β, TNF and antiapoptotic genes such as Bcl [8]. This feed-forward activation amplifies the inflammatory response together with tumorigenic environment. At the tumor site unregulated inflammation intensifies the proliferation rate and angiogenesis and also increases the mortality rate [9]. Thus, the need for anti-inflammatory drug of natural origin with less toxic effect in the treatment of cervical cancer is of prime importance.

Wedelolactone a coumestan, derived from Ecliptaprostrata and Wedeliachinensis is a natural drug involved in anti-cancer activity. It has been shown to inhibit NFκB because of its potent anti-inflammatory properties. Many studies have described the *in-vitro* and *in-vivo* anticancer properties of Wedelolactone in prostate, breast, hepatocellular and pituitary cancers [10, 11, 12, 13]. WDL is a multi-targeted compound and possesses inhibitory effect on multiple kinases. A study by Benes *et al.,* [14] emphasized the proapoptotic role of WDL on breast cancer cells by inhibiting topoisomerase IIα activity. The most important documentation is that WDL is a potent anti-inflammatory drug [15]. NFκB is the major pathway involved in immune cell activation in response to inflammation mediators such as TNFα and interleukins during the tumor initiation stage. Suppression of this pathway by WDL underlies the main mechanism of its inhibitory effect on chronic inflammation thereby hindering the tumorigenic environment and enhancing the chemotherapeutic response of cancer cells.

Apoptosis a stringently regulated process is a programmed cell death that initiates cell death via activation of various molecules. The induction of apoptosis in tumor cells exerts a promising way of cancer therapy [16]. Apoptosis is mediated by caspases which fall under cysteine protease family. Cytochrome C release from mitochondria is promoted by pro-apoptotic proteins such as Bax, Bad, Bak, and inhibited by anti-apoptotic proteins such as Bcl, Bcl-xL [17]. Cytochrome C release activates Caspase 3, which further activates PARP that executes nuclear apoptosis [18]. According to Mlynska *et al.,* [19] inflammation at the tumor site results in excess inflammatory cytokine production that ends up in abundant NFκB activation thereby preventing apoptosis of tumor cells. Descending down the expression of inflammatory cytokines by anti-inflammatory drugs might direct the tumor cells towards apoptosis and might improve the conventional chemotherapy regimen.

To the best of our knowledge, this is the first time study about the anti-inflammatory role of WDL in cervical cancer cells. Thus, the objective of this work was to ascertain the effect WDL on inflammatory cytokines IL-1β, IL-6, TGFβ and TNF-α mRNA expression in human cervical cancer cell line HeLa and to correlate these results with the gene expression of NF-κB. We also evaluated the apoptosis-related gene expression and cytotoxic effect of WDL in HeLa cell lines *in-vitro*. The current work aimed to provide an initial insight into the molecular mechanisms by which WDL could confer anti-cancer activity in HeLa cells. We explored that anti-inflammatory effect of WDL could be the possible mechanism adapted by HeLa cells to undergo apoptotic cell death.

MATERIALS AND METHODS:

Cell Culture and Reagents: HeLa cells obtained from National Centre for Cell Science (Pune, India) were maintained and grown in a humidified incubator at 37 °C with 5% CO_2. Cells were grown as a monolayer in plastic tissue culture flasks in Dulbecco's Modified Eagles Medium (DMEM) (GIBCO, Grand Island, New York, USA). The media was supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, New York, USA) and antibiotics (Penicillin 50 IU/mL, Streptomycin 3.5 mg/mL and Gentamycin 2.5 mg/mL) (GIBCO, Grand Island, New York, USA). Wedelolactone (WDL) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents were dissolved in Dimethyl sulphoxide.

Cell Viability Assay:

MTT Assay: HeLa cells were seeded in 96-well plates at a density of 5×10^3 cells/well in 200 mL DMEM containing 10% FBS and incubated overnight. Non-adherent cells were removed by

gentle washing after 24 h. Then cells were replaced with serum-free medium with varying concentrations of wedelolactone (0–25 µM). A negative control containing serum-free medium with DMSO was also evaluated. After 72 h of treatment, the plates were incubated with 20 mL 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) solutions (5 mg/mL) for 3 h at 37 °C. The formazan was dissolved in 150 mL/well Dimethyl sulfoxide (DMSO) and the absorbance was detected at 590 nm using microplate reader (Bio-Rad, USA). Cell viability was expressed as a percentage of untreated cells, which served as the negative control group and was designated as 100% [20]. The results were expressed as a percentage of the negative control. The median inhibitory concentration (IC_{50}) (defined as the drug concentration at which cell growth was inhibited by 50%) was assessed from the dose-response curves.

Trypan Blue Assay: Cell viability was determined by the Trypan blue dye exclusion assay; this dye excluded living cells and only penetrated the cell membrane of dead cells. HeLa cells were treated with wedelolactone (1–25 µM) for 72 h. Attached cells were then trypsinized and combined with Trypan blue reagent, and viable cells were counted by placing the plate on a hemocytometer. The percentage of viable cells was calculated. Results are representative of experiments carried out in triplicate.

RNA Extraction and cDNA Synthesis: Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and quantified in BioPhotometer (Eppendorf, Milan, Italy). For the first-strand cDNA synthesis, 1 µg of total RNA per sample was used, and the reaction was performed with IScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Polymerase chain reaction (PCR) amplifications were performed as follows: 35 cycles for IL-1β (94 ºC for 30 s, 6 5ºC for 60 s, and 72 ºC for 60 s), 40 cycles of IL6 (94 ºC for 60 s, 60 ºC for 60 s, and 68 ºC for 120 s), NFkB 40 cycles of (94 ºC for 60 s, 60 ºC for 60 s, and 68 ºC for 120 s) 35 cycles for TGF-β (94 ºC for 30 s, 62 ºC for 30 s, and 72 ºC for 60 s), 40 cycles for TNF-α (94 ºC for 60 s, 60 ºC for 60 s, and 68 ºC for 120 s), and 30 cycles for β actin (94 ºC for 35 s, 64 ºC for 45 s, and 72 ºC for 1 min). Then PCR product 10 µl was electrophoresed in 2% agarose gel and analyzed in gel doc XRS plus (Bio-Rad, USA). The densitometric analyses were carried out with image lab software (Bio-Rad, USA). The expression of each target gene was normalized with internal control. All reactions were performed in duplicate.

TABLE 1: OLIGONUCLEOTIDES FOR REAL-TIME PCR

TGF-β	TAGACCCTTTCTCCTCCAGGAGACG	GCTGGGGGTCTCCCGGCAAAAGGT
TNF-α	TCAGATCATCTTCTCGAACC	CAGATAGATGGGCTCATACC
IL-1β	AATCTGTACCTGTCCTGCGTGTT	TGGGTAATTTTTGGGATCTACACTCT
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
NFkB	GAAGGAATCGTACCGGGAACA	CTCAGAGGGCCTTGTGACAGTAA
β -actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG

Western Blotting: The cells were seeded in 6 well plates at a density of 5×10^4 cells/well in 3 mL of DMEM containing 10% FBS overnight. Non-adherent cells were removed by gentle washing after 24 h. Cells were then treated with different doses of wedelolactone to analyze the expression levels of Bcl, Bax, Caspase 3 and PARP. After 48 h of treatment, cells were lysed by the addition of cold RIPA buffer [150 mMNaCl, 50 mMTris HCl, 0.1% SDS, 1% Triton X-100, 1 mM PMSF, 2 mMNaF, β-glycerophosphate and 2 mM EDTA, and fresh protease inhibitor cocktail (Cat No. P8340, Sigma Aldrich)] and cell lysate was centrifuged at 14,000 rpm at 4 °C for 20 min. The supernatant was harvested and analyzed for protein content using BCA method (Cat No. 23227, Pierce, USA). Protein was denatured in sample buffer, then separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (semidry trans-blot system). The blots were blocked overnight at room temperature with Tris-Buffered Saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 4% bovine serum albumin. The blots were washed three times with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) and incubated with specific primary antibodies (1:1000 dilutions) at 4 °C overnight. The blots were incubated for 1 h at room temperature with secondary antibodies (1:5000 dilutions), and detected by ECL detection reagent. To ensure that equal amounts of sample protein were applied for electrophoresis, β -actin was used as an internal

control. Denistometric analysis was done using ImageJ software.

Statistical Data: Data were represented as Mean ± Standard error of the mean (SEM). Statistical significance was analyzed using Anova and Dunnett's multiple comparison test between the groups, using Graphpad Prism 7.0 software package. $P<0.05$ was considered to be statistically significant; all the experiments were done in triplicates.

RESULTS:

Cell Viability Assays:

MTT: To establish the impact of WDL on the survival of HeLa cells, cells were treated with 0.01-25 µm WDL, and after that cell viability was examined by MTT assay. As shown in **Fig. 1A**, cell viability was reduced after treating with 8µM WDL compared to the control. After 48 h, WDL showed high inhibition of cell population growth in a dose-dependent manner with IC_{50} value of 10 µM.

Trypan Blue Assay: Cell viability was further confirmed by Trypan blue exclusion assay. As shown in **Fig. 1B** WDL markedly decreased the viability of HeLa cells in a dose-dependent manner. It clearly exhibited a 50% reduction in cell viability at IC_{50} value of 10 µM **Fig. 1B**.

mRNA Expression Profiling –RT PCR: Using RT-PCR inflammatory cytokine specific mRNAs were detected in HeLa cells. Cytokine specific bands of TNFα, IL-1β, TGF-β, and IL-6 and NFκB transcripts were detected in untreated and compared with WDL dose-dependent treated cells. Our data showed a significant increase in the level of NFκB and cytokine mRNA expression in control.

At the same time decreased expression of NF-κB, TNFα, IL-1β and IL-6 were observed in WDL treated groups **Fig. 2** very little difference in the TGF-β mRNA expression observed in WDL treated cells.

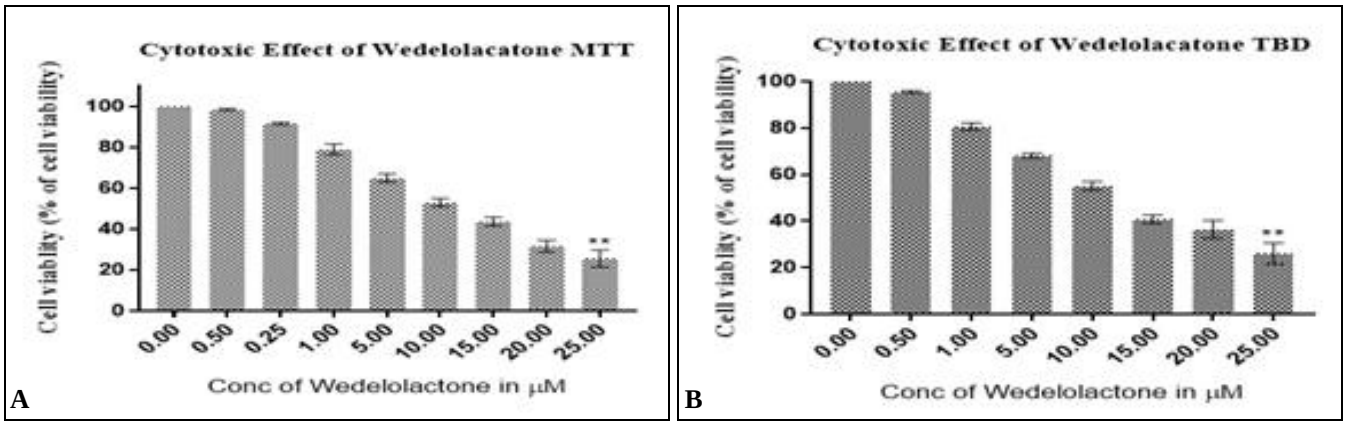

FIG: 1. EFFECT OF WEDELOLACTONE ON VIABILITY OF HeLa CELLS. CELLS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF WDL (0-25µM) FOR 48 h AND ANALYZED USING 1A) MTT ASSAY 1B) TRYPAN BLUE ASSAY. Data are mean ± SD of triplicate determinations.

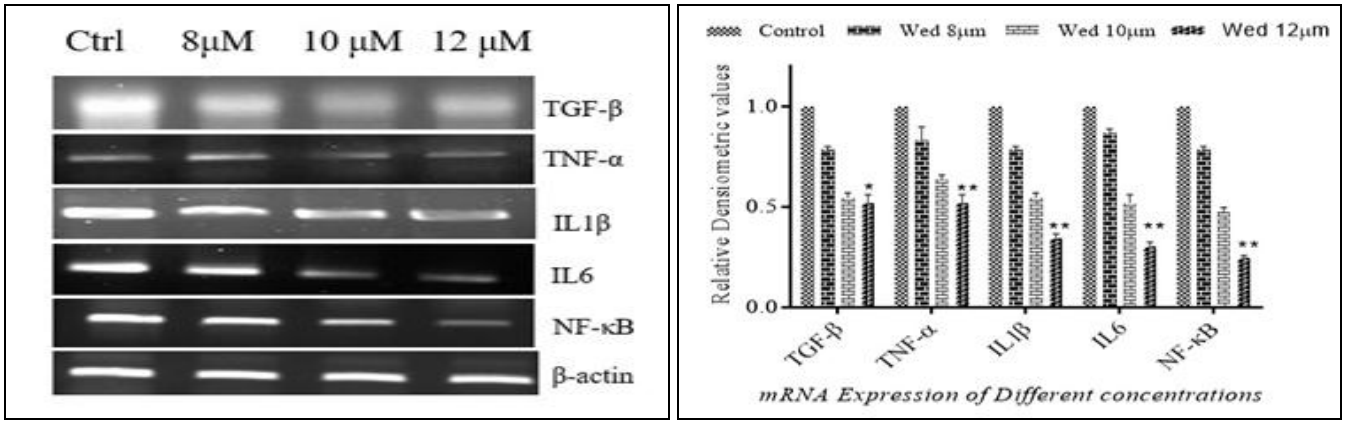

FIG. 2: EFFECT OF WDL ON INFLAMMATORY CYTOKINES, NF-κB mRNA EXPRESSION. Triplicates of each treatment group were used in each independent experiment. Results were expressed as the Mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, compared with control.

Immunoblot Analysis for Protein Expression: As shown in **Fig. 3**, the pro-apoptotic protein Bax was found to be elevated in WDL treated cells, whereas the expression of antiapoptotic protein Bcl-2 was repressed after 48 h of treatment with WDL. Our results also exhibited an increase in the protein expression of Cleaved Caspase 3 and Cleaved PARP in WDL treated cells compared to control.

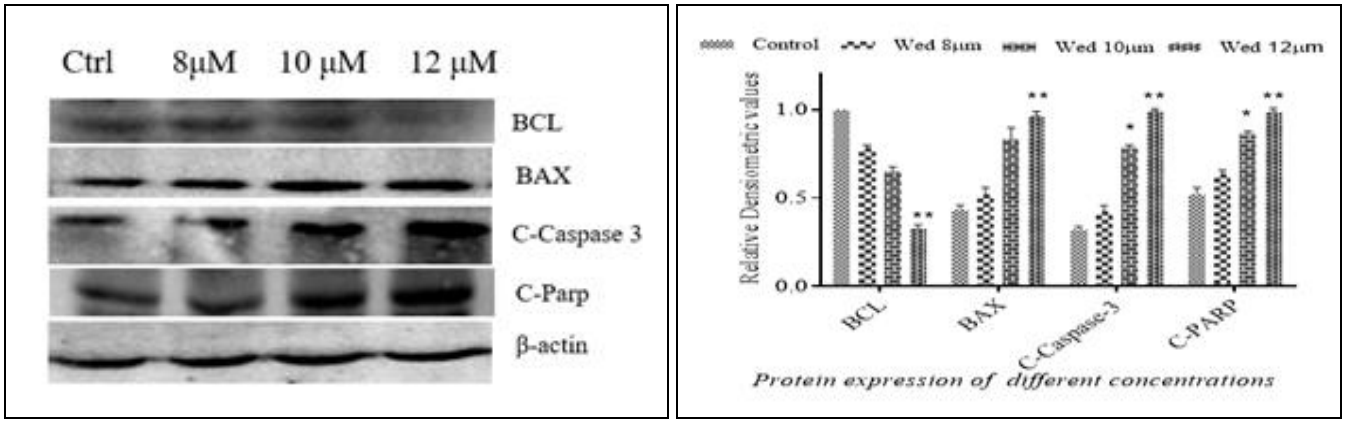

FIG. 3: HeLa CELLS WERE TREATED WITH DIFFERENT CONCENTRATIONS OF WDL (8µM, 10µM, 12µM) FOR 48 h. CELL LYSATES WERE ANALYZED BY WESTERN BLOT FOR APOPTOSIS-RELATED PROTEIN EXPRESSION: Bcl-2, BAX, CLEAVED CASPASE-3, AND CLEAVED PARP. Results were expressed as the Mean ± SD. $^{*}p<0.05$, $^{**}p<0.01$, compared with control.

DISCUSSION: Despite the existence of conventional chemotherapy for cervical cancer, poor clinical outcomes in cervical cancer patients were observed [21]. Inflammation at the tumor site repels towards apoptosis blocking the application of chemotherapeutic agents [22]. Increased production of proinflammatory cytokines promotes the condition from chronic inflammation to tumor. It has been documented that after the tumor development the continual production of inflammatory cytokines initiates signaling pathways such as NFκB that regulate the transcription of target genes involved in cellular proliferation and survival [23]. Eliminating inflammation after tumor development may lead to better strategies for cervical cancer therapy [24]. Drugs of natural origin that inhibit IL-1B, TNFα, IL-6, and TGFβ production may prove to be useful in treating cervical cancers as adjuvants in combination with more traditional chemo-therapeutic drugs. WDL is a natural drug that holds promising anti-inflammatory effect [25]. Its role against the inflammatory cytokines and NFκB in cervical cancer remains undefined. This *in-vitro* study is an attempt to reveal the dose-dependent anti-inflammatory effect of WDL on HeLa cells.

MTT is a mitochondrial activity-based assay that is used to analyze *in-vitro* drug efficacy at varying concentrations. The amount of formazon produced is comparable to the number of living cells that exist in the culture. Cell viability decreased with an increased dose of WDL treatment **Fig. 1A**. Our data are in line with previous studies [26] that indicated the cytotoxic effect of WDL derivative on breast, endometrial and ovarian cancer cell lines. Similarly cell viability assay using Trypan blue which is based on cytolysis also exhibited reduced viable cells in WDL treated compared to untreated control. WDL efficiently reduced the cell viability in a dose-dependent manner **Fig. 1B**.

Among various inflammatory cytokines TNFα, IL-1β, and IL-6 are critical links between inflammation and cancer cell proliferation, invasion through activation of NF-κB. Minimizing NFκB expression could be more beneficial than inhibiting IKK in cancer therapy. WDL was found to significantly suppress TNFα, IL-1β and IL-6 production in HeLa cells **Fig. 2**. To the best of our knowledge, this is the first study showing that WDL inhibits inflammatory cytokines in HeLa cells. Michels *et al.*, [27] provided evidence that in the tumor environment TNFα triggers NFκB activation which after that brings about gene expression of anti-apoptotic genes, TNFα itself and IL 1β. Activation of NF-κB also brings about IL-6 expression [28]. This, in turn, increases the tumor burden by promoting invasion and migration. Inhibition of TNFα, IL-1β, and IL6 by natural anti-

inflammatory drugs could contribute to the reduced expression of NF-κB [29]. Our proposition is supported by Yuan *et al*., [25] who reported that WDL inhibits inflammatory responses *via* suppression of the NFκB in RAW cells.

To further explore the molecular basis for WDL induced apoptosis in HeLa cancer cells, the expression of apoptosis-related proteins Caspase 3, PARP, Bcl-2, and Bax were analyzed by Western blot. This observation states that WDL induced apoptosis in HeLa cells was triggered by the downregulation of Bcl-2 and the up-regulation of Bax. In our study, there was a dose-dependent increase in ratio of Bax/Bcl-2 **Fig. 3**.

Similarly increased Caspase 3 and PARP expression in WDL treated HeLa cells show that apoptosis in cervical cancer is mediated through the mitochondria - associated apoptotic pathway. Caspase 3 is the major representative for cell death regulator that cleaves and activates many proteins including PARP. PARP is the enzyme that brings about DNA breakage during the process of programmed cell death [30]. Generally, the apoptosis mechanism involves multiple signaling pathways including cancer-elicited inflammatory pathways.

NFκB is the key mediator of inflammatory pathways. Evidence in the literature highlights that natural anti-inflammatory agents including WDL are all NFκB inhibitors [31, 32, 33]. NFκB inhibition paves way for the execution of cancer cell apoptosis. Targeting and eliminating inflammation, therefore, induces apoptosis that may lead to better strategies for cervical cancer therapy.

CONCLUSION: The demanding targets in the prevention and treatment of cancer are inflammatory proteins and their pathways. WDL is believed to suppress the inflammatory processes that lead to hyperproliferation. Though several signaling pathways are involved in tumor promotion, targeting inflammatory signaling pathway mediated by NFκB provides more efficacious treatment. Our results might refer the basic for the clinical study of NFκB and inflammatory cytokine inhibitor in cervical cancer therapeutics. Thus, inhibition of inflammation may, therefore, be an attractive approach supported by Wedelolactone for combination therapy.

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