Anti-proliferative effects of *Drynaria fortunei* in a model for triple negative breast cancer

NITIN T. TELANG[1], HAREESH B. NAIR[2] and GEORGE Y.C. WONG[3,4]

[1]Cancer Prevention Research Program, Palindrome Liaisons Consultants, Montvale, NJ 07645-1559, USA; [2]University of Texas Health Science Center, San Antonio, TX 78229, USA; [3]American Foundation for Chinese Medicine, New York, NY 10001, USA; [4]Breast Center, Maimonides Medical Center, Brooklyn, NY 11220, USA

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Abstract. Triple negative breast cancer (TNBC) is characterized by the absence of hormones and growth factor receptors. It is typically responsive to anthracycline/taxol-based conventional chemotherapy. However, major therapeutic limitations include systemic toxicity and acquired resistance to chemotherapeutics. To combat this, nutritional herbs from traditional Chinese medicine (TCM) with limited reported toxicity may represent treatment alternatives for TNBC. Such herbs can effectively target multiple signaling pathways in numerous breast cancer models. The efficacy of various nutritional herbs in a cellular model of TNBC is associated with the downregulation of retinoblastoma (RB) signaling through the cyclin D-CDK4/6-RB axis. Therefore, the present study was designed to examine the effects of *Drynaria fortunei* (DF) in the same cellular model of TNBC to identify potential mechanistic leads for its efficacy. DF is a nutritional herb that represents a common component of herbal formulations used in TCM. The estrogen receptor-negative, progesterone receptor-negative and human epidermal growth factor receptor-2-negative MDA-MB-231 human breast carcinoma-derived cell line was used as the cellular model for TNBC in the present study. Non-fractionated aqueous extract from the bark of DF represented the test agent. Quantitative end-point biomarkers for the efficacy of DF assessed in the present study included cell cycle progression, RB signaling and caspase 3/7 activity. Treatment with DF at cytostatic concentration induced S phase cell cycle arrest and inhibited RB signaling as evidenced by the downregulated expression of cyclin E, CDK2, E2F1 and RB phosphorylation. DF treatment increased pro-apoptotic caspase 3/7 activity which was inhibited by the pan-caspase inhibitor Z-VAD-FMK. DF treatment also exhibited increased expression of cleaved ADP-ribose) polymerase-1. These data identify potential mechanistic leads for anti-proliferative and pro-apoptotic effects of DF in the present TNBC model. The present experiments validated a mechanism-driven experimental approach to identify efficacious nutritional herbs and/or their bioactive constituents as treatment alternatives for TNBC.

Correspondence to: Professor Nitin T. Telang, Cancer Prevention Research Program, Palindrome Liaisons Consultants, 10 Rolling Ridge Road, Suite B, Montvale, NJ 07645-1559, USA
E-mail: ntelang3@gmail.com

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Introduction

Advanced stage metastatic breast cancer represents one of the major causes of mortality in women. In 2023 the American Cancer Society projected the estimated newly diagnosed breast cancer cases to be at 281,550 with the breast cancer-associated mortality to be at 43,600 (1). Clinical triple negative breast cancer (TNBC) is one type of breast cancer that lacks hormones and growth factor receptor expression which represents 15-20% of all cases of breast cancer incidence (2).

The status of receptor expression dictates the selection of appropriate chemo-endocrine therapy. The treatment option for TNBC includes the use of anthracycline, platin or taxane-based cytotoxic chemotherapy. However, these pharmacological agents frequently exert long-term systemic toxicity and cause spontaneous or acquired therapy resistance, leading to compromised therapy response and sub-optimal patient compliance. In addition, therapy resistance also favors metastatic progression of the disease due to the emergence of chemo-resistant cancer-initiating stem cell population (3-5). These aspects of clinical limitations for conventional chemotherapy emphasize the persisting demand for the identification of effective nontoxic testable alternatives.

Nutritional herbs constitute the main components of herbal formulations that are widely used in traditional Chinese medicine (TCM) for general health and hormonal issues in women. TCM represents a commonly used therapeutic alternative for therapy-resistant epithelial organ site cancers. Proven documented human consumption, low degrees of systemic toxicity and mechanistic leads for the preclinical efficacy of TCM provides a rationale for investigating the efficacy of Chinese nutritional herbs used in TCM as testable alternatives for therapy-resistant breast cancer. Chinese nutritional herbs have been documented to effectively target multiple signaling pathways (6,7).

However, the mechanism underlying the preclinical efficacy of Chinese nutritional herbs is likely to be context-dependent in cellular models of clinical breast cancer. In hormone receptor positive Luminal A subtype of breast cancer the growth inhibitory efficacy of various nutritional herbs such as *Cornus officinalis* (CO), *Epimedium grandiflorum* (EG) and *Lycium barbarum* (LB) has been found to be associated with altered cellular 17β-estradiol metabolism which generates anti-proliferative metabolites (8-10). By contrast, in the cellular model for TNBC the anti-proliferative and pro-apoptotic effects of several nutritional herbs such as *Dipsacus asperoides* (DA), CO and *Psoaralia corylifolia* (PC) have been found to be associated with inhibition of the retinoblastoma (RB), RAS, PI3K and AKT signaling pathways. These pathways are commonly associated with proliferative cancer cells, and provide growth advantage to primary tumor and to therapy-resistant cancer stem cells. Additionally, the pro-apoptotic effects of nutritional herbs have been reported to be associated with downregulation of anti-apoptotic BCL-2, upregulation of pro-apoptotic BAX and caspase-regulated apoptotic signaling pathways (11-14).

Accumulating evidence on effects of nutritional herbs emphasizes the importance of research into identifying the common and unique mechanisms of actions mediated by efficacious nutritional herbs and potential molecular targets. Such an experimental approach utilizing cellular models of various breast cancer subtypes can provide evidence for its applicability for future research directions focused on identifying mechanistic leads for growth inhibitory efficacy of bioactive agents present in natural products such as dietary phytochemicals and nutritional herbs used in TCM. Such research directions may also prioritize efficacious bioactive agents as potential drug candidates.

The nutritional herbs used in TCM, including Drynaria fortunei (DF), function as potent anti-oxidative, anti-angiogenic and immuno-modulatory agents, predominantly through modulation of several cell signaling pathways (6,7). DF is a common constituent used in herbal formulations in TCM. Multi-targeted efficacy of DF, leading to negative growth regulation, may benefit cancer growth inhibition. However, anti-cancer mechanisms for the growth inhibitory efficacy of DF on breast cancer have not been adequately documented. The multi-functional properties of DF and lack of sufficient evidence for the effects of DF against breast cancer provide a rationale for the present study.

The present study is focused on examining the effects of DF on a cellular model for TNBC to identify mechanistic pathways and potential molecular targets that may be responsible for its efficacy.

Materials and methods

Experimental model. Cellular models for clinical TNBC represent valuable experimental systems for mechanistic investigations that are focused on assessing the cancer growth inhibitory efficacy of pharmacological agents. The MDA-MB-231 cell line was originally isolated from a pleural effusion of a patient with metastatic breast carcinoma. These carcinoma cells lack the expressions of estrogen receptor-α (ER-α), progesterone receptor (PR) and amplified human epidermal growth factor receptor-2 (HER-2), representing an experimental model for TNBC (15,16). This cell line was obtained from American Type Culture Collection (ATCC). The cells were maintained in RPMI culture medium with L-glutamine and 5% fetal bovine serum (Life Technologies), following the protocol recommended by the supplier.

Test agent: Herbal formulations in TCM are commonly prepared as aqueous decoctions in boiling water. These decoctions are recommended to be consumed by the patients. To simulate patient consumption, non-fractionated aqueous extract of DF was prepared according to optimized protocol used in previous publications (11,12,14). Briefly, this protocol involves preparation of an aqueous extract of DF by boiling the herb in deionized water and concentrating the 500 x g supernatant by sequential centrifugations. The stock solution of the extract was reconstituted in the RPMI culture medium to obtain a concentration of 1 mg extract/1 ml. For the experiments stock solution was diluted in the RPMI culture medium to obtain the concentration range of μg/ml.

Dose response of Drynaria fortunei (DF). To determine the effective concentration range, dose response of DF was determined by measuring cell viability using Cell Titre Glo assay (Promega Corporation) in accordance with the protocol provided by the manufacturer. Cell viability was determined in cells treated with DF at the concentrations of 200, 400, 600, 800 and 1,000 μg/ml at day 7 after seeding, using Fluoroskan plate reader (Thermo Fisher Scientific Inc.). Cells maintained in the culture medium without any treatment represented the control. The data were expressed as the relative luminescent unit (RLU) and as % inhibition relative to untreated controls.

Anchorage independent (AI) growth assay. The AI growth formation represents a well-documented specific and sensitive *in vitro* surrogate end-point marker for *in vivo* tumor formation. This assay was performed following the optimized protocol (11,12,14). MDA-MB-231 cell suspension, at a density of $5x10^5$ cells per ml was prepared in 0.33% agar and treated with DF at the concentrations of 100, 200, 500, 1,000 and 5,000 μg/ml. Cells suspended in 0.33% agar without any treatment represented the control. Non-adherent colonies formed in 0.33% agar at day 21 after seeding were then counted at 10X magnification. The data were expressed as AI colony numbers.

Cell cycle progression. Monitoring the cell population at distinct phases of the cell cycle provides a quantitative measure of cell cycle progression. The cells were treated with 300, 400 and 800 μg/ml of DF. Cells maintained in the culture medium without any treatment represented control Cell cycle analysis was performed according to the optimized and published protocol (11,12,14). DNA content was determined using a Becton Dickinson FACSCAN Flow Cytometer (BD Biosciences) and analyzed using FACS Express software version 306 (De Novo Software). Distribution of individual cell population in the G_1 (quiescent), S and G_2 (proliferative) phases of the cell cycle was determined.

Western blot analysis. Quantitation of cellular proteins by Western blot analysis represents a commonly used assay. The

Western blot assay was performed according to the optimized and published protocol (11,12,14). The cells were treated with 300 and 800 μg/ml of DF. Cells without any treatment represented control. The cells were harvested and lysed in radio-immuno precipitation assay (RIPA) buffer containing protease inhibitors (Sigma-Aldrich), and were centrifuged at 10,000 x g for 15 mins at 4˚C. The protein content of the lysates was determined by the Bradford method and equal quantity of cellular proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE mini gels (Mini-PROTEAN TGX, Bio-Rad Laboratories). The gels were directly incubated with relevant primary and secondary antibodies (Table I). The chemo-luminescent signal was developed with ECL-plus reagent (Bio-Rad Laboratories), and detected by autoradiography. The signal intensity of proteins was quantified using molecular Image GS800 and Quantity One software (Bio-Rad Laboratories), and was presented as arbitrary scanning units (ASU).

Caspase assay. This assay represents a quantitative end-point for the mitochondrial associated apoptotic pathway. Caspase-3/7 activity was measured using Caspase-Glo assay kit (Promega Corporation), according to the optimized and published protocol (11,12,14). Cellular homogenate prepared from the cells treated with DF at 300, 400, 600 and 800 μg/ml, along with that prepared from cells without any treatment was used to measure the caspase 3/7 activity. The luminescence was measured using Luminometer (Thermo Fisher Scientific In.). The data were expressed as relative luminescent unit (RLU).

Statistical analysis. The experiments for dose response, caspase activity and pan-caspase inhibitor were conducted in triplicate and data were presented as mean ± SD. The experiments for AI colony formation cell cycle progression and RB signaling were conducted in duplicate and the data were presented as arithmetic means. Comparison of statistically significant differences among the common control and multiple treatment groups were analyzed using analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test as a post-hoc test using a threshold of α=0.05 using the Microsoft Excel 2013 XLSTAT-Base software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Dose response of DF. This experiment was conducted to examine the range of growth inhibitory effects of DF. Treatment of MDA-MB-231 cells with DF resulted in a concentration dependent reduction in cell viability. Specifically, DF concentrations of 200, 400, 600, 800 and 1,000 μg/ml resulted in a 6.7, 20.0, 60.0, 80.0 and 93.3% reduction in cell viability, relative to the untreated control (Table II). This dose response experiment identified 625 μg/ml as the 50% proliferation inhibitory concentration of DF.

Effect of DF on AI colony formation. This experiment was conducted to examine the effect of DF on anchorage independent (AI) colony formation. This *in vitro* end-point biomarker provides the data on the concentration dependent changes in the AI colony formation in response to treatment with DF (Table III). In response to DF concentration of 100, 200, 500, 1,000 and 5,000 μg/ml, the AI colony number was found to decrease with inhibition rates of 12.5, 25.3, 37.6, 50.5 and 96.1%, relative to the untreated control. This dose response experiment on AI colony formation identified 1,000 μg/ml as the 50% inhibitory concentration of DF.

Effect of DF on cell cycle progression. This experiment was conducted to examine the effect of DF on the proportion of cells in individual phases of the cell cycle and therefore, on cell cycle progression. The data presented showed that treatment with DF resulted in the S-phase arrest of cells. Additionally, the higher concentrations DF was found to abrogate the G2-phase of the cell cycle (Fig. 1A). Representative DNA histograms document the effects of untreated control and of 300, 400 and 800 μg/ml of DF on cell cycle progression. Consistent with the data presented in Fig. 1A, treatment with DF resulted in a concentration-dependent S-phase increase of 60.7 and 81.6%, relative to untreated control. In addition, higher concentration of DF abrogated G2 phase of the cell cycle (Fig. 1B-E).

Effect of DF on RB signaling. The RB signaling pathway serves an essential role in transition of cells in S and G2 phases of the cell cycle wherein expression status of cyclin E, CDK2, phosphorylated RB (pRB) and E2F1is critical. This experiment was therefore conducted to examine the effect of DF on RB signaling activity. Treatment with DF was found to suppress the expressions of cyclin E, CDK2, pRB and E2F1 levels in a dose-dependent manner (Fig. 2A). The data were also presented as total protein: β-actin ratio which demonstrated that in response to treatment with DF at its maximally effective concentration of 800 μg/ml, the expressions of cyclin E, CDK2 and E2F1 were inhibited by 35.8, 38.7 and 38.5% (P=0.04) respectively, relative to untreated control (Fig. 2B). The data expressed as pRB: RB ratio demonstrated that DF at its maximally effective concentration of 800 μg/ml inhibited this ratio by 74.7% (P=0.01), relative to the untreated control due to a reduction in pRB levels (Fig. 2C).

Effect of DF on cellular apoptosis. Caspase 3/7 activity represents a specific and sensitive marker for cellular apoptosis. The data presented in Fig. 3A demonstrated that the treatment with DF resulted in a concentration-dependent 2X, 4X, 10X and 17X increase in caspase 3/7 activity, relative to the untreated control. The experiment conducted to examine the effect of the pan caspase inhibitor Z-VAD-FMK demonstrated that treatment with DF at a concentration of 800 μg/ml induced the caspase 3/7 activity by16.8 X (P=0.01), relative to control. Treatment with DF + 10 μM Z VAD FMK reduced the caspase 3/7 activity by 61.8% (P=0.04), relative to DF treated cells (Fig. 3B). The experiment conducted to examine the effect of DF on the expression of cleaved poly(ADP-ribose) polymerase-1 (PARP-1) demonstrated that treatment with 800 μg/ml DF increased the expression of cleaved PARP by 1.9X (P=0.01), relative to control (Fig. 3C).

Discussion

TNBC is considered to be an aggressive subtype of breast cancer, with an incidence of 15-20% and disparity towards

Table I. Antibodies used for western blotting.

Antibody	Dilution	Cat. no.	Vendor
Primary antibody			
Cyclin E	1:200	SC 48420	SCB
CDK2	1:200	SC 6248	SCB
E2F1	1:100	SC 137059	SCB
pRB (Ser 780)	1:100	3590	CST
RB	1:100	SC 74562	SCB
PARP-1	1:200	SC 8007	SCB
Cleaved PARP-1	1:200	SC 56196	SCB
β-actin C4	1:200	SC 47778	SCB
Anti-rabbit secondary antibody			
IgG-HRP	1:1,000	SC 2337	SCB

CDK2, cyclin dependent kinase2; E2F1, member of the E2F family of transcription factors; pRB, phosphorylated retinoblastoma; RB, retinoblastoma; PARP-1, poly (ADP-ribose) polymerase-1; IgG-HRP, IgG horse radish peroxidase conjugate; SCB, Santa Cruz Biotechnology, Inc; CST, Cell Signaling Technology, Inc.

Table II. Dose response of *Drynaria fortunei*.

Treatment	Concentration (μg/ml)	Relative luminescent unit	Inhibition (% control)
Control	-	1.5±0.1	-
DF	200	1.4±0.3	6.7
	400	1.2±0.1	20.0
	600	0.6±0.3	60.0
	800	0.3±0.2	80.0
	1,000	0.1±0.05	93.3

Data expressed as mean ± SD from three independent experiments per treatment groups. Control vs. DF 600 μg/ml, P=0.04. Control vs. DF 800 μg/ml, P=0.01. Control vs. DF 1,000 μg/ml, P=0.01. DF, *Drynaria fortunei*.

Table III. Effect of DF in anchorage independent colony formation.

Treatment	Concentration (μg/ml)	AI colony number	Inhibition (% control)
Control	-	649	-
DF	100	568	12.5
	200	485	25.3
	500	406	37.6
	1,000	321	50.5
	5,000	25	96.1

Data expressed as arithmetic means from two independent experiments per treatment group. Control vs. DF 1,000 μg/ml, P=0.04. Control vs. DF 5,000 μg/ml, P=0.01. AI, anchorage independent; DF, *Drynaria fortunei*.

the African American ethnic population (2). TNBC lacks ER-α, PR and HER-2 expression which is noted to readily acquire resistance to a range of long-term conventional cytotoxic chemotherapy such as anthracyclines (including doxorubicin), platins (such as carboplatin) and taxols (such as paclitaxel). Treatment options using cytotoxic conventional chemotherapeutic are based on documented preclinical efficacy of these agents in TNBC models. TNBC has also been known to harbor a therapy-resistant cancer-initiating stem cell population, which proliferates following chemotherapy (5). The TNBC subtype is notable for tumor heterogeneity predominantly due to extensive cellular

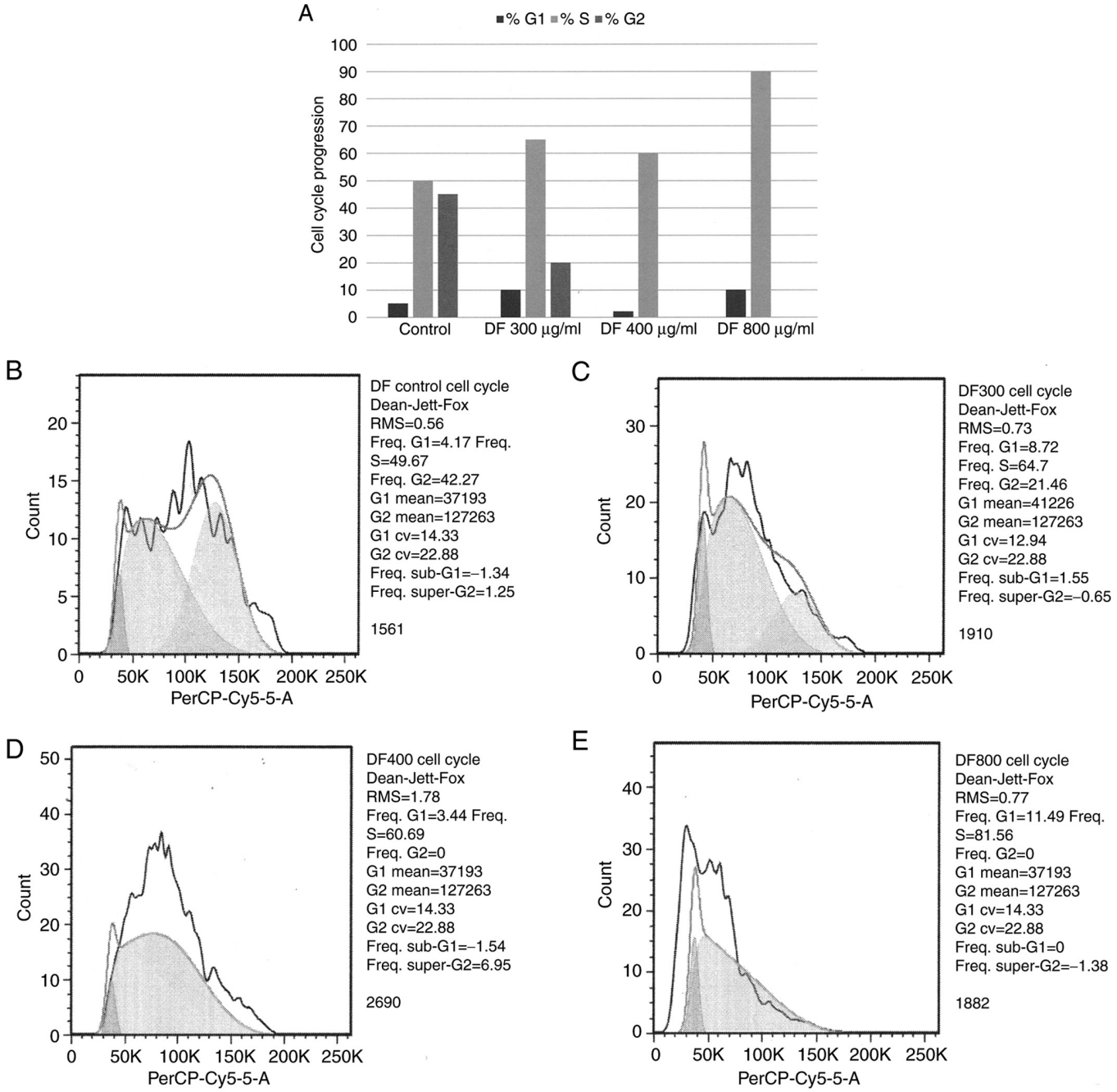

Figure 1. (A) Effect of DF on cell cycle progression. Data presented as arithmetic means from two independent experiments. Treatment with DF resulted in growth arrest of cells in the S phase of cell cycle. Control %S group vs. DF 300 μg/ml %S group, P=0.04; Control %S group vs. DF 800 μg/ml %S group, P=0.01. At the higher concentration, DF treatment abrogated the G_2 phase of the cell cycle. (B-E) Representative DNA histograms for (B) untreated control (S-phase 49.67%), DF treatment at (C) 300 μg/ml (S-phase 64.70%), (D) 400 μg/ml (S-phase 60.69%) and (E) 800 μg/ml (S-phase 81.56%) showing increased S-phase arrest and abrogation of the G_2 phase of the cell cycle at higher concentrations of DF. DF, *Drynaria fortunei*

plasticity and phenotypic diversity. Amongst the multiple subtypes of TNBC the M TNBC subtype has documented the presence of putative chemo-resistant cancer-initiating stem cells. The MDA-MB-231 cell line model for TNBC belongs to the M TNBC subtype (17). Established cellular models for the M TNBC subtypes include cell lines derived from clinical breast carcinoma such as MDA-MB-157, BT 549, SUM 159PT and MDA-MB-231. These cell lines have been documented to exhibit hyper-proliferation *in vitro* and tumorigenesis *in vivo*, and thereby, provide valuable experimental approaches for cell line based and tumor transplant-based investigations on growth inhibitory efficacy of nutritional herbs. Published evidence on the MDA-MB-231 model has been documented to exhibit growth inhibitory efficacy of several mechanistically distinct nutritional herbs used in TCM (11-14).

Experiments in the present study were designed based on the MDA-MB-231 model for clinical TNBC to examine effects of DF, a nutritional herb that represents a common component of herbal formulations used in TCM.

Inhibitory effects of DF on triple negative MDA-MB-231 cells were found in the present study, as evidenced by the effective

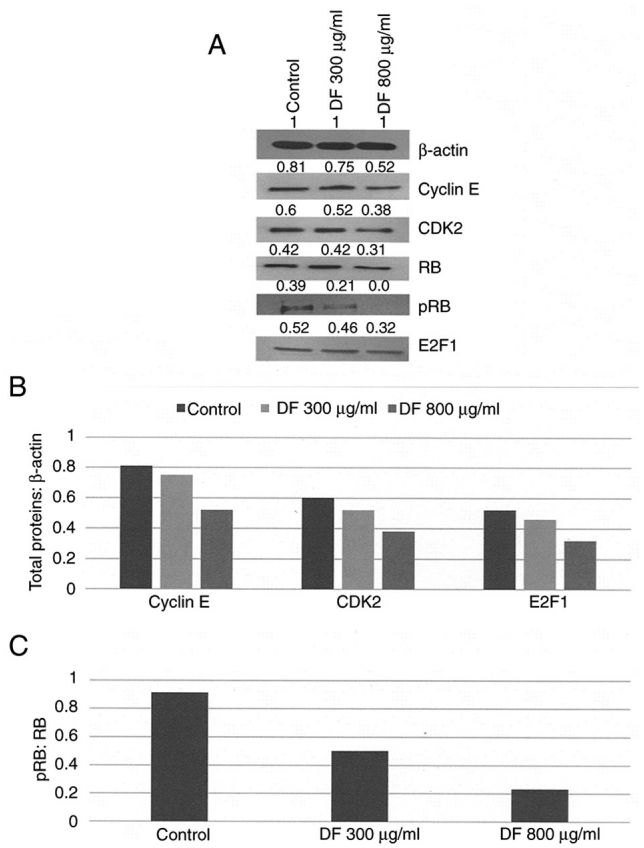

Figure 2. (A) Effects of DF on RB signaling. Treatment with DF resulted in a concentration dependent inhibition of RB signaling proteins. Data are obtained from two independent experiments. Representative western blotting is presented. Internal control is represented by β-actin protein. Signal intensity of proteins are quantitated from densitometric scans and presented as arbitrary scanning unit. Protein expression normalized from (B) β-actin or (C) total RB. (B) Data presented as arithmetic means from two independent experiments. Total proteins:β-actin ratio for cyclin E, CDK2 and E2F1. Respective control groups vs. 800 μg/ml DF for Cyclin E, CDK2 and E2F1, all P=0.04. (C) pRB:RB ratio, data presented as arithmetic means from two independent experiments. Control vs. 800 μg/ml DF group, P=0.01. CDK2, cyclin dependent kinase 2; E2F1, member of the E2F family of transcription factors; pRB, phosphorylated retinoblastoma protein, RB, retinoblastoma protein; DF, *Drynaria fortunei*.

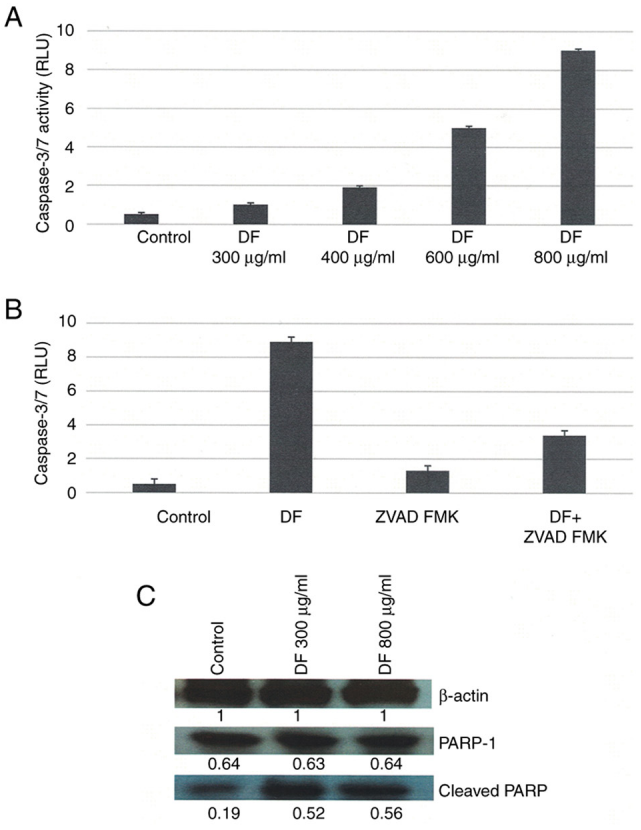

Figure 3. (A) Effect of DF on caspase 3/7 activity. Treatment with DF resulted in a concentration dependent increase in caspase 3/7 activity. Data expressed as mean ± SD from three independent experiments per treatment groups. Control versus DF 400 μg/ml group, P=0.04; Control vs. DF 600 μg/ml, P=0.01; control vs. DF 800 μg/ml group, P=0.01. (B) Effect of pan caspase inhibitor on caspase 3/7 activity. Treatment with the pan-caspase inhibitor ZFAD FMK inhibited DF-induced caspase 3/7 activity. Control vs. DF group, P=0.01. DF vs. DF + Z-VAD-FMK P=0.03. (C) Effect of DF on expression of cleaved PARP-1. The data are presented as arithmetic means from two independent experiments. Control vs. DF 800 μg/ml P=0.01. DF, *Drynaria fortunei*; RLU, relative luminescent unit; PARP-1, poly (ADP-ribose) polymerase-1.

reduction of cell viability in adherent culture and reduction in the number of AI colonies formed in a non-adherent culture. AI colony formation represents an *in vitro* marker for *in vivo* tumor formation. Collectively, these data provide evidence of the susceptibility for growth inhibition mediated by DF in the present experimental model and for the effective reduction in cancer risk. The present preclinical evidence provides a proof of concept for possible benefit of DF for prevention of clinical breast cancer.

The RB signaling represents one of the major tumor suppressor pathways responsible for the regulation of cellular proliferation, differentiation and apoptosis. The tumor suppressive function of RB has been documented to be compromised in cases of clinical TNBC and is associated with accelerated cell cycle progression due to the inactivation of negative growth regulatory proteins and the inhibition of cellular apoptosis (18). RB signaling targets G1 - S phase transition through the cyclin D1-CDK 4/6- pRB-E2F axis where p16 ^{INK4} functions as a major CDK inhibitor. Additionally, RB signaling targets the S and/or G2/M phases of the cell cycle through the cyclin E-CDK2- pRB-E2F axis where p21 $^{CIP1/waf1}$ and p27 ^{KIP1} function as the major CDK inhibitors. In the RB signaling cascade expression of E2F family of transcription factors represents a critical event preceding expression of RB target genes (19,20). In the present study DF at the concentration of 800 μg/ml represented the maximally effective dose that could inhibit the expression of cyclin E, CDK2 and E2F1 and RB phosphorylation.

The growth inhibitory efficacy of nutritional herbs CO, DA, PC in the present TNBC model has been documented to associate with inhibition of RB signaling through the cyclin D1-CDK4/6-pRB axis (11,12). Additionally, treatment with DA also resulted in inhibition of RAS, PI3K and AKT signaling which have also been reported to be constitutively activated in hyper-proliferative cancer cells (14). By contrast, growth inhibition by DF in the present study was associated with the inhibition of Cyclin E, CDK2, E2F1 expression and RB phosphorylation. Therefore, individual nutritional herbs may affect the expression of distinct proteins involved in the RB signaling pathway. Collectively the data on nutritional herbs suggest that distinct mechanisms of action and distinct molecular targets may be responsible for their respective inhibitory efficacy.

It is conceivable that constitutive bioactive agents present in nutritional herbs may target several signaling pathways for effective growth inhibition (7). The effects of nutritional herbs on cellular growth appear to be context dependent. Extract prepared from the rhizomes of DF containing flavonoids has been documented to exert proliferation promoting effects in estrogen responsive breast cancer cells and in osteoblastic cells through the osteopontin-related pathways possibly regulated by the functional estrogen receptor (21,22). DF has also been reported to promote proliferation of human umbilical vascular endothelial cells through VEGF and MMP related pathways to induce angiogenesis which was demonstrated through the chicken chorio-allentoic membrane assay (23). Network pharmacology and transcriptomic analysis for the effects of DF has demonstrated the targeting efficacy of multiple relevant genes such as TP53, AKT1, immune function related STAT1, STAT 3, IL6 and IL10, and estrogen receptor genes ESR1 and ESR2 coding for ER-α and ER-β, respectively (23-25). Proliferative inhibitory effects of DF found in the present ER negative TNBC model suggest that the distinct proliferation-modulating effects of DF may be related to ER function.

The pro-apoptotic effects of nutritional herbs CO. DA, PC are evidenced by increases in the Sub G0 (apoptotic) phase of the cell cycle, inhibited expression of anti-apoptotic BCL-2 protein, upregulated expression of pro-apoptotic BAX protein and increased caspase 3/7 activity (11-14). In the present study treatment with the maximally effective dose of DF at 800 μg/ml is associated with an increase in caspase 3/7 activity and its reduction by the pan-caspase inhibitor Z-VAD-FMK. Apoptotic cells are known to exhibit cleavage of the full-length PARP-1 protein to the truncated PARP-1. Treatment with DF resulted in increased PARP-1 cleavage. Therefore, the pro-apoptotic effects of DF are highly likely to be supported by modulation of additional apoptosis-related markers.

Collectively, the data on the effects of DF on RB signaling, induction of caspase 3/7 activity, its inhibition by the pan caspase inhibitor Z-VAD-FMK and increased cleavage of PARP-1 presented in the present study substantiated the mechanistic leads for the growth inhibitory efficacy of DF. Furthermore, the present experimental approach facilitates future research on cellular models for M TNBC using *in vitro* experiments on cell lines and *in vivo* tumor transplant experiments focused on identifying additional nutritional herbs and putative molecular targets for their growth inhibitory efficacy.

In conclusion, investigations into the growth inhibitory efficacy of nutritional herbs on the various models of TNBC including the present model for TNBC has identified mechanistic pathways mediating anti-proliferative and pro-apoptotic processes (11-14). Therefore, it is conceivable that multiple bioactive agents in the non-fractionated aqueous extracts may be effective in an interactive manner, affecting cancer cell survival specific PI3K, AKT and m TOR pathways, and through their effects on MAPK, MEK, ERK signaling pathways (6,7).

The experimental approach using the present TNBC model provided several scientifically robust rationales for future investigations. Major bioactive agents present in DF include flavones, anthocyanins, terpenes, polyphenolic acids and lignans (22,24). These agents may be responsible for growth inhibition, either individually or in combination. Reliable cancer stem cell models may represent valuable experimental approaches for examining cancer stem cell-targeting efficacy of bioactive agents present in natural products (26,27). Cancer stem cell specific telomerase activity (28), epigenetic modulation (29), and stem cell plasticity through epithelial-mesenchymal transition (30) represent valuable therapeutic targets. Pharmacological agents or natural products that target these processes may also represent potential novel drug candidates (31-33).

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contribution

NTT designed the experimental protocols, analyzed and interpreted the primary data, and prepared the manuscript. HBN conducted the experiments and participated in the preparation of the manuscript. GYCW initiated research projects on preventive efficacy of Chinese nutritional herbs on breast cancer. The efforts of GYCW on translating clinical aspects of herbal medicine into mechanism-based evidence for preclinical efficacy has provided a scientifically robust rationale for current research directions. GYCW also recommended the nutritional herb for the present study, analyzed and interpreted the data and participated in the preparation of the manuscript. NTT and HBN confirm the authenticity of all the raw data. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' information

Professor Nitin T. Telang, ORCID ID: 0000-0002-9059-8995.

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