Original Article Open Access

Archives Of Breast Cancer

DOI: 10.32768/abc.202183203-209

Growth Inhibitory Efficacy of Tabebuia Avellanedae in a Model for Triple Negative Breast Cancer

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ARTICLE INFO

Received:
02 March 2021
Revised:
28 March 2021
Accepted:
02 April 2021

Key words:
Triple negative breast cancer,
Tabebuia avellanedae,
cell cycle,
RB signaling,
cellular apoptosis,
caspase 3/7 activity

ABSTRACT

Background: Tabebuia avellanedae (TA) is a tree that is indigenous to the Amazon rainforest. The experiments in the present study were designed to examine the inhibitory effects of TA, and to identify mechanistic targets for its efficacy in the estrogen-α receptor (ER-α), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) negative MDA-MB-231 model for triple negative breast cancer (TNBC).

Methods: Non-fractionated aqueous extract from the inner bark of TA was used in the experiments. Anchorage dependent growth, anchorage independent (AI) colony formation, cell cycle progression, and expressions of relevant regulatory proteins represented quantitative end points.

Results: Long-term treatment for 21 days with the maximum cytostatic concentration of 2.5% TA resulted in a 90% inhibition (P=0.014) in AI colony number. Short-term treatment for 2 days with 1.0% TA (IC_{50}) resulted in about a 1.3 fold increase (P=0.014) in G_1: $S+G_2/M$ ratio, about a 1.48 fold increase (P=0.010) in the sub G_0 (apoptotic) cells and about a 3.2 fold increase (P=0.014) in the pro-apoptotic caspase 3/7 activity. Mechanistically, the short-term treatment with 2.5% TA decreased Cyclin D1 expression by about 83.3%, and pRB expression by about 73.3%.

Conclusion: TNBC represents an aggressive cancer notable for its resistance to conventional and targeted therapy. Non-toxic natural substances may represent testable alternatives. This study identifies potential mechanistic leads for TA as a novel naturally occurring testable alternative for secondary prevention/therapy of TNBC, and validates a novel mechanistic approach to evaluate efficacious non-toxic phytochemicals and herbs as testable alternatives against therapy resistant breast cancer.

Introduction

Therapy resistant invasive breast cancer accelerates disease progression and represents a predominant cause of mortality in the US. The American Cancer Society projections for the breast cancer incidence and mortality estimate 246,660 newly diagnosed breast cancer cases and 40,450 breast cancer related deaths in women in 2020.[1] The mortality rate is due to the incidence of advanced metastatic cancer. Clinical triple negative breast cancer (TNBC) represents an aggressive subtype that lacks the expression of ER-α, PR and HER-2. TNBC is notable for its resistance to conventional endocrine or HER-2 targeted therapy.[2-4] Current treatment options for the TNBC subtype are mostly restricted to anthracyclin, taxol and platinum based conventional chemotherapy. In addition, selective inhibition of

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poly (ADP-ribose) polymerase (PARP), phosphoinosine-3-kinase (PI3K) or molecular target of rapamycin (m-TOR) pathways via small molecule based targeted therapy has documented clinical efficacy.[5] These conventional and targeted treatment options are frequently associated with long-term systemic toxicity, de novo or acquired tumor resistance and emergence of therapy resistant cancer stem cell population.[6] These limitations emphasize a need to identify novel, less toxic options as testable alternatives to existing treatment strategies.

Complementary and alternative approaches utilizing herbal medicines are being used for general health issues and for palliative care of breast cancer in women.[7,8] Efficacious non-toxic natural phytochemicals and nutritional herbs may offer testable alternatives as a novel approach to eliminate therapy associated systemic toxicity and/or to reduce the emergence of therapy resistant tumor cell phenotype. Non-fractionated aqueous extracts from several nutritional herbs have documented growth inhibitory efficacy via distinct mechanisms in a human breast carcinoma derived cellular model for ER-α^+/PR^+/HER-2- Luminal A subtype[9-11], and in a model for ER-α^-, PR^- and HER-2^- triple negative subtype.[12,13]

TA is a tree that is indigenous to the Amazon rainforest. A tea made from the inner bark of TA, also known as Taheebo or Pau d'Arco, has been traditionally used by the people in that region to treat a variety of ailments including bacterial, fungal and viral infections.[14] Non-fractionated aqueous extract from TA represents a source for furano-naphthoquinines, quinines, naphthoquinones and flavonoids, and has documented efficacy in preclinical model for colon cancer CT-26.[15, 16] Since the relevant part of TA for medical usage is the inner bark, the abbreviation TA in this manuscript refers to the inner bark of this tree.

Global gene expression profiling to monitor differential gene expressions for growth inhibitory has demonstrated that non-fractionated aqueous extract of TA decreases the expression of positive growth regulatory cyclins, while increasing the expression of genes relevant to cellular apoptosis in the MCF-7 model for the Luminal A molecular subtype of breast cancer[9], and decreases the aromatase activity in the aromatase expressing MCF7-AROM model for post-menopausal breast cancer.[11] Growth inhibitory efficacy of aqueous extracts of Chinese nutritional herbs *Dipsacus asperoides* and *Cornus officinalis* via the RB signaling pathway have been demonstrated in the MDA-MB-231 model for TNBC molecular subtype of breast cancer.[12, 13] These data on the Luminal A and TNBC models identify potential mechanistic leads. However, growth inhibition and the molecular mechanisms for efficacy of TA in the TNBC model are not identified.

The TNBC subtype frequently leads to progression of therapy resistant metastatic disease.[3,5] The experiments in the present study are designed to evaluate growth inhibitory effects of TA, and to identify susceptible mechanistic pathways and molecular targets for its efficacy in a cellular model for TNBC.

Methods

Experimental model

The TNBC cell line MDA-MB-231 is ER-α negative, PR negative and HER-2 non-amplified.[17,18] This cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and was maintained in RPMI medium with L-glutamine and 5% fetal bovine serum (Life Technologies, Grand Island, NY) following the protocol recommended by the vendor.

Tabebuia avellanedae (TA)

The test agent is provided by Taheebo Japan Co., Ltd., Osaka, Japan in the form of water soluble powder prepared from the inner bark of the TA tree. This product is commercially available under the name of Taheebo NFD Essence. The aqueous extract of TA typically contains about 100 µg of naphthofuran dione (NFD, 3.9 µM, Molecular Mass: 258) and about 0.4µg of β-lapachone (β-LAP, 1.6 nM, Molecular Mass: 242.27). These compounds represent known bioactive agents of TA (Personal Communication: Prof. Fukuda, Taheebo Japan Co. Ltd. Osaka, Japan).[15-17]

The Stock solution of TA was prepared by dissolving 500 mg of the powder in 100 ml of double distilled water using the boiling extraction protocol.[9,11] The stock solution was serially diluted using the culture medium to obtain the concentrations of 2.5%, 2.0%, 1.5%, 1.0% and 0.5% TA for the dose response experiments.

Anchorage Independent (AI) Growth Assay

This assay was performed following the optimized protocol.[11-13] TA treated and untreated control cells were suspended in RPMI medium containing 0.33% agar and were overlaid on the basement layer of 0.6% agar. The cultures were incubated at 37°C in a CO_2 incubator for 21 days. The AI colonies were stained with 0.005% crystal violet and colony counts were determined at 10X magnification. The data were expressed as AI colony number.

Cell Cycle Progression

Cells were monitored for cell cycle progression following published protocol.[11-13] Cellular DNA content was analyzed using a Becton Dickinson FACSCAN Flow Cytometer (BD Biosciences, Research Triangle Park, NC, USA) and analyzed with FACS Express software (De Novo Software,

Glendale, CA, USA). The cell cycle progression was presented as % of cells in G_1, S and G_2/M phases of the cell cycle, and as G_1: S+G_2/M ratio.

Western Blot Analysis

Cellular proteins were separated by 10% SDS-PAGE (Mini-PROTEAN TGX, Bio-Rad Laboratories), transferred onto a nitrocellulose membrane (Bio-Rad Laboratori3es), and incubated with primary and secondary antibodies according to the published protocol.[13] Antibodies against the following proteins were used: β-actin, cyclin D1 and RB (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). CDK4 and phospho-Rb Ser 780 (Cell Signaling Technology, Inc. Danvers, CA, USA). The chemo-luminescent signal was developed with ECL-plus reagent (Bio-Rad, Hercules, CA, USA), and detected by autoradiography. The data were quantified by arbitrary scanning units (ASU).

Caspase Assay

Caspase-3/7 activity in the MDA-MB-231 cells was measured using Caspase-Glo assay kit (Promega, Madison, WI, USA) following the protocol provided by the vendor. The luminescence was measured using Luminometer (Thermo Scientific Co, Waltham, MA, USA). The data were expressed as relative fluorescence units (RLU).

Statistical Analysis

The experiments for dose response, AI growth, cell cycle progression, and Caspase 3/7 activity were conducted in triplicate. The data were expressed as mean ± SD. Comparison of statistically significant differences between the common control and multiple treatment groups was analyzed using analysis of variance and Dunnett's multiple comparison test as a post-hoc with a threshold of α=0.05. The data were analyzed using the Microsoft Excel 2013 XLSTAT-Base software.

Results

Growth Inhibitory Effects of TA

The data on the dose response of TA on MDA-MB-231 cells are presented in Table 1. Treatment with TA resulted in a dose dependent cytostatic growth arrest of MDA-MB-231 cells, and identified IC50 as 1.0%, and IC90 as 2.5%, respectively. Statistical analysis revealed that 1% TA induced a 52.0% inhibition (P=0.037) and 2.5% TA induced a 90.0% inhibition (P=0.014) in the viable cell number, relative to the control. Treatment with TA at concentration higher than 2.5% resulted in a viable cell number that was lower than the initial seeding density of 1.0X105. Therefore, this concentration was considered toxic (data not shown).

The data on the effect of TA on AI colony formation are presented in Table 2. Treatment with TA within the cytostatic range of 1.0% and 2.5% induced a 50.9% reduction (P=0.037) and a 90.2% reduction (P=0.014) in the number of AI colonies, relative to the control.

Effects of TA on Cell Cycle Progression

The data presented in Table 3 examined the effect of TA on the cell cycle progression of MDA-MB-231 cells. In response to the treatment with 1.0 % TA, the cells exhibited a 1.3 fold increase (P=0.014) in the G_1: S+G_2/M ratio, relative to the control. This increase was due to an inhibition of G_1 to S phase transition and resultant G_1 arrest.

The data presented in Figure 1 examined the effect of TA on the status of select cell cycle regulatory proteins. In response to treatment with 1% and

Table 1. Growth inhibitory effect of TA on MDA-MB-231 cells

Treatment	Concentration (%)	Viable cell number ($10x^5$) [a]	Inhibition (% control)
Control	----	25.0±2.5	----
TA	0.5	18.0±3.2	28
	1.0	12.0±1.4*	52
	1.5	11.0±1.4	56
	2.0	7.0±0.7	72
	2.5	2.5±0.3**	90

[a] determined at day 7 seeding of $1.0x10^5$ cells by trypan blue dye exclusion test. Mean ±SD, n= 3 per treatment group. * P= 0.037, ** P= 0.014. Data analyzed using ANOVA with Dunnett's post-hoc multiple comparison test (α=0.05). TA, *Tabebuia avellandae*; ANOVA, analysis of variance.

Table 2. Effect of TA on anchorage independent growth in MDA-MB-231 cells

Treatment	Concentration (%)	AI colony number [a]	Inhibition (% control)
Control	----	255±76	----
TA	1.0	125±37*	50.9
	2.5	25±5**	90.2

[a] determined at day 21 post-seeding of $5.0x10^5$ cells. Mean ±SD, n=3 per treatment group.
* P=0.037, ** P= 0.014. Data analyzed using ANOVA with Dunnett's post-hoc multiple comparison test (α=0.05). AI, anchorage independent; TA, T*abebuia avellandae*; ANOVA, analysis of variance.

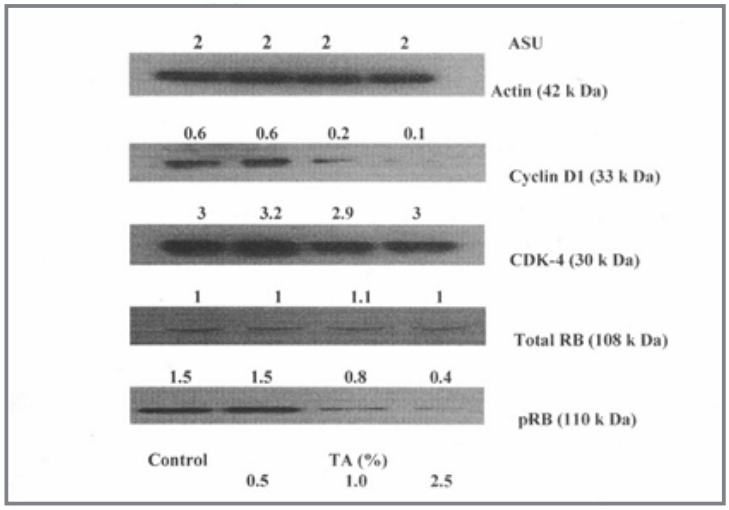

Figure 1. Western blot analysis of select proteins of the RB pathway. The expressions of Cyclin D1 and p-RB exhibit a dose dependent inhibition in response to treatment with TA. ASU, arbitrary scanning unit; CDK4, cyclin dependent kinase-4; RB, retinoblastoma; pRB, phosphorylated RB; TA, *Tabebuia avllanedae.*

2.5%TA, expression of cyclin D1 was reduced in a dose dependent manner by about 66.7% and 83.3%, respectively, relative to the control. Similarly, the expression of pRB was reduced by about 46.7% and 73.3% respectively, relative to the control.

Effects of TA on Cellular Apoptosis

This experiment examined the effect of TA on cellular apoptosis in MDA-MB-231 cells. Treatment with 0.5% and 1.0% TA resulted in about a 50.0% increase (P=0.037), and about a 1.43 fold increase (P=0.010) in apoptotic cells that were represented as the sub G_0 phase of the cell cycle (Figure 2A). Additionally, treatment with 1.0% TA resulted in about a 3.2 fold increase (P=0.014) in caspase 3/7 activity (Figure 2B).

Discussion

Human tissue derived cell culture models offer valuable mechanistic approaches to identify clinically translatable leads. Published comparative studies on non-tumorigenic triple negative 184-B5 cells, and on the carcinoma derived MDA-MB-231 cells have revealed that in contrast to 184-B5 cells, MDA-MB-231 cells exhibit aberrant hyper-proliferation, accelerated cell cycle progression,

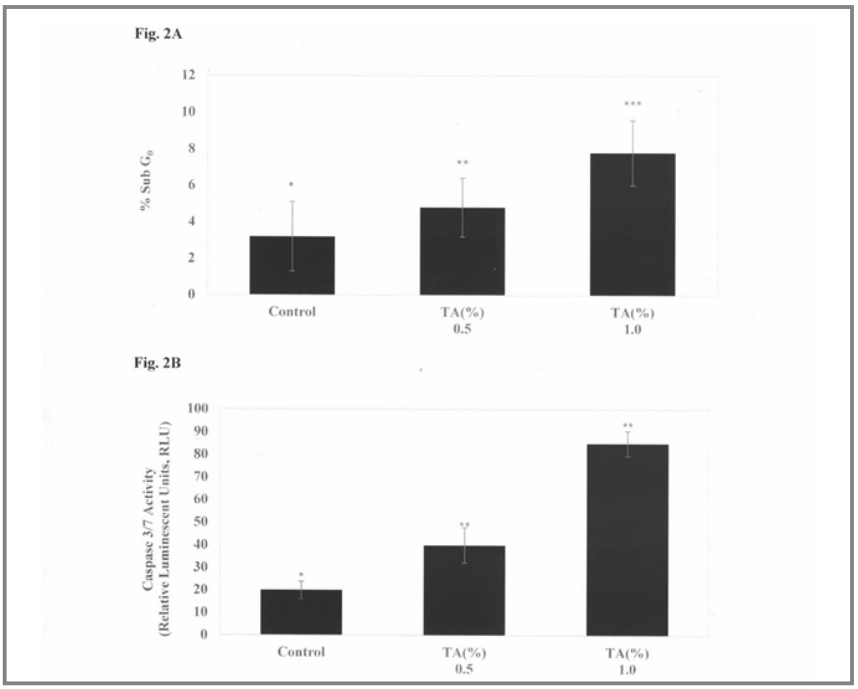

Figure 2. Induction of cellular apoptosis. A. Incidence of cells in subG_0 (apoptotic phase) exhibits an increase in response to treatment with TA. *-** P=0.037, *-*** P=0.010. Data analyzed by ANOVA with Dunnett's multiple comparison post-hoc test. B. Increase in caspase 3/7 activity in response to treatment with TA. *-** P=0.040, *-***P=0.014. Data analyzed by ANOVA with Dunnett's multiple comparison post-hoc test. TA, Tabebuia avellanedae; ANOVA, analysis of variance.

Table 3. Effect of TA on cell cycle progression in MDA-MB-231 cells

Treatment[a]	Concentration (%)	Cell cycle phase [b]			
		% G_1	% S	% G_2/M	G_1: S+G_2/M
Control	----	29.9±5.0	11.9±0.6	41.7±2.9	0.6±0.3
TA	0.5	39.4±2.0	12.2±0.6	36.2±1.8	0.8±0.4
	1.0	49.2±3.5*	22.2±1.1	12.8±0.6	1.4±0.3**

[a] TA treatment for 48 hr. [b] determined by flow cytometry based FACS assay. Mean ± SD, n=3 per treatment group. *P=0.02. **P=0.014. Data analyzed using ANOVA with Dunnell's post-hoc multiple comparison test (α=0.05). TA, *Tabebuia avellandae*; FACS, fluorescence assisted cell sorting.

down-regulated cellular apoptosis and AI growth in vitro.[14] These data suggest loss of homeostatic growth control and retention of cancer risk in MDA-MB-231 cells. This aspect is also documented in the ER-α+/PR+/HER-2- Luminal A model.[9,10,19] Furthermore, it is notable that AI colonies are observed in carcinoma derived MDA-MB-231 cells, but not in non-tumorigenic 184-B5 cells. Thus, AI growth represents an important in vitro surrogate end point marker for in vivo tumor development, and AI growth provides a quantifiable marker for cancer risk.

Treatment options for TNBC are associated with long-term systemic toxicity, acquired tumor resistance and emergence of drug resistant stem cells.[2,5,6] These limitations emphasize a need to identify effective, non-toxic alternatives as testable therapeutic options. Nutritional herbs *Dipsacus asperoides and Cornus officinalis* have documented growth inhibitory efficacy in the present model for TNBC.[12,13] It is noteworthy that there are several cellular models for clinical TNBC, and that the MDA-MB-213 model represents one such model. The present study outcome on the MDA-MB-231 model provides a mechanistic proof of concept for investigations on other TNBC models to examine the efficacy of naturally occurring phytochemicals and herbal extracts as testable alternatives for prevention/therapy of TNBC.

Little published evidence is available for clinical efficacy of TA on cancer patients. Anecdotal evidence on a limited number of patients demonstrates the effect of TA on the status of quality of life in advanced metastatic multiple organ site cancers.[20] In addition, the effects of NFD have been documented in three head and neck cancer patients and in one patient with lung metastasis from rectal cancer.[20,21]

In the studies discussed above, TA and NFD were administered as aqueous solutions. In traditional Chinese medicine, herbal formulations are commonly administered to patients in the form of herbal tea that is prepared by boiling the herbs in water. Thus, non-fractionated aqueous extracts of herbal formulations represent a commonly used method in patients. To simulate patient consumption of Taheebo tea, non-fractionated aqueous extract from TA was used in the present study.

At the mechanistic level, NFD, a major bio-active component of TA, has documented efficacy as a small molecule inhibitor of recombinant dual-specificity phosphatase Cdc-25 that regulates cell cycle transition. In addition, NFD induced G_1/S and G_2/M arrest and inhibited the proliferation of PC3 prostate carcinoma derived cells and MDA-MB-435 breast carcinoma derived cells at μM concentrations.[22]

PARP inhibitors represent a clinical option for targeted therapy of TNBC. Monotherapy with PARP inhibitors induces systemic toxicity and therapy resistance.[5] Combined treatment with PARP inhibitors and a naturally occurring quinone β-LAP has demonstrated synergistic interactions resulting in enhanced therapeutic efficacy of PARP inhibitors in pre-clinical xenograft models of lung, pancreatic and breast cancer.[23, 24] In response to treatment with TA within its cytostatic range, MDA-MB-231 cells exhibited a dose dependent growth inhibition and reduction in the number of AI colonies. The effective half-maximum concentration (IC_{50}) of TA was determined at 1.0%. This concentration was estimated to contain 0.039 μM of NFD. The maximum cytostatic concentration (IC_{90}) of TA was determined at 2.5%. This concentration is estimated to contain about 0.097 μM of NFD. Thus, the data on cytostatic growth arrest and reduction in the number of AI colonies suggest that TA may have effectively reversed the loss of homeostatic growth control and inhibited cancer risk in part, due to the presence of NFD in the present model for TNBC. In addition to NFD, β-LAP represents a minor constituent of TA.[15,16] The β-LAP content of 1.0% and 2.5% TA was estimated to be 0.016 nM and 0.040 nM, respectively. Thus, these concentrations represent non-effective low concentrations of β-LAP. Additionally, preclinical in vivo studies on the effects of TA in mice transplanted with Ehrlich's ascites carcinoma cells have shown that the levels of β-LAP are non-detectable as measured by thin layer chromatography based, and high pressure liquid chromatography based assays.[16] High μM concentrations of β-LAP inhibit growth and induce BCL-2 and caspase-dependent apoptosis in the T24 model of bladder cancer.[25] However, despite the documented efficacy of β-LAP at the higher pharmacological concentrations, these effective high μM concentrations of β-LAP are of limited relevance in the present study where concentrations of TA are low, and consequently, those of β-LAP.

Non-fractionated aqueous extract of TA also has documented growth inhibitory efficacy in ER-$α^+$/PR^+/HER-2- MCF-7 cells that represent a model for endocrine therapy responsive Luminal A

molecular subtype of clinical breast cancer. In this model, TA within the cytostatic dose range inhibited cell cycle progression and induced cellular apoptosis. At the mechanistic level, the biological effects of TA were associated with up-regulated expression of select proliferation specific genes, modulated expression of apoptosis specific genes and enhanced expression of genes specific for xenobiotic metabolism.[9] In the present study, the effect of TA on cell cycle progression revealed a dose dependent G_1 phase blockade and inhibition of S+G_2/M phases of the cell cycle. These alterations in the cell cycle resulted in a dose dependent increase in the G_1: S+G_2/M ratio.

The cell cycle regulatory function of RB via the cyclin D1-CDK4/6-p-RB axis is compromised in therapy resistant basal-like and triple negative subtypes of clinical breast cancer.[26, 27] The data from the experiment designed to examine the effect of TA on the RB pathway clearly demonstrated that TA inhibited the expression of cyclin D1 and p-RB in a dose dependent fashion, while, intriguingly, the expression of CDK4 remained essentially unaltered. Kinase-dependent site specific phosphorylation of RB represents a critical post-translational modification for the tumor suppressive function via inactivation of cell cycle progression and promotion of cellular apoptosis.[28] Thus, the present data on inhibition of cyclin D1 and p-RB provide mechanistic lead to suggest that the RB pathway might represent a molecular target for the efficacy of TA in the present model system.[29,30]

Mitochondria-mediated intrinsic apoptotic pathway involves altered membrane permeability, cytochrome-c release, and apoptosome-mediated activation of Caspase 9 and subsequently of Caspase 3/7.[31, 32] The experiment designed to examine the effect of TA on cellular apoptosis demonstrated that TA produced a dose dependent progressive increase in the Sub G_0 (apoptotic) phase of the cell cycle, and an increase in the pro-apoptotic Caspase 3/7 activity. Collectively, these data on cellular apoptosis provide mechanistic leads to support the evidence that induction of cellular apoptosis by TA may function via caspase dependent mechanism in the present model system.

Differential gene expression profiling in the MCF-7 model revealed that TA resulted in downregulation of cell cycle regulatory genes, cyclins B1 and E and cyclin dependent kinases CDK2, CDK4 and CDK6 and in upregulation of apoptosis related genes, caspase 4, caspase 7 and caspase 8.[9] Thus, TA appears to be effective via mechanistically distinct pathways in cell culture models for Luminal A and TNBC subtypes of clinical breast cancer, thereby supporting the concept that growth inhibitory effects of TA may be independent of expressions of hormone receptors. In this context, it is notable that mechanistically distinct nutritional herbs demonstrate differential growth inhibitory efficacy in a model where isogenic phenotypes exhibit modulated ER function.[33]

In conclusion, the results of the present study exhibiting anti-proliferative and pro-apoptotic effects of TA, together with the published evidence for the efficacy of extracts from several nutritional herbs on the ER-α positive, PR positive and HER-2 non-amplified (Luminal A) model[9-11], and on the ER-α negative, PR negative, HER-2 non-amplified negative (TNBC) model[12, 13] may provide a mechanistic rationale for future preclinical and clinical investigations.

Conflict of Interest

The authors declare that there is no conflict of interests. This study was supported by philanthropic contributions to the American Foundation for Chinese Medicine from the Randall and Barbara Smith Foundation, the Saint Agatha Foundation and the Sophie Stenbeck Family Foundation.

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