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RESEARCH ARTICLE

Gallic Acid, a Promising Chemopreventive Agent, Antagonizes Breast Cancer: Molecular Mechanisms of Action from Cell Culture Studies

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Abstract

Objective the present consideration converged on trailing the underlying molecular mechanism of gallic acid in striking the distinctly invasive breast cancer cell line; MDA-MB-231 *in vitro*. Methods the cytotoxic influence of gallic acid and 5-fluorouracil (5-FU) on MDA-MB-231 cells was stipulated by MTT assay. The expression levels of apoptosis, proliferation, progression, invasion and metastases-related genes were identified by RT-PCR. Results Our data specified that gallic acid and 5-FU displayed modest growth suppression with IC_{50} values of 251 and 367.171 µM contra MDA-MB-231 cells respectively after 48 h. Otherwise, treatment of MDA-MB-231 cells with gallic acid inspired significant downregulation in the expression level of survivin, STAT3, IL-6, VEGF, Slug, Snail, MMP7 and Notch-4 genes. Whereas, 5-FU instigated significant downregulation in the expression level survivin, STAT3, IL-6 and VEGF genes correlated with significant upregulation in the expression level of Snail and Notch-4 genes. Likewise, 5-FU provoked insignificant upregulation in the expression level of Slug and downregulation in the expression level MMP7 genes. Conclusion the attained data throw light on the distinct mechanism by which gallic acid could engage breast cancer. Gallic acid manifested chemo-preventive potentiality against breast cancer aggressiveness through rectifying several dysregulated molecules implicated in cancer cell survival, proliferation, progression, angiogenesis, chemo-resistance and metastasis.

Keywords: *MDA-MB-231 cell line, Gallic acid, 5-Flurouracil, Molecular pathways, In vitro.*

Introduction

Breast cancer is a popular and a convoluted pernicious ailment recognized by a variety of divergences at the genomic and molecular levels, which evident in dysregulated signalling pathways implicated in the evolution, progression and metastasis of the cancer [1]. It is the ultimate ordinarily distinguished pernicious tumor in females of all races and is the second dominant factors of cancer dying in females of most races. Breast cancer is usually managed by a set of surgery, chemotherapy, radiotherapy, and hormone therapy. Alongside examination and continual establishment of novel adjuvant medications, present 5-year survival rates are virtually 90%. Regardless of the enhancements, the long term recurrence rates can be as high as 20%, and metastasis at recognition and metastatic retrogression after the initial handling endure an untreatable ailment and the prime factor of death of breast cancer [2].

Growing data elucidate the prominence of the epithelial-mesenchymal transition (EMT) in breast cancer invasion and metastasis. EMT is a convoluted procedure in which epithelial cancer cells acquire a mesenchymal phenotype and discard their epithelial characteristics. In this procedure, the epithelial cells acquire elevated migratory and invasive features to become mesenchymal cells through the EMT. In breast cancer, a chain of EMT processes can result in cell detachment, migration, invasion and colonization at secondary locations [3].

Cumulating clue specified that tumors are greatly constituted of various cell kinds and their instigation and development are driven by a subset of cells, designated cancer stem cells (CSCs) or tumor-initiating cells. CSCs are a distinct set of cancer cells, which are accompanied with tumor instigation, progression, migration, invasion, opposition to chemotherapy and radiation therapy, and relapse [4]. It has been registered that EMT leads to the elevation of CSC-like features in several kinds of cancer, comprising breast cancer, whilst suppressing the process of EMT may restrain CSC origination [5]. CSCs collaborate particular features with native stem and/or progenitor cells. They have conglomerated oncogenic mutations and missing normal restrictions on growth regulation, and can be preferentially opposed to chemo-radiotherapy. Cancer management that fails to eradicate CSCs may authorize retrogression and even diaspora of the indigenous tumor [6].

5-Fluorouracil (5-Fu) plays an important role in standard chemotherapy protocols for a variety of solid tumors including breast cancer. But it is limited in clinical application due to the resistance [7]. Antimetabolite drugs work by inhibiting essential biosynthetic processes, or by being incorporated into macromolecules, such as DNA and RNA, and inhibiting their normal function. The fluoropyrimidine 5-fluorouracil (5-FU) does both. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase [8]. The 5-FU metabolite; fluorouridine triphosphate (FUTP) is extensively incorporated into RNA, disrupting normal RNA processing and function. Significant correlations between 5-FU misincorporation into RNA and loss of clonogenic potential have been shown in human breast cancer cell lines [9].

Lately, mounting concern of many investigators is converged on the application of dietary polyphenols in cancer prevention and chemotherapy [10]. Among polyphenol compounds manifesting antitumor peculiarities in *in vitro* studies are gallic acid (GA). GA is a polyhydroxyphenolic compound [3, 4, 5-trihydroxybenzoic acid, C_6H_2 (OH) $_3$ COOH] that is vastly present in grapes vegetables and many fruit (Fig. 1) [11].

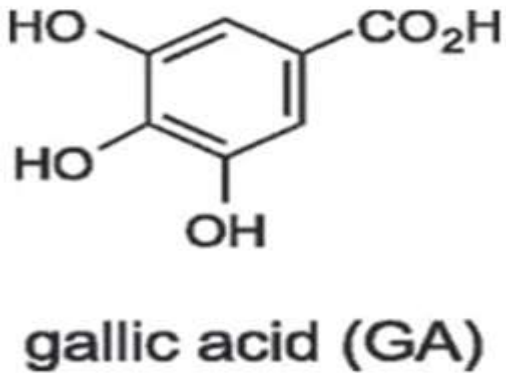

Fig.1: Chemical structure of gallic acid [11]

GA exhibits anti-inflammatory, anti-virus, and free radical scavenging activities. Furthermore, it is registered that GA can suppress expansion of tumor cells by motivating apoptosis *via* the ROS-mediated pathways [12]. GA represses P815 cell metastasis to the liver [13] and activator protein-1 (AP-1) transcriptional activity [14] as well as defeats tumor angiogenesis [15]. However, although there are some motivating data evidenced the efficacy of GA against cancer cells, up to now little is recognized about its peculiarities towards multidrug resistance breast cancer cells [16]. Perceiving the molecular mechanisms implied the malignant attitudes of breast cancer cells would allocate lead molecules for targeted therapy [17]. The present investigation was intended to gain an insight into the potency of gallic acid in targeting multiple molecules implicated in the signalling pathways of cancer cell survival, proliferation, progression, angiogenesis, chemoresistance and metastasis in breast cancer cell line (MDA-MB-231) that is well known to be enriched with cancer stem cells.

Materials and Methods

Cell Propagation and Maintenance

MDA-MB-231 human breast cancer cells were obtained from VACSERA (The holding company for biological products & vaccines), Egypt. They were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Belgium) supplemented with 10 % fetal bovine serum (FBS; Lonza) and 1% penicillin-streptomycin (Lonza) at 37°C humidified atmosphere with 5% CO_2. The cells were harvested after trypsinization (0.025 % trypsin (Lonza) and 0.02 % EDTA) and washed twice with Dulbecco's phosphate-buffered saline (DPBS; Lonza). When the cell density reached approximately 80%, cells were split for further culture.

Cytotoxicity Assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Sigma, USA) is based on the conversion of MTT into formazan crystals by living cells, which reflects cytotoxicity based on mitochondrial activity [18]. The cytotoxic impact of the gallic acid (Sigma-Aldrich, USA) was measured by MTT assay using MDA-MB-231 cells. The cells were incubated with various concentrations of the compound (10, 25, 50, 100, 200, 400, 800 and 1000 μM) for 24 h, 48 h and 72 h at a cell density of 1 x 10^4 cells/well of 96 well plate.

After different incubation, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml, and the samples were incubated at 37ºC for 4 h. After 4 hours, the medium was decanted and dimethyl sulfoxide (DMSO) was added to each well, including blank wells and left for 30 minutes to dissolve formazan crystals that formed during MTT cleavage in actively metabolizing cells. Absorbance of formazan in each plate was measured at 570 nm, using a microplate reader (Model 500; BIORad Instrument Inc., USA). Using the relation between the used concentrations and the mitochondrial activity (%), IC_{50} of the tested compound was calculated. For the untreated cells (negative control), medium was added instead of the test compound. A positive control 5-Fluorouracil (5-FU, Mr=130.08, Sigma-Aldrich, USA) was used as a cytotoxic natural agent giving 100% inhibition. All tests and analyses were done in triplicate and the results were averaged.

Gene Expression Analysis

Cells were seeded at a cell density of 3×10^4 cells/well and were treated for 48 h. Total RNA was isolated from cell samples using the RNAeasy mini Kit (Qiagen, USA) then the concentration and purity of total extracted RNA were determined using NanoDrop 2000c spectrohphotometer (Thermo Fisher Scientific, USA). RNA of each treatment was converted to first-strand cDNA according to the manufacturer's instructions using Revert Aid First Strand CDNA Synthesis Kit (Thermo Scientific, USA). Specific primer sequences **(Table 1)** for survivin, STAT3, IL6, VEGF, Snail, Slug, MMP7, Notch-4 and housekeeping gene GAPDH were used for quantitative real time PCR.

The reaction conditions were as follows: 95 °C for 10 min, 95 °C for 15 s, 60° C for 30 s (Survivin, IL6, VEGF, Snail) and at 56 °C for 30 s (Slug, MMP7, Notch-4) and at 58 °C for 30 s (STAT3) and 72 °C for 30 s with a total of 40 cycles of amplification. Survivin, STAT3, IL6, VEGF, Snail, Slug, MMP7, Notch-4 expression levels were normalized with respect to GAPDH transcript and calculated by $2^{-\Delta\Delta CT}$ method. DNA Technology Detecting Thermocycler DT Lite 4S1 (Russia) was used for gene expression quantitation.

Table 1: Sequences of the primers used in the RT- PCR analysis

Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Ref.
Survivin	AGTGAGGGAGGAAGAAGGCA	ATTCACTGTGGAAGGCTCTGC	**[19]**
STAT3	TGAGACTTGGGCTTACCATTGGGT	TCTTTAATGGGCCACAACAGGGCT	**[20]**
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	**[21]**
VEGF	TCGGGCCTCCGAAACCATGA	CCTGGTGAGAGATCTGGTTC	**[22]**
Slug	TGTTGCAGTGAGGGCAAGAA	GACCCTGGTTGCTTCAAGGA	**[23]**
Snail	TACAAGGCCATGTCCGGACCCA	TGTGGAGCAGGGACATTCGGGA	**[24]**
MMP7	AGATGTGGAGTGCCAGATGT	TAGACTGCTACCATCCGTCC	**[25]**
Notch-4	AACTCCTCCCCAGGAATCTG	CCTCCATCCAGCAGAGGTT	**[26]**
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	**[27]**

Statistical Analysis

The attained results are delineated as means ± standard deviations. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare the significance between groups. The level of significance was set at P<0.05.

Results

In Vitro Appraisal of Gallic Acid Cytotoxic Efficacy

In the current investigation, we specified the extent of cytotoxicity of gallic acid on the highly aggressive and invasive breast cancer cell line (MDA-MB-231) with the elevated proportion of cancer stem cells at concentrations 10, 25, 50, 100, 200, 400, 800 and 1000 µM for several time intervals. The suppression of proliferation of MDA-MB-231 cell line was affirmed through MTT assay, which is depend on the diversion of MTT into formazan crystals by living cells, that indicates cytotoxicity based on mitochondrial activity. After 48 h incubation time, gallic acid displayed modest growth suppression with IC_{50} value of 251 µM contra MDA-MB-231 cells as compared to control cells. At 24 and 72 h incubation time, gallic acid didn't demonstrate any cytotoxicity **(data not shown)**. Furthermore, 5-FU manifested modest growth suppression with IC_{50} value of 367.171 µM contra MDA-MB-231 cells compared to control cells after 48 h incubation time.

Gene Expression Levels of Chosen Molecular Pathways

Fig. (2) clarified the impact of gallic acid and 5-FU on survivin, STAT3, IL-6 and VEGF genes expression level in MDA-MB-231 cell line. Handling of MDA-MB-231 cell line with gallic acid or 5-FU experienced significant (P < 0.05) downregulation in the expression level of survivin, STAT3, IL-6 and VEGF genes as compared to control cells. Over and above, gallic acid exposed cells displayed significant (P < 0.05) downregulation in the expression level of IL-6 gene in MDA-MB-231 cells comparative to 5-FU exposed cells.

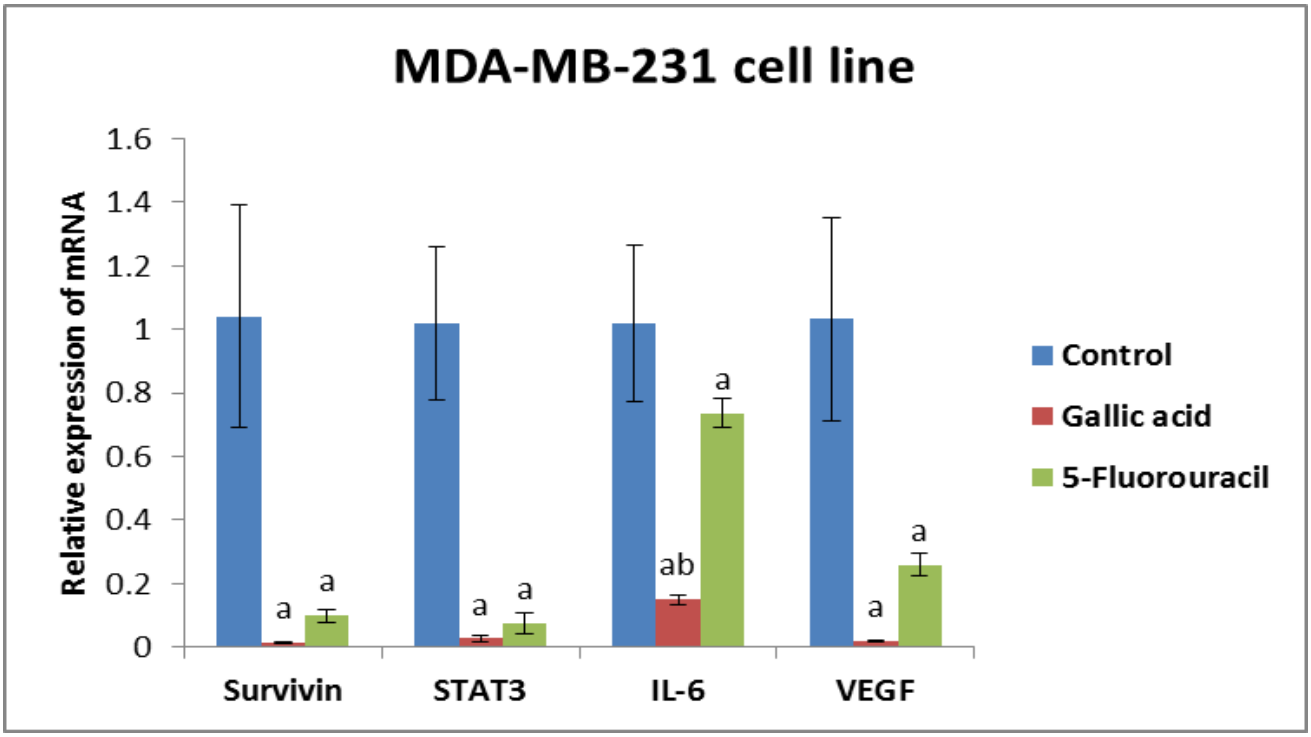

Fig.2: Influence of gallic acid and 5-FU on the mRNA expression level of survivin, STAT3, IL-6 and VEGF in MDA-MB-231 cell line. Data are depicted as mean ± SD, Data were reproducible. [a]Significant change at P< 0.05 compared with control cells, [b] Significant change at P< 0.05 compared with 5-FU

Exposure of MDA-MB-231 cell line to gallic acid provoked significant (P < 0.05) downregulation in the expression level of Slug, Snail, MMP7 and Notch-4 genes *versus* control cells and 5-FU treated cells (Fig. 3). On the other hand, 5-FU exposed cells displayed insignificant (P>0.05) upregulation in the expression level of Slug gene as compared to control cells. Moreover, 5-FU triggered significant (P < 0.05) upregulation in the expression level of Snail and Notch-4 comparative to the control cells.

Eventually, 5-FU exposed cells manifested insignificant (P > 0.05) downregulation in the expression level of MMP7 gene as compared to control cells.

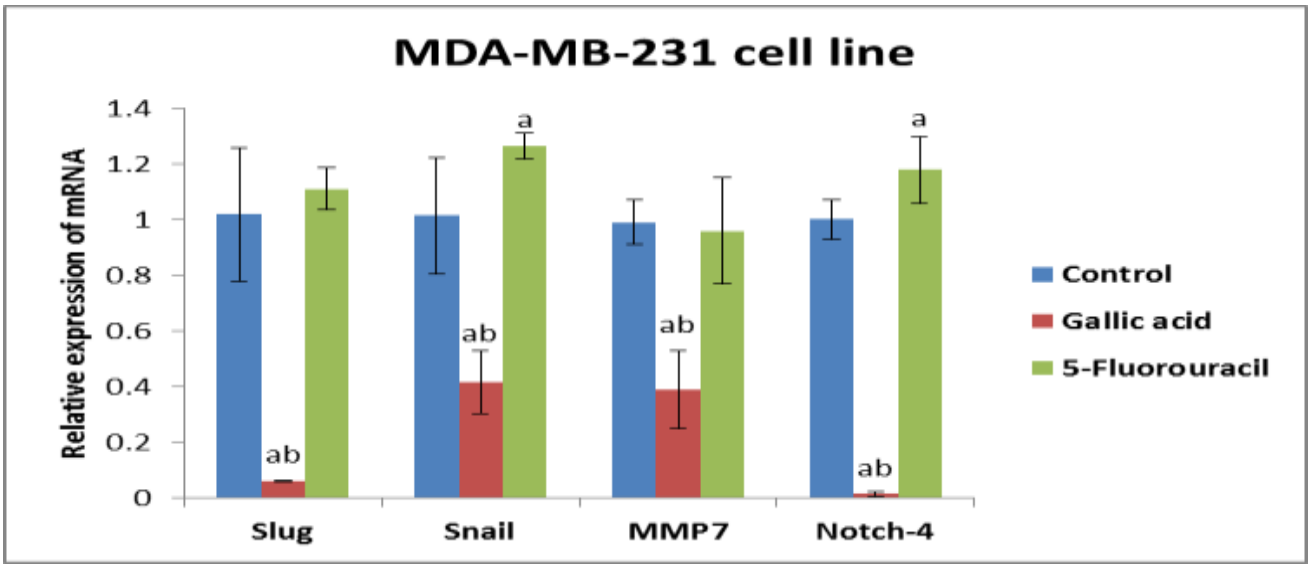

Fig.3: Influence of gallic acid and 5-FU on the mRNA expression level of Slug, Snail, MMP7 and Notch-4 genes in MDA-MB-231 cell line. Data are depicted as mean ± SD, Data were reproducible. [a]Significant change at P<0.05 compared with control cells, [b] Significant change at P< 0.05 compared with 5-FU

Discussion

Gallic acid was recorded to significantly downregulate survivin expression level in MDA-MB-231 cells, which concurs with the outcomes of Kim et al. [28] who announced that ellagic acid can downregulate the expression level of survivin gene in MDA-MB-231 cells. This signalizes that one of the targets of ellagic acid is IAP family. Survivin is a member of IAP family; IAP family can link to the cell death proteases like caspase 3 and caspase 7 and suppresses caspase action and cell death in cells subjected to apoptotic stimulant. In breast cancer, survivin is a considerable foreteller of survival in poor prognosis breast cancer patients [29].

Ellagic acid remarkably diminished the viable cells, motivated G0/G1-phase detain of the cell cycle and apoptosis. Ellagic acid too augmented p53 and p21 and reduced CDK2 gene expressions that may cause the G0/G1 detain of human bladder cancer (T24) cells [30]. Consequently, ellagic acid could prompt apoptosis in a cell cycle dependent way. Commonly, the processes contributing to DNA restoration encompass upregulation of p21 through p53-dependent mechanisms. In cervical carcinoma (CaSki) cells, ellagic acid could not stimulate a considerable elevation in the level of p53 mRNA and protein, even if a higher promotion of p21 mRNA and protein was disclosed after treatment with ellagic acid. The motivation of p21 by ellagic acid could be either *via* a p53-independent way, or by a p53-dependent way not implying the alterations in the level of p53 protein [31]. In commitment to these outcomes, Chen et al. [32] declared that cyclins (cyclin A2 and cyclin E2) are downregulated in ellagic acid-treated MCF-7 cells, whilst cyclin-dependent kinase (CDK) suppressors (p21, Cip1, p15 and p19) are amplified. It has been recognized that factors implicated in survivin repression at the post-translational level encompass CDK suppressors and Hsp90 suppressors as these factors have the capacity to oppose mitotic phosphorylation of survivin at Thr^{34}, herewith hastening survivin retrogression. Interestingly, administration of CDK suppressors leads to downregulation of survivin expression and motivation of apoptosis in an *in vivo* study [33].

5-Flurouracil in the present investigation was applied as a reference medicament for treatment of breast cancer cell lines MDA-MB-231. 5-FU was found to significantly downregulate survivin gene expression in MDA-MB-231 cells. 5-FU, a generally utilized anticancer chemotherapeutic factor, is an analog of pyrimidine which interposes with thymidylate synthase action, prohibiting thymidylate (the precursor of thymidine triphosphate) generation. 5-FU prospectively elicits in target cells, DNA- and RNA-pointed activities. Various considerations that

converged on the anticancer impact of 5-FU have established that 5-FU motivates cell cycle arrest and/or apoptosis in several forms of tumors comprising breast, oral, gastric, and colon tumors [34]. Commonly, p53 is found to exert a fundamental part in apoptosis stimulation and 5-FU sensibility in human colon cancer cells [35]. The investigation of Liu et al. [36] registered that 5-FU enhances the cyclin dependent kinase suppressor p21WAF1/CIP1 expression without p53 cumulation in either laryngeal squamous cell carcinoma (UMSCC12 or UMSCC11A) cells. Both UMSCC11A and UMSCC12 cells displayed no elevation in the expression of Bax nor suppression of Bcl-2, with the intense cumulation of p21 advocating that the apoptotic response to 5-FU could be p21-dependent. These examiners noted that a rise in p21 expression is escorted by an elevated concentration of pRb in laryngeal carcinoma cells exposed to 5-FU, designating an affirmative correlation between them. In regulating the cell cycle control, an elevated concentration of p21 can retain pRb in a hypophosphorylated event which is competent to detain E2Fs and restrain the cell cycle [36]. Therefore, the augmentation of cyclin D suppressors by 5-FU may be one of the mechanisms beyond the diminution of survivin gene expression in the present study.

The other proposed mechanism may be related with the repression of nuclear factor-kappa B (NF-κB) action by 5-FU which results in the suppression of the expression of the anti-apoptotic molecules, TRAF-2 and CIAP-1 that eliciting the promotion of caspase-8 and caspase-3 leading eventually to human salivary gland cancer cell apoptosis [37]. NF-κB promotion is a prevalent event in cancers, which leads to the aberrant expression of NF-κB target genes and results in malignant diversion, metastatic pervasion, abnormal cell proliferation or opposition to cell death. NF-κB can motivate cell cycle progression and minify apoptosis by augmenting survivin expression, herewith elevating cellular proliferation. In the exploration of Cui et al. [38], they have described that survivin acts as a downstream arbitrator of NF-κB signalling in bladder cancer malignant progression. Wang et al. [39] documented that the suppressor of NF-κB can restrain the expression of survivin in glycochenodeoxycholate-stimulated hepatocyte apoptosis.

Gallic acid in this investigation was observed to significantly downregulate signal transducer and activator of transcription 3 (STAT3) expression level in MDA-MB-231 cells which is in conformity with Heidarian et al [40] who registered that gallic acid can lessen the level of pSTAT3 cellular signalling protein in PC-3 prostate cancer cells in a dose-dependent way. IL-6 has been noted to motivate various downstream signalling molecules, encompassing STAT3, which consequently boosts inflammatory reactions and aids the cancer cells to retain convenient homeostasis [41], thus hastening the lung tumorigenesis process [42]. Ellagic acid exhibit anti-inflammatory characteristics owing to its impact on the isoform of NO synthase, cyclooxygenase-2, tumor necrosis factor alpha and IL-6 attenuation *via* the suppression of NF-κB [43]. Heidarian et al. [40] declared that IL-6 downregulation and reduced IL-6 protein level in PC3 cells by GA leads to minimized pSTAT3, pERK, and pAKT signalling proteins which resulted in the lowering of the cell survival, propagation, and invasion in PC3 cells.

In the present research, 5-FU was observed to significantly downregulate STAT3 expression level in MDA-MB-231 cells, which is in convention with Pandey et al [44] who found that 5-FU can inspire moderate dose-dependent suppression in the STAT3 pool in gastric cancer cells. STAT3 is recognized as a potential transcription factor which transfers several growth signals from the cell membrane to the nucleus. It is implicated in numerous cellular processes encompassing proliferation, immune responses and survival. In a diversity of human malignancies, constitutive promotion of STAT3 is coordinated with poor prognosis and tumor progression [45]. 5-FU administartion prohibited the phosphorylation of STAT3 and its attachment to the promoter part of human telomerase reverse transcriptase (hTERT) [46]. Mounting studies propose that telomerase regulate not only telomeric elongation, but also modulate the cancer stem cell origination and conservation *via* the cellular reprogramming process [47]. In colorectal cancer cells (HCT116), these interrogators observed that upon 5-FU treatments the binding affinity of STAT3 is diminished to 0.7 fold relative to the untreated control. Deactivated STAT3 led to reduced attaching to the hTERT promoter

and consequently decreased telomerase activity which is serious to retain chromosomal stability over repeated cell division [46].

Gallic acid in the current consideration was noticed to significantly downregulate IL-6 expression level in MDA-MB-231 cells which coincides with Heidarian et al. [40] who introduced that gallic acid can significantly downregulate IL-6 expression in PC-3 prostate cancer cells at the gene and protein level. Increased levels of IL-6 in cancer patients could be allied to enhanced expression in typical immune cells as a consequence of chronic (systemic) inflammation, and/or from stromal or cancer cells within tumors. The generating motivation of the STAT3 transcription factor in cancer cells is essential for IL-6-dependent oncogenic actions like reinforcing cancer cell proliferation and survival [48].

STAT3 heightening has been noted to assist cancer cell survival in the conditions of growth factor deficiency [49] and to motivate chemoresistance in cancer cells subjected to hypoxia [50], proposing that STAT3 may be a potentially paramount cancer cell survival factor in cancers treated with antiangiogenic medications. Thus, IL-6, a multifunctional cytokine, which is implicated in the expansion and differentiation of several kinds of malignant cancers, encompassing breast, ovarian, lung, cervical, and prostate carcinomas [51]. Ellagic acid suppresses the generation of inflammatory mediators by hindering NF-κB promotion *in vitro* [52]. NF-κB and inflammation designate a positive feedback loop in the milieu of inflammatory sites to prompt cellular and DNA damage herewith persuades cell proliferation, ultimately leading to cancer progression [53]. NF-κB has been recognized to exert crucial roles in regulating the genes expression accountable for inflammation, proliferation and apoptosis. A growing body of evidence hypothesized that NF-κB modulates inducible enzymes and cytokines iNOS, COX-2, TNF-α, IL-2 and IL-6 expressions [54]. Thus, the anti-inflammatory property of ellagic acid is related to diminished NF-κB expression in 1, 2-dimethylhydrazine-inspired rats, which is one of the considerable mechanisms for the attenuation of colorectal cancer by ellagic acid [43].

Various clues have indicated that TNF-α and IL-6 expression levels are dependent on the stimulation of NF-κB which activates inflammation [55], thence initiating a positive auto-regulatory loop that can exaggerate the inflammatory reaction and elongate the period of chronic inflammation. These outcomes propose that suppression of NF-κB action by ellagic acid adjusts inflammatory processes *via* inhibition of inflammatory cytokines. Thus, ellagic acid is capable of hindering the colonic inflammation *via* the involvement of NF-κB pathway, therefore, consequently reducing the inflammatory factors COX-2, iNOS, TNF-α and IL-6. These impacts could be allied to the antioxidative nature of ellagic acid, which reveals its chemopreventive potential through regulation of inflammation-related mediators which are one of the major generators for colorectal cancer development [43].

In the present approach, 5-FU was found to significantly downregulate IL-6 expression level in MDA-MB-231 cells. Shibata et al [56] declared that 5-FU can insignificantly minify serum IL-6 level of mice having colon cancer. Eventually, they also documented that BRMS1 knock down augments IL-6 expression and motivates these processes; stipulating that the repressive impacts of BRMS1 on IL-6 expression are dependent on NF-κB [57]. Aota et al [58] observed that across management of a human salivary gland cancer cell line (cl-1) with 5-FU, the NF-κB action is repressed in a time-dependent way. This suppression was arbitrated by a prohibition of the disintegration of the repressory IkB-α protein. More specifically, a reduction in either the kinase activity of IkB kinase (IKK), or the ubiquitination or proteasome-mediated disintegration of IkB-α could be accountable for the 5-FU-mediated suppression of NF-κB action [59]. Therefore, the suppression of NF-κB by 5-FU could be the mechanism accountable for the attenuation of IL-6 gene expression in the present study. As, Karin [60] mentioned that through the capacity of NF-κB to amplify the expression of tumor promoting cytokines, like IL-6 or TNF-α and survival genes, like BCL-X_L it offers a pivotal connection between inflammation and tumor.

A large body of literature proposes that IL6 motivates STAT3 and in addition, the study of Eichten et al. [61] implies a critical function for the IL6/STAT3 pathway in opposition to anti-VEGF medications. Remarkably, this research group has converged on autocrine IL6/STAT3 signalling in the tumor cells. Autocrine IL6 signalling has been recorded in several tumors, encompassing multiple myeloma [62], lung cancer [63], and breast cancer [64].

In the current research, gallic acid was recorded to significantly downregulate VEGF expression level in MDA-MB-231 which is in conformity with Vanella et al. [65] who annotated that gallic acid can repress the VEGF protein level in prostate cancer cells. Angiogenesis process is coordinated by equilibrium between pro- and anti-angiogenic mediators. Over breast cancer development, angiogenesis transpires when the action of enhancers overrides that of suppressors. Angiogenesis is a multistep process, controlled by equilibrium between motivatory and repressory mediators secreted by the cancer and its microenvironment. Thence, angiogenesis process could be a substantial target to attenuate tumor growth and metastasis. Angiogenesis is desired at almost every step of tumor progression and metastasis, and tumor vasculature has been specified as a powerful prognostic marker for tumor grading [66].

After invasion of vessel into tumor masses of breast, there are at least six diverse angiogenesis-related growth factors released, among which VEGF is the most dominant angiogenesis enhancer [67]. VEGF, the most paramount angiogenic signal protein, motivates tumor neo-angiogenesis by augmenting mitogenic and survival characteristics of vascular endothelial cells. VEGF exemplifies the eminent angiogenic factor that boosts the proliferation, survival, migration and tube origination of endothelial cells [65]. Two kinds of receptor tyrosine kinases (RTKs), namely VEGFR-1 (Flt-1) and VEGFR-2 (KDR in human/ Flk-1 in mice) are fundamentally interposed with high affinities the particular maneuver of the VEGF on the endothelial cells. From these receptors, VEGFR-2 exerts a greater crucial part in mediating the permeability of endothelial cells and mitogenesis. Promotion of VEGFR-2 contributes to phosphorylation of various downstream signals encompassing AKT, JNK, and ERK that in turn motivates proliferation, migration, and tube origination of endothelial cells [68].

Thus, suppression of angiogenesis may exemplify a portentous therapeutic modality for cancer. Several phytochemicals could have a potential as anti-angiogenic factors fit to regulate cancer development and metastasis [66]. Wang et al. [68] cited that ellagic acid exhibits effective anti-angiogenesis potentialities by particularly targeting VEGFR-2 and its signalling pathway in breast cancer.

The in-silico analysis was performed to examine the structure-based interaction between ellagic acid and VEGFR-2. The conclusion of this analysis uncovered that ellagic acid considerably restrains a chain of VEGF-stimulated angiogenesis processes encompassing proliferation, migration, and tube origination of endothelial cells. What's more, it promptly restrains VEGFR-2 tyrosine kinase action and its downstream signalling pathways encompassing PI3K/Akt and MAPK in endothelial cells.

Furthermore, the neo-vessel origination in the chick chorioallantoic membrane and sprouts formation of chicken aorta is distinctly blocked by ellagic acid. The study of breast cancer xenografts disclosed that ellagic acid considerably hinders the growth of MDA-MB-231 and the expression of P-VEGFR2. Molecular docking imitation specified that ellagic acid can form aromatic interactions and hydrogen bonds within the ATP-binding site of the VEGFR-2 kinase unit. Conclusively, ellagic acid could exert anti-angiogenesis impacts through VEGFR-2 signalling pathway in breast cancer [68].

By functionally coupling with VEGF, VEGFR-2 would experience autophosphory-lation fundamentally at Tyr1175 regions within its intracellular kinase domain and consequently commence a chain of downstream signal transductions to endothelial cells [69]. The observations Wang et al [68] uncovered that ellagic acid can block Tyr1175 phosphorylation of VEGFR-2 triggered by VEGF as well as VEGFR-2 tyrosine kinase action. Moreover, these investigators elucidated that various MAPK signalling arbitrators comprising p-AKT, p-JNK, p-ERK, and eNOS are prohibited *via* ellagic acid. Additionally, it was also observed that ellagic acid dose dependently

blocks MMPs action, affirming the former outcomes that reduced action of MMPs might be also accountable for hindering with the binding of VEGF to VEGFR-2, and subsequently suppressing the neo-angiogenesis process [70].

5-FU in the current consideration was noticed to significantly downregulate VEGF expression in MDA-MB-231 cells, which is in conformity with the findings of Kensara et al [37] who declared that 5-FU can suppress the protein level of VEGF in colorectal cancer tissue of rats. 5-FU has been recorded to block the NF-κB pathway and NF-κB-arbitrated NO generation in cancer cells. Moreover, selective suppression of NF-κB and COX-2 has manifested to motivate the *in vitro* cytotoxic impacts of 5-FU on colorectal carcinoma cells and attenuate VEGF generation and vascular density in tumor tissues [71]. Besides, IL-6 in specially manifests a familiarity with HIF-1 and VEGF, which is principally driven by STAT3 signalling. For instance, applying chromatin immunoprecipitation assays, Niu et al. [72] documented that STAT3 connects promptly to the VEGF promoter and an energized STAT3 mutant (STAT3C) could efficaciously augment VEGF and tumor angiogenesis. Furthermore, close correlations have been specified between IL-6 and hypoxia inducible factors (both HIF-1 and HIF-2), which advocate an alternative connection between this pleiotropic cytokine and VEGF regulation [73]. Therefore, the down expression of VEGF gene by 5-FU in the present study may be linked with the downregulation of the STAT3/IL-6 axis.

Gallic acid in the present research was registered to significantly downregulate Slug expression level in MDA-MB-231 cells which coincides with Takahashi et al [74] observations who cited that epigallocatechingallate can considerably prohibit the upregulated expression level of Slug in the human non-small cell lung cancer cell lines H1299 and Lu99. Promotion of EMT programs is triggered by various biological molecules, like TGF-ß and TNF-α, and also by mechanical stimuli, like perturbation of cell–cell adherent co-junction and strength of extracellular matrix (ECM) in the tumor microenvironment [75]. The mechanical stimuli encouraged the expression of mesenchymal proteins (vimentin and N-cadherin) and transcription mediators (Slug) in a subpopulation of cells at a prime edge of scratch [76]. Vimentin, Slug, and N-cadherin are mesenchymal proteins incorporating to the molecular phenotypes of EMT. The investigation of Takahashi et al [74] registered that epigallocatechin gallate (EGCG) prohibits EMT molecular phenotypes, like the suppression of cell motility and expression of vimentin and Slug, and elevate in cell stiffness generating rigid elasticity of cell membrane. The expression of Slug and vimentin in human non-small lung cancer cell lines Lu99 and H1299 is correlated with reduce Young's modulus and high migration prospect. These events have been recorded to be blocked with EGCG and other green tea catechins, preserving the mesenchymal proteins steady-state level. Thus, the suppression of mechanical and biochemical EMT phenotypes with EGCG is a mechanism-dependent repression of cancer metastasis.

As is recognized, ß-catenin intercedes the promotion of Slug and overexpression of Slug will, in turn, oppress E-cadherin, the epithelial junction protein, which has a fundamental part in preserving the epithelial integrity [77]. TGF-ß enhanced the binding of ß-catenin to the Slug promoter, thus motivating Slug expression. It has been declared that ellagic acid considerably reduced ß-catenin in colon cancer cells [78]. Besides, gallic acid has been recorded to significantly diminished TGF-ß in rat modeled liver fibrosis [79]. These findings lead us to speculate that gallic acid could downregulate Slug gene expression in MDA-MB-231 cell line *via* prohibiting TGF-ß-stimulated ß-catenin nuclear translocation and the binding to the Slug promoter; thus, it could extremely revoke the interactions between ß-catenin and Slug. This elucidates the fundamental part of the Slug in the anti-metastasis impact of gallic acid.

In the current investigation 5-FU was recorded to insignificantly upregulate Slug expression in MDA-MB-231 cells. Chung et al [46] declared that Slug control is sensible to 5-FU in DLDI and HCT116 colon cancer cell lines. ß-catenin is a multifunctional protein that has been proposed as a predominant molecule for loosening cell-cell interactions in malignant switched epithelial cells. Adherent cell-cell connections are based on α-

catein/cadherin communication and ß-catenin/α-catenin binding [80].

Across destabilization of cell-cell adhesion and lack of E-cadherin expression, membranous ß-catenin is released into the cytoplasm. The increased cumulation of ß-catenin in the cytoplasm leads to its nuclear translocation. ß-catenin behaves as a cofactor of transcriptional controllers, like T-cell factor/lymphocyte enhancing factor (TCF/LEF). This pathway causes the upregulation of several target genes, like the zinc-finger protein Slug, vimentin, and matrix metalloproteinase-9, which are required for dysregulation of cell-cycle progression, tumor progression, migration and invasion. It has been noted that, the attenuation of secreted cytosolic ß-catenin in HPV16-positive squamous cell carcinoma (SCC) displays no significance after handling with 5-FU [81]. In addition, for head and neck squamous cell carcinoma (HNSCC) cell lines 11A (HNSCC11A), there was no significant depression of ß-catenin expression levels by 5-FU regardless of the utilized drug concentration or incubation period relative to the negative control. Thus, the insignificant upregulation in Slug gene expression level in the present work could be attributed to the insignificant impact of 5-FU on ß-catenin expression.

Gallic acid was recognized for downregulate Snail expression level in MDA-MB-231cells in the present consideration which is in comparable with the outcomes of Huang et al [82] who recorded that the EtOAc (ethyl acetate) extracts of Polyphenol-rich *Avicennia marina* leaf downregulate Snail protein expression in MDA-MB-231 cells after 24 h. Besides, ellagic acid has been observed to amplify the expression of E-cadherin and block the expression of Snail, matrix metalloproteinase MMP-2 and MMP-9 in human glioblastoma (U87) xenograft tissues [83]. The EMT motivating transcription mediators Snail and Slug are often theorized as exhibiting comparable impacts; even so, they control multiple non-overlapping genes. Genome-wide analysis of Snail and Slug promoter recruitment indicates that Snail binds at ~8000 promoter regions while Slug binds at about 1500. The Slug is expressed mostly in the basal cells of the typical mammary duct while Snail is expressed in stromal fibroblasts, suggestive of their diverse biological functions in normal mammary gland biology [84].

The TGF-ß pathway is a main regulator of EMT owing to its capability to motivate numerous transcriptional pathways that substantially harmonize to boost a cell towards a mesenchymal phenotype. In canonical TGF-ß signalling, TGF-ß joins the TGF-ß receptor I, a serine/threonine receptor kinase, which transactivates TGF-ß receptor II to both recruit and phosphorylate Smad2 and Smad3. Activated Smad2/3 translocates into the nucleus along micro-tubules in a dynein-dependent manner. Phosphorylated Smad2 and Smad3 demand dynein-light chains km23-1 and km23-2, respectively, for their nuclear relocation. In the nucleus, stimulated Smad2/3 congregates with Smad4 to control target gene expression, comprising Snail1, Snail2, Zeb1, and Twist1, genes predominant to EMT [85]. Therefore, the suppression of TGF-ß by ellagic acid might be the implicating mechanism by which gallic acid could cease the premier step in the transcriptional modules that impose the cell towards the mesenchymal phenotype with consequential down regulation of Snail expression.

In the present work, 5-FU was noticed to significantly upregulate Snail expression in MDA-MB-231 cells. 5-FU-manifested motivation of TGF-ß pathway has been registered in human colorectal cancer cells both *in vitro* and in a xenograft model [86]. It has been recorded that TGF-ß instigated epithelial to mesenchymal transition (EMT) in PC-3 prostate cancer cells in parallel with activation of Snail, twist, and Slug gene expression [87].

Gallic acid in the present investigation was found to significantly downregulate MMP-7 expression level in MDA-MB-231 cells, which is in regularity with the data of Nowakowska and Tarasiuk [16] who mentioned that GA (30, 60, 100 and 120 μM) and EGCG (5, 10 and 20 μM) elicit a comparable concentration-based suppression of MMP-2 and MMP-9 activity in MCF7/DOX and MCF7/DOX500 cells. It is excessively recognized that cancer cells enduring EMT may manifest the elevation of particular secretion mediators, like cytokines, growth factors and chemokines, which could be favorable for tumor progression [88]. As a family of zinc-dependent proteases, MMPs

are in a locality to humble the components of ECM, which motivates tumor invasion. In the study of Wang et al. [83], it was noted that EA represses glioblastoma cell invasion in the matrigel invasion assay. Furthermore, EA blocks glioblastoma invasion in the U87 xenografted model by motivating E-cadherin and prohibiting the expression of transcription mediators such as Snail. Although there are some data elucidating the efficacy of GA in matrix MMPs downregulation in several tumors [89], up to now very little is known in the literature about this activity in regard to opposed breast cancer. Nevertheless, the findings of Nowakowska and Tarasiuk [16] documented the capacity of this polyphenolic compound in minifying the activity of MMP-2 and MMP-9 in MCF7/DOX and MCF7/ DOX500 cells.

AP-1 is a substantial transcription mediator that contributes to MMP-7 expression. It has been recorded that EGCG alone motivates AP-1 DNA binding activity and GA curbs that activity. These outcomes propose that the suppressory impacts of GA on EGCG-stimulated MMP-7 may be related with downregulation of the AP-1 transcription activity [90].

From another point of view, the ADAM family belongs to one of the Zn-dependent metalloproteinases and ADAMs is well specified as ectodomain sheddases, and their domains work as metalloproteases. ADAM17 is recognized as a fundamental member of the ADAM family that is implicated in the release of several integrins from the cell surface and proteolysis of collagen IV of the ECM, proposing that ADAM17 impacts the invasive action of diverse cells encompassing glioma cells. For several EGFR pro-ligands, ADAM17 is considered as a major upstream component [91]. EGFR joining with ligands subsequently motivates PI3K/Akt and MEK/ERK pathways, which contribute to invasiveness and other malignant phenotypes [92]. Gallic acid considerably attenuates the phosphorylation of members of both Ras/MAPK and PI3K/Akt signal transduction pathways, which have been involved in cell proliferation, survival and invasion. These findings propose that repression of ADAM17 through gallic acid may be accountable for reduced invasiveness *via* the downregulation of Ras/MAPK and PI3K/Akt pathways.

Currently, in this study, 5-FU was observed to insignificantly downregulate MMP-7 gene expression level in MDA-MB-231 cells. This finding coincides with Fang et al. [93] who declared that the treatment of HT29 (colon cancer cells with high metastatic potential) with (0, 6.25, 12.5, 25, 50 μM) 5-FU for 72 hours display a trend of reduced expression of MMP-7 in a concentration based way. Crawford et al. [94] mentioned that there is a positive correlation between MMP-7 transcription and nuclear ß-catenin protein levels in colorectal cancer. Umbreit et al. [81] annotated that for HNSCC there is slight (insignificant) diminution of nuclear ß-catenin expression level by 5-FU when compared to the negative control. Thus, our results could be explained as that 5-FU has a slight effect on ß-catenin expression and hence it triggers insignificant downregulation of MMP-7 expression level in MDA-MB-231 cells.

Gallic acid in the current investigation was shown to significantly downregulate Notch-4 expression level in MDA-MB-231 cells, which harmonizes with the findings of Liu et al. [95] who announced that gallic acid can diminish Notch-1 protein expression in human glioblastoma. Notch signalling pathway exerts a major role in tumor cell survival, differentiation, and proliferation. In malignant tumors, the Notch signalling pathway has been elucidated to work with both oncogenic and anticarcinogenic impacts, according to various cell types [96]. An increasing amount of evidence has manifested the prominence of the modified Notch signalling pathway in the growth, apoptosis and differentiation in diverse malignant tumors [83]. Clue from the previous study indicated that the suppression of Notch expression can reduce PI3K/Akt activity [97]. Thus, repression of the Notch signalling pathway alone or in combination with PI3K/Akt may be conducive to gain maximum beneficial impacts. In the study of Wang et al. [83], it has been noted that ellagic acid hampers tumor growth *via* the repression of the Notch-1 and Akt signalling pathways in human glioblastoma U87 xenografted tissues.

In this research work, 5-FU significantly upregulated Notch-4 gene expression level in MDA-MB-231 cells. Various mechanisms are accountable for drug opposition in human cancers, comprising motivation of Wnt, sonic

hedgehog, and Notch signalling pathways [98]. Leong and Karsan [99] reveals that the Notch signalling network, is frequently dysregulated in several human malignancies, including breast, gastric, colon, and liver cancers. 5-FU has been recorded to significantly increase Notch-1 expression in gastric CSCs and these data propose that Notch signalling is implicated in autophagy-mediated chemoresistance [100].

Conclusion

The present research provides a solid foundation that gallic acid is a prominent chemopreventive lead compound in repressing the aggression of breast cancer. Targeted blockage of crucial signalling pathways compromised in breast cancer proliferation, differentiation, survival and metastasis is thought to be the molecular mechanism behind its promising anticancer prospective. Gallic acid works far better than 5-FU in stoppage of these molecular passages in the triple negative breast cancer cell line MDA-MB-231.

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