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# Regulation of Keratin-17 Expression and Cell Proliferation through *in vivo* Combination Therapy

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**Running title**: Modulation of Keratin-17 and cellular proliferation in skin cancer

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## Abstract

The attention on chemoprevention of non-melanoma skin cancer (NMSC) is expanding due to its rising occurrence. In this study, a mouse model of two-stage chemically induced skin cancers was used to assess the effects of topical combination therapy with six distinct compounds: celecoxib, 1,3-diaminodihydrochloride (DAP), ginger oil, grape seed proanthocyanidins extract, Avastin, and 5-flurouracile (5-FU). Nine groups of 108 mice each were established: as a normal control, DMBA + croton oil as the cancer group, single treated groups with each drug alone, and finally a combination group with all used drugs together. At first, a topically applied single dose of 100µg of DMBA dissolved in acetone (100µL). For two weeks, mice received two applications of 1% croton oil at a volume of 100 µL/mouse. All treatments were then applied topically for 17 weeks. The current findings indicated that tumor size was affected by single-drug treatments. On the other hand, combination therapy did not reduce the mice's body weight, but it did significantly slow the growth of tumors and increase animal survival. Additionally, the combined treatment downregulated the expression of the keratin-17 (K-17) protein in all treated groups and synergistically inhibited the proliferation of tumor cells, which may be related to the halting of cell growth.

**Keywords:** Non-melanoma skin cancer, DMBA, croton oil, combination therapy, Keratin-17, PCNA.

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## Introduction

Skin cancer ranks fifth globally in terms of incidence and is one of the deadliest cancers of the current decade (1). The World Health Organization (WHO) predicts that over 1.7 million new cases of melanoma and NMSC will occur worldwide by 2025 (2). The two primary subtypes of skin cancer are melanoma and non-melanoma skin cancer, which account for more than 90% of all skin cancers and are diagnosed in one out of every three cases worldwide (3).

The basal cells of complex epithelia, such as sebaceous glands and hair follicles, contain type I keratin 17 (K-17). Normal skin does not exhibit K-17 expression, but it can be caused by cancer, psoriasis, viral infections, stress, and tissue damage. Mateescu et al. (4) claim numerous biological

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processes are regulated by the multi-function protein K-17, including skin inflammation, cell division and proliferation, and skin appendage differentiation. Moreover, K-17 is regarded as an oncogenic protein, and a poor prognosis for various cancer types is frequently linked to its high expression (5).

One of the characteristics of tumor progression is altered rates of cell proliferation (6). Since Proliferating Cell Nuclear Antigen (PCNA) is a marker of cell proliferation, it is thought to be a helpful method for quickly determining the proportion of proliferating cells in tumors. Important for the S phase synthesis of chromosomal DNA, it binds with cellular proteins that regulate checkpoints and the cell cycle, and functions as an auxiliary protein of DNA polymerase. The nuclear non-histone antigen PCNA is found in the cell nucleus later in the G1 phase. In the S phase, it rises, and in the G2 and M phases, it lowers. According to various studies, PCNA expression can be used as a standard for early cancer detection and is a sign of cancer.

To increase the effectiveness of chemotherapy and lessen its side effects, recent studies have found that traditional Chinese medicine is a new source of neoadjuvant chemotherapy and anticancer medications (7). A common spice and traditional treatment for gastrointestinal problems, discomfort, and inflammation is ginger (8). Moreover, antibacterial, antiviral, and antifungal qualities have been discovered in essential ginger oil. (9,10). Flavonoids, sesquiterpenes, terpenoids, and phenylpropanoids are among its anti-cancer constituents (11,12). It has been demonstrated that the bioactive substances in this oil cause cancer cells to undergo apoptosis (13). Grape seed proanthocyanidins (GSP) exhibit chemopreventive and/or chemotherapeutic effects and are considered anti-cancer agents because they have been shown to prevent the growth of some cancer cells in both in vitro and in vivo settings (7).

In the synthesis of polyamines, ornithine decarboxylase (ODC) is the initial and rate-limiting enzyme. It has been demonstrated that ODC overexpression as an oncogene is indicative of tumor growth and progression in both humans and laboratory animals (14). Cancer could be avoided by manipulating cell cycle checkpoints with inhibitors that target the oncogenic ODC (15). One strong ODC inhibitor is 1,3-Diaminopropane dihydrochloride (DAP). Furthermore, a variety of cancers are treated with pyrimidines like 5-FU (16). Despite 5-FU's FDA-approved uses, doctors frequently apply it topically to treat a range of other skin disorders, including keratoacanthomas, vitiligo that is resistant to treatment, and melanoma's metastatic skin lesions. In dermatology, 5-FU can also be used to lessen keloids and hypertrophic scars (17).

The enzyme cyclooxygenase-2 (COX-2) is thought to be a key target for the development of novel cancer medications (18). Pain and inflammation associated with cancer are commonly treated with non-steroidal anti-inflammatory drugs (NSAIDs). By stopping COX-2, certain NSAIDs have been demonstrated to prevent the development and spread of skin, lung, breast, prostate, and colorectal cancers (19). A specific inhibitor of COX-2 called celecoxib has anti-cancer effects on a number of cancers, as ovarian cancer and adenomas. Bevacizumab (Avastin) is a humanized monoclonal antibody that targets VEGF A. By keeping nutrients from getting to tumor cells, it also has an antitumor effect. Furthermore, it enhances the mechanism of drug delivery into the tumor and normalizes the remaining blood vessels, enabling the drugs to reach the tumor in higher concentrations (20).

Thus, to investigate the potential multi-target combination therapy of various conventional chemotherapeutic agents, including 5-FU, celecoxib, Avastin, and DAP, which acquire multivariate genetic target pathways, this study examined potential synergistic profiles with

common nutraceuticals of ginger and GSPE against DMBA/croton oil–induced skin carcinogenesis *in vivo*.

## Materials and methods

### Chemicals

The natural oil from ginger (CAS No: 8007-08-7) was from Sigma-Aldrich in St. Louis, Missouri, USA. With CAS Number 84929.27.1, Mepaco Medifood Company, Sharkeiya, Egypt, supplied a very pure GSPE (purity 99.5% on HPLC). The supplier of DMBA was Sigma Chemicals, located in St. Louis, Missouri. Furthermore, a 1% dilution solution was made by mixing croton oil with acetone. The HPLC-grade celecoxib was imported from Sigma-Aldrich in St. Louis, Missouri, in the United States. The bevacizumab (Avastin®) 100 mg/4 ml was provided by Hoffmann-La Roche Ltd. 5-FU was acquired from the Australian company EBEWE Pharma Ges.m.b.H. Nfg.KG, located in A-4866 Unterach, (250 mg<5 ml). The DAP 98% at CAS Number-10517-44-9 was bought from the US mainland's Sigma-Aldrich in St. Louis, Missouri.

### Animals and Husbandry

Male Balb/c mice, between six and seven weeks, weighing 20 ± 5 g, were used in this investigation. They were purchased from Biological Products & Vaccines Holding Company (VACSERA) in Giza, Egypt. A 12-hour light/dark cycle, 25 ± 2 °C, and 65 ± 5% relative humidity were the normal laboratory settings maintained for the mice in this study. The mice were given a regular pellet diet and kept in polypropylene cages. Since the relevant ethics committees have approved either the human cell lines or mice used in this study, it has been carried out in compliance with the ethical guidelines established by the 1964 Declaration of Helsinki and its subsequent revisions. Under approval number IACUC-SCI-TU-0147, the protocol for the experiment was accepted by Tanta University Faculty of Science's Institutional Animal Care Committee and Research Ethical Committee (REC). This experiment did not use any human subjects.

### Experimental Design

Two days before the carcinogen was applied, 108 mice (12 per group) were divided into nine groups. After a 2 $cm^2$ section dorsal shaving of the mice, the following process was used: The normal control is G1. G2 (DMBA /Croton oil), while G3, G4, G5, G6, G7, G8, G9 treated with the same dose of DMBA+croton oil as in G2. Then each group was separately treated with each drug alone as follows: Ginger, GSPE, DAP, Avastin, 5-FU, and celecoxib, respectively, and finally a combination of all used drugs as G9. As initiator, DMBA was topically applied once at 100µg/100µL of acetone (100 µL/mouse), and as a promoter 1% croton oil was applied twice at 100 µL/mouse for two weeks. DAP (g/l) in drinking water (23), ginger (4 mg/mice) (21), and GSPE (120 mg/$cm^2$) (22) were applied topically twice weekly over the experiment time. Dissolving celecoxib (0.25 mg per 200 µL of acetone) and combining it with KY-gel to produce a concentration of 2 mg/200 µL for topical application in a volume of 200 µL three times a week (25). Avastin was diluted with sterile saline at a dose of 5 mg/kg twice weekly intraperitoneally (24). Water-based gel (KY-gel) was used to dilute the 5% 5-FU cream to 0.5%.

Skin tumors lasting at least two weeks and larger than 1 mm in diameter were recorded and included in the overall cumulative data. Additionally, Fialkow and Reddy noted the quantity of tumors in each cage as well as the delays in tumor appearance for every group (26). After the experiment, intraperitoneal sodium pentobarbital anesthesia (300 mg/kg b. wt.) was used to sacrifice all the mice (27). Tumor volumes (v) were computed using the formula V= (L X W2/2) following the use of calipers to measure each tumor mass's long (L) and short (w) dimensions (mm) (28). The tumors and specific skin sections were kept in buffered

formalin (10%) for standard histopathological analysis following sterile necropsy.

### Histological evaluation

A part of the affected skin tissues and the tumors were removed and stored in 10% buffered formalin before being embedded in paraffin. Tissue sections were cut into 4 µm-thick sections and embedded in paraffin. Following H&E staining, the skin sections and tumors were histopathologically diagnosed by at least two animal pathologists using a light microscope following Turusov et al. (29).

### Immunohistochemistry (IHC) for K-17

The following K-17 protein expression was found in tissues by IHC analysis: Sections at 4 µm thick were cut from the paraffin-embedded tissues. Following two hours of baking at 70°C, deparaffinization in xylene, and hydration in graded alcohol, the sections were exposed to 3% hydrogen peroxide for 20 minutes to inhibit the activity of the endogenous peroxidase. After being microwave-treated for antigen retrieval, the slides were incubated for 20 minutes with 10% normal goat serum. Then, tissue slides were incubated with a rabbit K-17 monoclonal antibody (ab109725, Abcam, Cambridge, MA, UK) on the tissue sections for the whole night at 4°C. Finally, a secondary antibody conjugated with biotin (SA00004-2, ProteinTech Group, Inc., Wuhan, China) was used to incubate the tissue slides for an hour at room temperature.

As previously stated, the sample was assessed using how intense the stain is and the proportion of positive cells (30). Staining intensity was evaluated using the following criteria: negative = 0, weak-positive = 1, moderate-positive = 2, or strong-positive = 3. Positive areas between 0 and 5% were stated by zero, those between 6 and 25% by one, those between 26 and 50% by two, those between 51 and 75% by three, and those between 76 and 100% by four. This scheme was used to assess the positive rate. Multiplying the coloration intensity by the positive values yielded the final value.

### Immunohistochemistry (IHC) for PCNA

Using the avidin-biotin complex technique, PCNA was prepared in 4 µm sections of skin tissue (31). The sections underwent a series of graded ethanol hydrations and xylene dewaxing before being rinsed in sodium citrate buffer (pH 6.0), autoclaved until boiling, and cooled to enable antigen retrieval. Later, sections were left overnight at 4°C in a solution of 0.3% H2O2, normal horse serum, and a rabbit polyclonal anti-PCNA antibody diluted at 1:500 (Product No. SAB2108448, Sigma). Each section underwent a TBS-T wash, 0.3% hydrogen peroxide treatment, and ABC peroxidase treatment (ABC Kit; Vector Laboratories) in that order. As a negative control, normal serum was employed in place of the primary antibody. The chromogen for all IHC reactions was 3,3'-diaminobenzidine tetrahydrochloride (DAB**)**. The PCNA labeling indices LI % was calculated by counting the number of positively stained cells or nuclei in each layer of each section and dividing that number by the total number of nuclei X100. Two pathologists who were not aware of the experimental protocol evaluated IHC in a blind manner.

### Statistical analysis

The results are presented as means ± standard deviation, and each experiment was conducted in triplicate. For the statistical analysis and data plotting, the assessment of ANOVA or the two-tailed t-test was utilized. Data presented as percentages were examined using the Chi-squared (X2) test in Microsoft Excel®. $P < 0.05$ was established as the cutoff point for statistical significance.

## Results

**Average body weights:**

The body weight of the mice over the duration of the 20-week period is shown in Figure 1. Body weight, the presence of papillary tumors on the

depilated skin, and mortality were recorded weekly. There were no treatment-related deaths in any group until the study was discontinued. There were no detectable statistical differences between the treated groups and normal growth rates. Finally, the body weights of all groups remained unchanged.

**Tumor morphological changes during chemically induced skin carcinogenesis in a group**

Weekly observations were made of the mortality rate and the presence of papilloma on the depilated skin. The first skin papilloma was found eight weeks after DMBA + croton oil was applied to every experimental group. Only a high and persistent tumor incidence was seen in G2 mice administered DMBA + croton oil (Figure 2). Furthermore, during the course of the experimental weeks, the volume or incidence of tumors significantly decreased in all treated groups (Figure 3).

In group 2, which was treated with DMBA and croton oil, only 10 out of 12 mice (80% incidence) developed skin tumors, with an average of 6.8 tumors per mouse (tumor multiplicities). Following ginger oil treatment (G3), GSPE (G4), DAP (G5), Avastin (G6), 5-FU (G7), and celecoxib (G8), tumor numbers were lower than in G2. In certain mice injected with DMBA + croton oil, the first sign of gross tumor masses was a tiny, stiff, nodular growth that grew quickly. Tumor volumes decreased by treatment with either a single medication or a combination of medications.

**Histopathological examination**

The control group's skin tissue normally appeared, with well-differentiated epidermis, dermis, and subcutis, per histopathological analysis of the skin sections (Figure 2, F). Large keratin pearls, a few papillomas, epidermal hyperplasia, and a variety of well-differentiated squamous cell carcinomas (SCC) were observed in the carcinogen-only treated group, on the other hand, there were also a number of tumors with dysplastic epithelium and anomalous epithelial cell proliferation that extended into the dermis (Figure 2, G-J). In G3 and G4, ginger and GSPE treatment resulted in fewer moderately differentiated papillomas and SCCs with fewer keratinized cells and hyperplasia (Figure 3).

**Following drug usage, either alone or in combination, decreased Keratin-17 expression in skin tissues**

The immunoreactivity of keratin-17 IHC staining in each group is illustrated in Figure 5. Compared to normal, the K-17 gene was highly expressed in the skin tissue of mice in group 2. Furthermore, following DMBA + croton oil, the skin tissue of mice in group 9 treated with each drug individually or in combination showed significantly lower K-17 expression after DMBA + croton oil than that of mice in group 2. Furthermore, K-17 expression was lowest in group 9 compared to all treated groups.

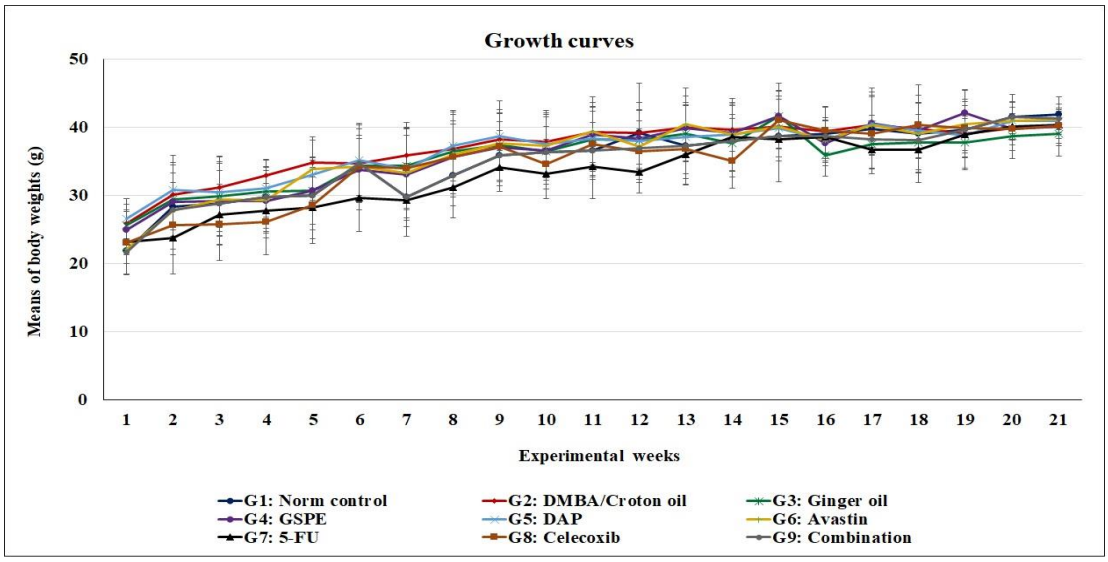


**Figure 1:** Growth curve of all experimental groups.

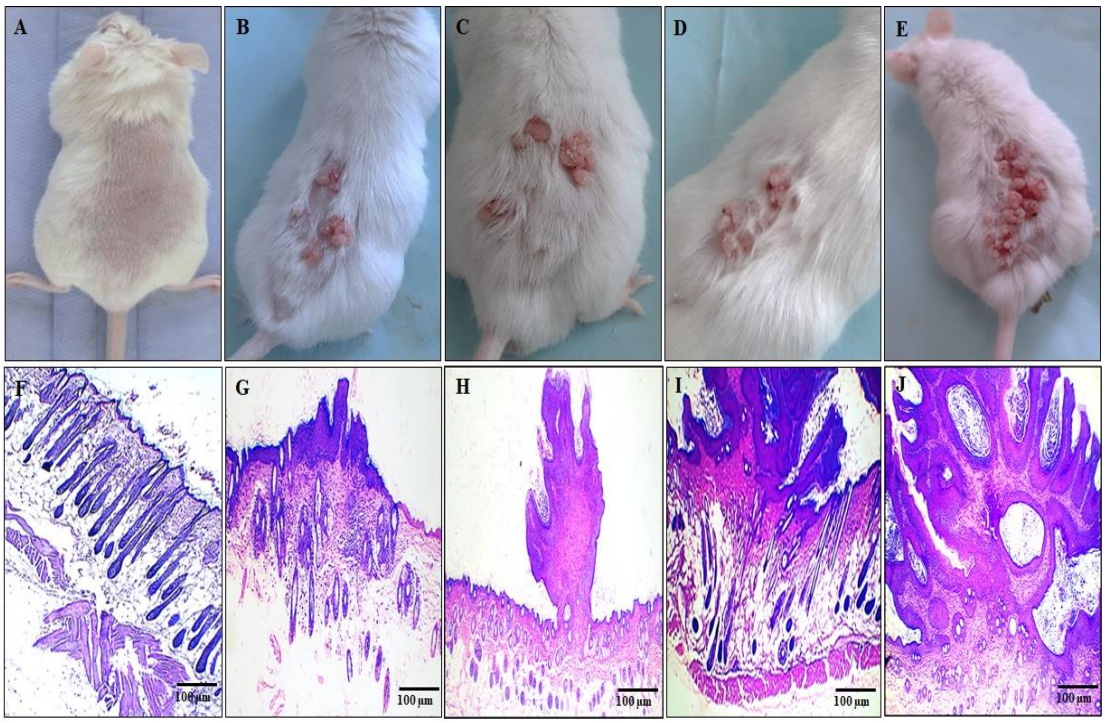


**Figure 2:** A-E: Morphological variations in tumor appearance during DMBA/Croton oil induced skin carcinogenesis. A: Normal control or vehicle-treated mice (G1); B-E: G2: DMBA+ Croton oil; F-J: Histopathological examination of H&E-stained skin section, X100. F: showing well-differentiated skin layers, epidermis, dermis, and subcutis in normal control (G1); G: Hyperplasia in DMBA+ croton oil skin cancer induced in group 2 (G2). H: Papilloma in G2; I: Keratoacanthoma and multiple Papillomatosis growth in G2; J: squamosa cell carcinoma in G2.

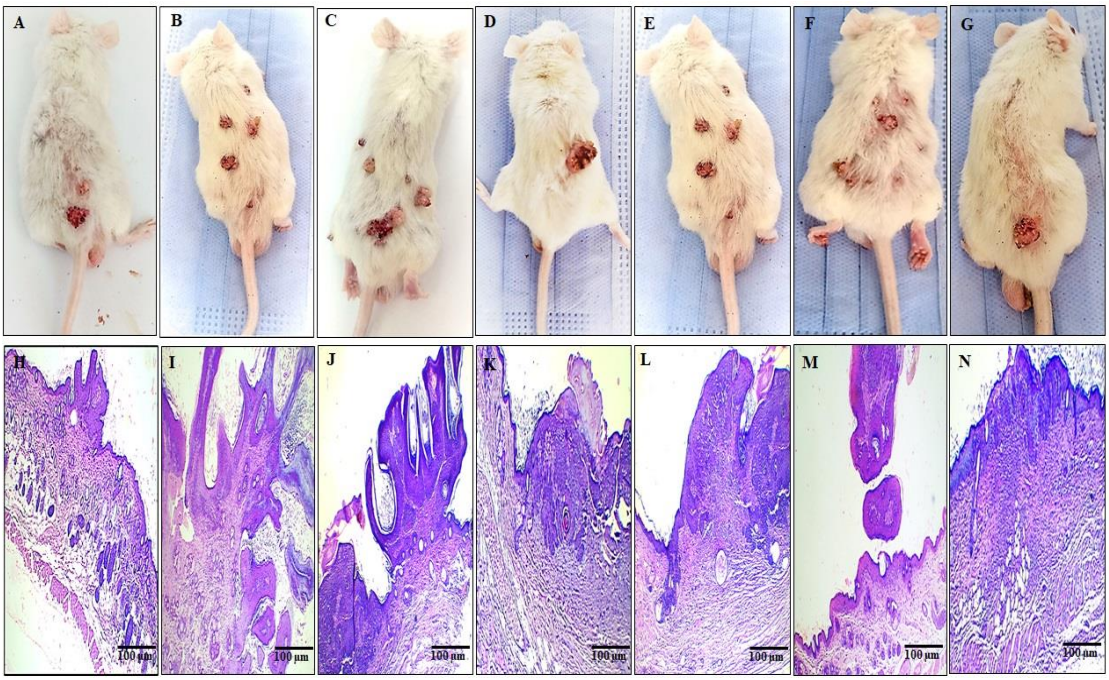


**Figure 3:** A-G: Morphological variations in tumor appearance during treatment with either a single drug or their combination after DMBA/Croton oil skin cancer induction. A: G3: ginger oil; B: G4: GSPE; C: G5: DAP; D: G6: Avastin; E: G7: 5-FU; F: G8: celecoxib; G: G9: Combination of all used drugs. H-N: The tumor size was only slightly affected by the single drug or its combination in the histopathological analysis of H&E-stained skin sections from the various treatment groups, but the tumor growth was significantly slowed down by combination therapy, X100.

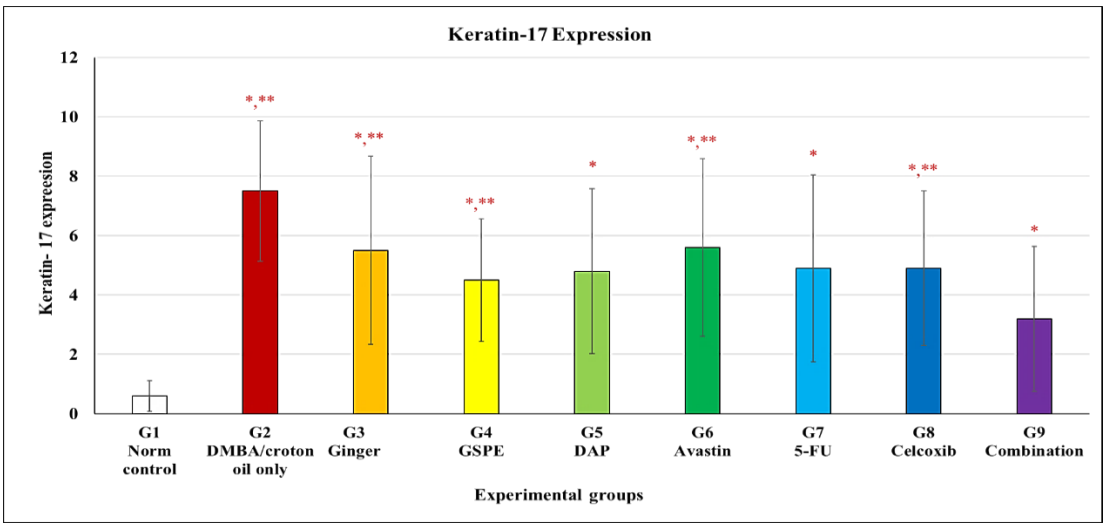


**Figure 4:** Quantification of immunostaining showed significantly few K-17 (IHC) expressions as compared to the (G2, $P<0.05$) (*), most significant low value compared to (G9, $P< 0.05$; *)

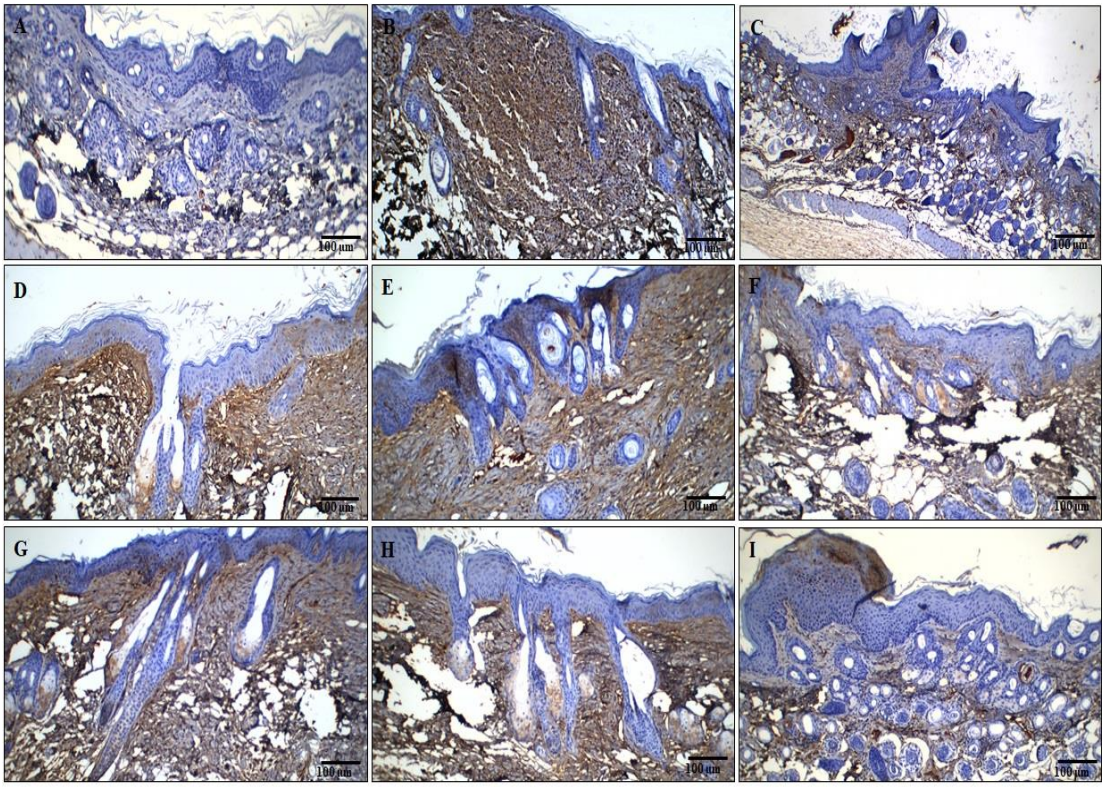


**Figure 5: Representative** images of expression patterns of Keratin-17, during chemical-induced skin carcinogenesis in different groups; A: weak expression of k-17 in normal control (G1); B: High intense of k-17 in (G2) DMBA+ Croton oil; C-H: Moderate expression of K-17 in treated groups, C: G3: ginger oil; D: G4: GSPE; E: G5: DAP; F: G6: Avastin; G: G7: 5-FU; H: G8: Celecoxib; I: G9: Weak expression in Combination of all used drugs. K-17, X100.

**Reduction of PCNA Labeling index (%) levels in either single or combination-treated groups in skin tissue**

Figure 7 displays the immunoreactivity of PCNA IHC staining in each group. Ten mice from each group had their skin tissue stained with PCNA IHC, which primarily showed nuclear brownish staining of varying intensities. Hematoxylin counterstaining caused PCNA-negative nuclei to appear blue. PCNA- LI (%) was computed separately in the skin of normal controls, in the tumors that developed in various groups receiving the carcinogen with or without treatment, and in the normally occurring hyperplasia and papilloma of DMBA/Croton oil-treated groups. In comparison to all other groups, the average LI (%) in normal control skin tissue (G1) was primarily lower.

Compared to normal skin, the skin of mice that received DMBA + croton oil in G2 showed many strongly positively stained PCNA nuclei. Furthermore, the mice's skin tissue in all groups treated with a single drug or a combination of drugs in G9 following DMBA/Croton oil exhibited noticeably lower PCNA-LI (%) compared to G2. Furthermore, mice in group 9 had the lowest PCNA-LI (%) across all tissues when compared to mice in groups 3, 4, 6, 7, and 8.

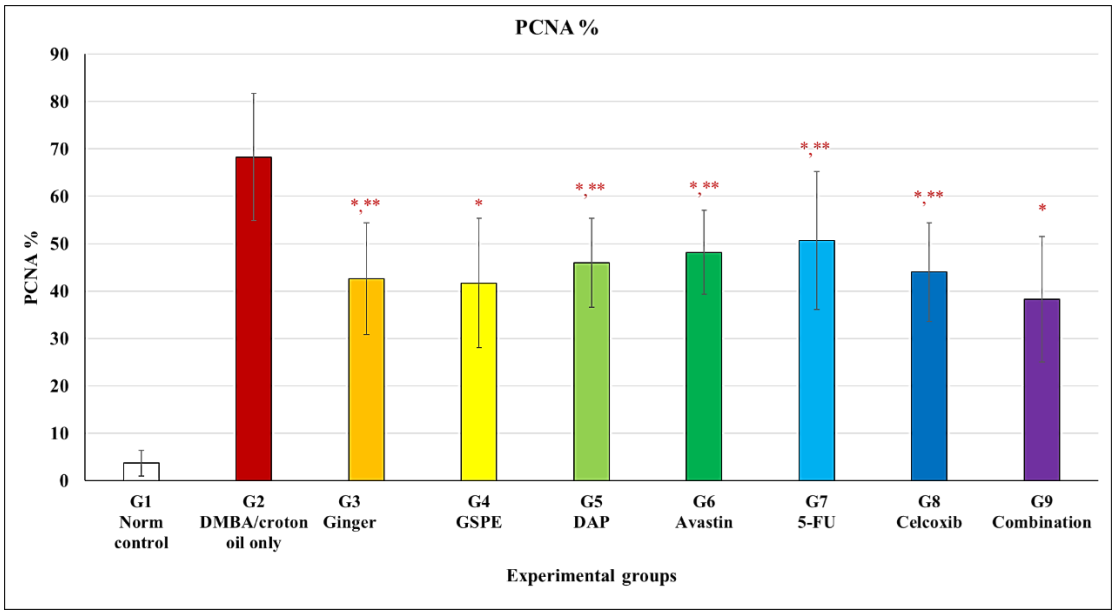


**Figure 6:** PCNA-LI (%) in the groups of the experiment; *: Significant vs G2 (*P<0.05*); **: Significant vs G9 (*P< 0.05*).

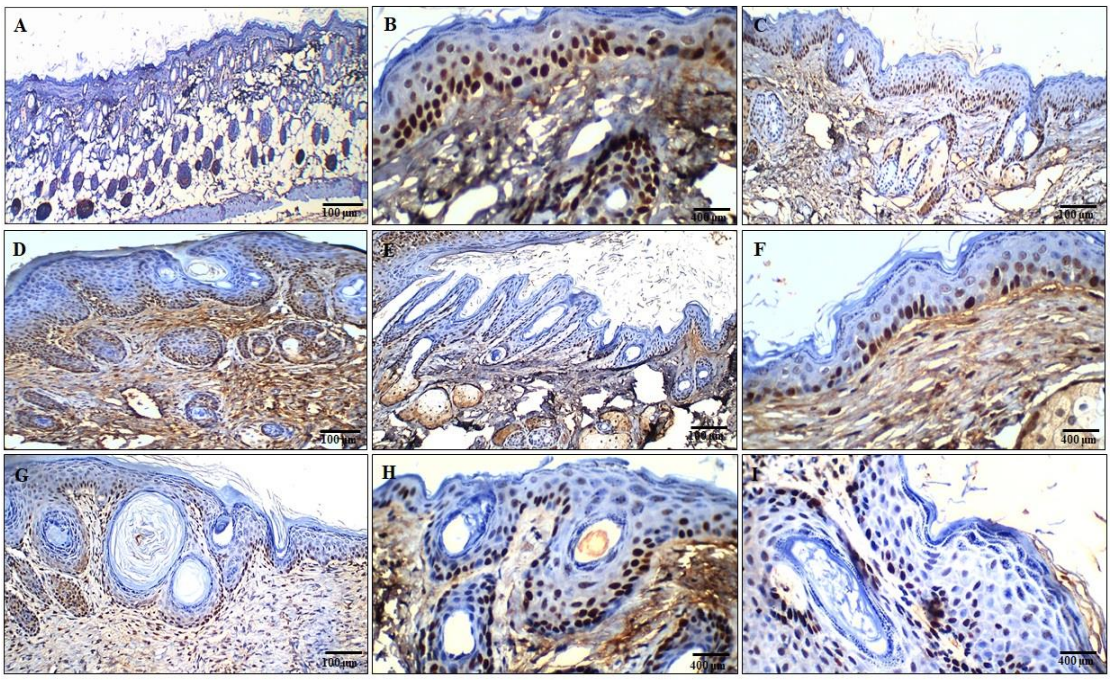


**Figure 7:** An IHC PCNA stained skin sections of chemical-induced skin carcinogenesis in different groups; A: less expression in normal control (G1); B: strong positive intensity in (G2) DMBA+ Croton oil; C: G3: ginger oil; D: G4: GSPE; E: G5: DAP; F:G6: Avastin; G: G7: 5-FU; H: G8: celecoxib; I: G9: Combination of all used drugs with weak expression. PCNA, X100&400.

## Discussion

Cancer of the Skin is expected to rise dramatically over the next 20 years if it is not detected early, endangering the global healthcare system. Drug resistance, clinical success, and novel drug identification pose significant challenges, even though the condition is curable at an early stage. Because of their effectiveness and safety record, natural products can be used to treat both malignant and inflammatory skin conditions. These anti-cancer drugs are often made from natural sources, such as marine, zoonotic, and botanical sources. Natural compounds should have anticarcinogenic properties through a variety of mechanisms, as cell proliferation inhibition, apoptosis potentiation, and metastasis suppression (32).

Our findings showed that K-17 expression was either moderate or weak in the groups that received either single or combination treatment, and only very strong in G2 (DMBA + Croton oil). For many types of cancer, K-17 is a prognostic, diagnostic, and predictive marker. Its high expression often correlates with tumor aggressiveness and has a poor prognosis (33). Moreover, the size, depth of invasion, and metastasis of the tumor are all correlated with high expression of keratin 17 in gastric cancer (34). Furthermore, it was demonstrated to predict a weak response to treatment in patients with HNSCC, especially those undergoing immune checkpoint blockades (35). K-17 is a poor prognostic biomarker for several cancers, including lung squamous cell carcinoma (LSCC) and pulmonary adenocarcinoma (LUAD) (36). Khanom et al. (37) state that K-17 has been shown to both cause and promote the growth of oral squamous cell carcinoma (OSCC). Furthermore, in non-small cell lung cancer (NSCLC), downregulating K-17 has encouraged colony formation, invasion, and cell proliferation (38).

Given that a rise in PCNA immunoreactivity is related to an increase in the proliferation of cells. This study evaluated the connection between PCNA expression and combination treatment for skin cancer. With an average of 70%, PCNA LI (%) in G2 (DMBA and croton oil) was the highest intense, whereas in treated groups, it was moderate or weak. This is in line with earlier research that demonstrated a favorable correlation between PCNA expression and OSCC histologic grading. PCNA was found to be expressed differently in normal and malignant lesions and normal and dysplastic epithelium. Furthermore, PCNA expression and histologic grading were positively correlated (6).

Applying ginger topically prevented mice from developing chemically induced carcinogenesis (Sreedhar et al., (39). Before application, ginger was topically applied to the skin, which significantly reduced the production of cyclooxygenase, lipoxygenase, and epidermal ornithine ODC activities triggered by 12-O-tetradecanoylphorbol-13-acetate (TPA). According to this, ginger extracts may have anti-tumor-promoting qualities (39). Furthermore, 6-gingerol has antiproliferative qualities against epidermoid carcinoma, inhibits growth, and triggers apoptosis. 6-gingerol kills epidermoid carcinoma cells by causing apoptosis, but it also prevents melanoma tumors from growing by disrupting the venous supply to the tumor (40).

Dietary GSPs prevented TPA-induced edema, hyperplasia, leukocyte infiltration, myeloperoxidase, COX-2 expression, and PGE-2 production in mouse skin, according to Meeran et al. (41). Targeting of COX-2 is a potentially effective treatment line for SCC in humans, as Gao et al. (42) showed that there is a potent anti-cancer effect against SCC in vivo from both chemical and genetic inhibition of COX-2. Celecoxib decreases deoxynivalenol-induced proliferation, inflammation, and protein kinase C translocation in mouse skin by changing downstream targets (43). Either *in vitro* or *in vivo* treatment of oral squamous cell carcinoma with 5-FU and bevacizumab produced synergistic and significant antitumor

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effects in HSC-2 tumor xenografts and induced apoptosis of tumor cells, according to Itashiki et al. (20). Furthermore, this combination treatment reduced the ability of cells to proliferate and micro vessel density (MVD) while increasing apoptosis in the remaining tumors.

In conclusion, our results provide more evidence that PCNA and Keratin-17 expressions may be sensitive and specific biomarkers for cell proliferation and differentiation in skin cancer. Furthermore, multi-targeted combination therapies are more effective than single treatment approaches.

## Abbreviations:

DMBA, 7,12-Dimethylbenzene[a] Anthracene, Keratin-17, K-17, NMSC, non-melanoma skin cancer, PCNA, Proliferating cell nuclear antigen.

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## Declarations

### Data availability

All data generated or analyzed during this study are included in this published article. If detailed data are required, they can contact the corresponding author of the study

### Ethics approval and consent to participate:

Since the relevant ethics committee has approved all the human cell lines and mice used in the study, it has been carried out in compliance with the ethical guidelines established by the 1964 Declaration of Helsinki and its subsequent amendments. The Research Ethical Committee (REC) and the Institutional Animal Care Committee at Tanta University's Faculty of Science's Zoology Department approved the experimental protocol (No. REC/IACUC/SCI/TU/0147).

### 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.

### Author Contributions

EIS, conceptualization of the research idea, EIS, EMY & MS experiments, methodology development, data collection, interpretation of results, and writing review & editing. EIS, MMH, EMY & MS methodology development, interpretation of results, data collection, writing-review & editing. All authors read and approved the final manuscript.

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