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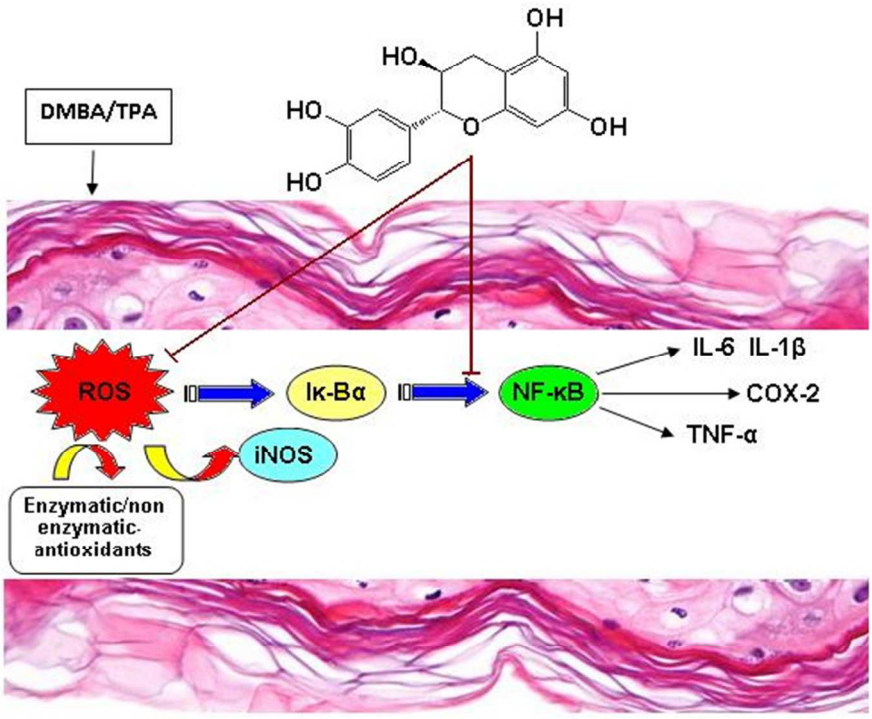

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Topical (+)-catechin emulsified gel prevents DMBA/TPA-induced squamous cell carcinoma of skin by modulating antioxidants and inflammatory biomarkers in BALB/c mice

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Abstract

An emulsified gel of (+)-catechin was developed and evaluated topically against 7,12-dimethylbenz(a)anthracene-induced and 12-*O*-tetradecanoylphorbol-13-acetate-promoted (DMBA-induced and TPA-promoted) squamous cell carcinoma of the skin in BALB/c mice. The biological evaluation outcomes indicated that the (+)-catechin emulsified gel increased the activity of oxidative stress biomarkers glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx), whereas it decreased the level of malondialdehyde (MDA). The mechanistic study showed that genes implicated in the inflammation and cancer, such as cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-κB), and inducible nitric-oxide synthase (iNOS) were down-regulated by (+)-catechin emulsified gel while inhibiting an inflammatory mediator prostaglandin E_2 (PGE_2). The (+)-catechin emulsified gel further suppressed the activity of pro-inflammatory cytokines, *viz.* tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). The *In vitro* permeation study revealed that release of (+)-catechin from an emulsified gel base was reached at steady-state after 6 h, while pH of the entire emulsified gel was found to be in the range of between 6.2 and 6.5 that falls well within the normal pH range of the skin.

***Keywords*:** Topical (+)-catechin emulsified gel base, DMBA/TPA-induced squamous cell carcinoma, oxidative stress biomarkers, Cyclooxygenase-2, Nuclear factor-kappa B, Cytokines.

Introduction

A number of environmental and genetic factors are involved in skin cancer initiation and promotion; however, exposure to chemical carcinogens and solar ultraviolet (UV) radiation are primarily responsible for skin cancer.[1] In recent years, it has been found that oxidative stress plays a major role in the development of skin cancer and therefore, efforts are being made to identify naturally occurring antioxidants that can prevent and slow down or reverse the process of carcinogenesis.[2] Chemoprevention of skin cancer by consumption of naturally occurring botanicals appears a practical approach and hence, world-wide interest is considerably increasing to use these botanicals.[3] Polyphenolic compounds constitute one of the largest groups of phytochemicals and are important part of the human diet, with flavonoids and phenolic acids being the most common in the food.[4] These polyphenols have been found to exert antioxidant,[5,6] anticancer,[7,8] antiviral,[9,10] antibacterial,[11,12] and anti-inflammatory[13,14] actions in both *in vitro* and *in vivo* models. One such polyphenol, which has attracted a lot of interest in the last few years is (+)-catechin (Fig. 1). The (+)-catechin is widely distributed in various plants like *Camellia sinensis* (tea) and *Acacia catechu* Willd.[15] In our previous research, we found that (+)-catechin rich aqueous extract of *Acacia catechu* showed chemopreventive effects against skin,[16] liver,[17] and breast cancers.[18] At the same time, we reported the growth inhibition and apoptosis induction potential of (+)-catechin against hepatocellular[19] and breast carcinoma.[20] These results indicated the potential of (+)-catechin as the cancer chemopreventive agent. But unfortunately, dietary polyphenols are subjected to first-pass metabolism by the gastrointestinal tract and liver, which results in extensive modification of the administered compounds and decreased bioavailability.[15] The topical delivery of polyphenols is an attractive approach to bypass the first-pass metabolism and at the same time, it can be directly applied to the area of interest.[21] Keeping aforementioned

summary in mind, we have developed and biologically evaluated a topical emulsified gel of (+)-catechin against DMBA-induced and TPA-promoted squamous cell carcinoma of the skin in mice.

Materials and Methods

Chemicals and general experimental methods

Chemicals for the formulation of (+)-catechin emulsified gel were purchased from Loba Chemie, Spectrochem, and Finar, India. HPLC solvents were purchased from Fischer Scientific and Merck, India, while reagents and chemicals for *in vitro* and *in vivo* biological studies were purchased from Sigma Life Sciences, Sigma-Aldrich, and HiMedia, India. Antibodies against COX-2, NF-κB, and iNOS were obtained from Santa Cruz Biotechnology, Santa Cruz, CA (USA). For thin layer chromatography, Merck TLC plates silica gel 60 F_{254} (Merck, Germany) were used. The melting point of isolated (+)-catechin was recorded on a digital melting point apparatus (Indosati scientific lab equipments, India). FT-IR spectrum was acquired with a PerkinElmer spectrum 400 FT-IR and FT-NIR spectrometer. For ^{1}H and ^{13}C NMR recording, a Bruker AVANCE II 400 NMR spectrometer was used at 400 and 100 MHz, respectively, while using $CDCl_3$ as a solvent and tetramethylsilane as an internal standard. The mass spectrum of (+)-catechin was obtained with a Waters Micromass Q-T of micro Mass spectrometer using an electrospray ionization at 70 eV. For HPLC studies, a Waters HPLC system with an empower software system 2.1 was used.

Extraction of *Acacia catechu* heartwood and isolation of (+)-catechin

Acacia catechu heartwood was collected in the month of September, 2011 from Hamirpur, Himachal Pradesh, India,. The plant material was taxonomically recognized and validated by Dr. Sunil Dutta, Scientist, National Medicinal Plant Board, Ayush, New Delhi, India. A voucher specimen (AC-2011) has been submitted in the herbarium of Department of Biotechnology, Bioinformatics, and Pharmacy, Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India.

1 kg of powder dried *A. catechu* heartwood was taken in a stainless steel pot containing 10 L of distilled water. The mixture was boiled for 5 h, kept at room temperature for 24 h. The resulting solution was filtered through a fine muslin cloth to discard the suspended materials. The filtrate obtained was subjected to evaporation and in this process; residue left behind was air-dried to yield a partially purified solid mass of (+)-catechin (224 g, 22.4% w/w).

150 g of solid mass obtained in the previous step and 1 L of distilled water were taken in a stainless steel container, boiled till complete dissolution, followed by filtration. The filtrate was evaporated to volume of 500 mL and allowed to stand for 24 h. The aqueous filtrate was discarded and the residue remained behind was solubilized in ethyl alcohol. The alcoholic solvent was evaporated to dryness under reduced pressure and the solid achieved was further dissolved in 500 mL of hot water and kept at room temperature for 24 h. The precipitates of (+)-catechin thus appeared were collected, air-dried, and further re-crystallized thrice with distilled water to give white crystals of (+)-catechin.

Yield: 39 g (3.9 % w/w). M.p.: 95–97 °C IR (ν max, cm^{-1}): 3305.00 (broad, five O–H stretch), 2933.57, 2854.25 (C–H stretch), 1628.55, 1610.07, 1520.72 (C=C stretch), 1469.15, 1286.88,

1146.94, 1030.30. ^{1}H NMR ($CDCl_3$+ DMSO.d_6 mixture, δ, ppm): 8.7826 & 9.0315 (4H, two broad signals, four OH groups), 6.7355–6.7403 (1H, d, *J* = 1.92 Hz, C-2′-H), 6.6756–6.6958 (1H, d, *J* = 8.08 Hz, C-5′-H), 6.5774–6.6027 (1H, dd, *J* = 8.16, 1.96 Hz, C-6′-H), 5.8837–5.8894 (1H, d, *J* = 2.28 Hz, C-7-H), 5.6976–5.7032 (1H, d, *J* = 2.24 Hz, C-9-H), 4.4670–4.4856 (1H, d, *J* = 7.44 Hz, C-2-H), 4.0228 (1H, broad signal, C-3-OH), 3.7993–3.8514 (1H, m, C-3-H), 2.6536–2.7070 (1H, dd, *J* = 16.08, 5.36 Hz, C-4-H_A), 2.3361–2.3961 (1H, dd, *J* = 16.04, 8.08 Hz, C-4-H_B). ^{13}C NMR ($CDCl_3$+ DMSO.d_6 mixture, δ ppm): 156.33 (C-8), 156.05 (C-6), 155.23 (C-10), 144.72 (C-4′ & C-3′), 130.50 (C-1′), 118.27 (C-6′), 114.92 (C-2′), 114.31 (C-5′), 98.96 (C-5), 95.04 (C-7), 93.80 (C-9), 80.98 (C-2), 66.39 (C-3), 27.67 (C-4). ESI-MS (negative-ion mode, *m/z*): Calculated exact mass: 290.1; Found: 290.1 (M^-) & 289.0 (M^-–1) (100%). The HPLC purity chromatogram of isolated (+)-catechin is shown in **Fig. S6**.

Development of (+)-catechin emulsified gel

The preparation of the gellified emulsion includes the preparation of the two phases, *i.e.* emulsion phase, followed by the addition of an emulsion into the aqueous solution of the gelling agent to form a semisolid emulsifying gel formulation. The oil phase of the emulsion was prepared by dissolving the 0.5 g of Span 60 in 4 mL of olive oil, while 2 mL of Tween 20 was dissolved in 4 mL of distilled water to obtain the aqueous phase. The (+)-catechin (120 mg) was dissolved in 5 mL of propylene glycol, and then 0.03 g of methyl paraben, 0.01 g of propyl paraben, and 0.1 g of disodium EDTA were added to the aqueous phase. Butylated hydroxy toluene (0.01 g) was added to the oil phase and both the oil and aqueous phase were heated to 60–65 °C, followed by the addition of an oil phase into the aqueous phase with constant stirring, until it cooled down to room temperature. The obtained emulsion was mixed with 1.5 g of the carbopol 940 to yield an elegant emulsified gel of the (+)-catechin.

pH determination of (+)-catechin emulsified gel

The pH of the prepared emulsified gel was determined by employing a digital pH meter. 1 g of the emulsified gel was stirred in distilled water, until a uniform dispersion was formed. It was kept aside for 2 h. The volume was then made up to 100 mL and pH measurement was performed. The test was performed in triplicate and the mean was calculated.

HPLC studies

The stock solutions were prepared by dissolving the 5.0 mg of standard (+)-catechin in 7 mL of diluents (0.1% formic acid in water and methanol, 3:7 v/v) with sonication for 15 min. The final volume of the solution was then diluted to 10 mL at room temperature. The solution was centrifuged at 12,000 × *g* for 2 min to obtain a clear solution and was filtered through a 0.22 µm filter. The quantification of isolated (+)-catechin was carried out by comparing the HPLC peak area of it with the standard (+)-catechin. The reverse-phase HPLC system, comprising of Waters HPLC 717 Autosampler, Waters C18 spherisorb symmetry column (5.0 µm, 250 mm × 4.6 mm), and photodiode array detector (Waters 2996) was used for HPLC studies. The mobile phase consisting of 0.1% trifluoroacetic acid (TFA) and acetonitrile (ACN) in a ratio of 85:15 v/v was used. Before HPLC run, the mobile phase was degassed using an ultrasonic water bath and filtered through a membrane filter of 0.22 µm pore size. The column was eluted in the isocratic mode at a flow rate of 1.0 mL/min. The injection volume was 20 µL and the (+)-catechin was detected at an absorbance wavelength of 279 nm. The run time of analysis was 25 min at 30 °C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards.

In vitro skin permeation study

An essential parameter in the evaluation of the drug delivery is the rate at which the drug is released from the carrier. The skin permeation studies of (+)-catechin emulsified gel were carried out by using a modified Franz diffusion cell. The cellulose membrane was mounted on modified Franz diffusion cell, maintained at 37 °C. A known quantity of the formulation was spread in the donor compartment, while phosphate buffer saline solution (pH 7.4) was used as the receptor medium. At predetermined time intervals (1 to10 h); samples (each of 3 mL) were withdrawn from the receptor medium and were suitably diluted. The samples were filtered for an analysis, while filling receptor medium with an equal amount of buffered solution to maintain a constant volume. The samples withdrawn were subjected to HPLC studies for the estimation of drug contents.

Animals

6–7 weeks old, male BALB/c mice, each weighing 25 ± 2 g, were housed in the departmental animal facility at conditions temperature 22 ± 3 °C, humidity 65 ± 5%, and 12-h light/dark cycle. The animals were fed a standard diet and water *ad libitum* and were acclimatized for 1 week, prior the experiments. The animal care and handling were performed as per protocol set by the World Health Organization (WHO, Geneva, Switzerland) and the Indian National Science Academy (INSA, New Delhi, India). The experimental protocol was approved by the Institutional Animal Ethics Committee (CPCSEA No. 13/RKN/BNCP-06).

Experimental design, tumor induction, and analysis

Following 1 week of adaptation, the mice were randomly separated into three groups (n = 10). The group 1 served as a control and was topically administered gel base devoid of (+)-catechin.

The group 2 received topical administration of DMBA for initiation of skin tumors (100 nmol/100 mL of acetone) for 2 weeks succeeded by TPA (1.7 nmol/100 mL of acetone) 3 times a week for 20 weeks. The group 3 mice along with the treatment of DMBA/TPA, also received topical administration of (+)-catechin gel (≈ 1 mg/cm^2 skin area) 3 times a week. In group 3, topical application of (+)-catechin was commenced 1 week prior to the topical application of DMBA. The percent of tumor bearing animals or tumor incidence rate, mean count of papillomas per mouse, average body weights of mice, and percent of mice, those survived the 20-week period were recorded at weekly interval. The papillomas, those existed for two weeks or longer were only taken into consideration for the final assessment of the tumor development.[22]

Biochemical parameters

The skin papillomas were excised and meticulously washed with cold phosphate buffered saline of pH 7.4. A 10% tissue homogenate (w/v) was prepared from the sample (skin papillomas) in 0.15 M Tris-HCl buffer of pH 7.4 using a mechanical homogenizer. Aliquots of skin papilloma homogenates were stored at 4 °C for the assessment of GSH, while the remaining homogenates were centrifuged in cold conditions at 10,000 x *g* for 30 min. The supernatant appeared on centrifugation was used for the quantification of MDA, SOD, CAT, GST, GR, and GPx.

Estimation of skin GSH

The level of GSH was quantified in terms of total non-protein sulfhydryl groups as per protocol reported earlier.[23] Homogenates (proteins) were readily precipitated with 0.1 mL of 25% trichloroacetic acid and precipitates were removed after centrifugation at 15,000 x *g* for 10 min, to obtain the protein-free supernatant. In free SH group estimations, to a 1 mL of the supernatant, 2 mL of 0.6 mM DTNB (prepared in 0.2 M sodium phosphate buffer of pH 8.0) was added, and

the optical density was read at 412 nm. Reduced GSH was used as a reference standard. The GSH content is presented as nmol/mg protein.

Quantitative estimation of skin lipid peroxidation (estimation of MDA)

Levels of lipid peroxides were quantified as per protocol described previously.[24] In short; thiobarbituric acid (0.8%), SDS (0.1%), and acetic acid (20%) were mixed with 100 µL supernatant of 10% tissue homogenate. The solution was then subjected to heat for 30 min, cooled, extracted with *N*-butanolpyridine, and the absorbance of MDA was measured at 532 nm. The level of MDA is presented as nmol/mg protein.

Estimation of skin SOD activity

The level of SOD in the skin cytosol was measured as per previously discussed method.[25] In this method, photo-oxidation of hydroxylamine hydrochloride generates superoxide anions, leading to reduction of nitroblue tetrazolium into the blue formazon, which is measured at 560 nm. The activity of superoxide dismutase is presented as U/mg of protein; where, 1 IU is identified as the quantity of enzyme inhibiting the increase in absorbance by 50%.

Estimation of skin CAT activity

The CAT activity was measured as per protocol described earlier.[26] To a 100 µL of the supernatant (prepared from 10% tissue homogenate, as discussed above) in 50 mM phosphate buffer of pH 7.0, 30 mM of H_2O_2 was added and the change in optical density was recorded at 240 nm. The level of the CAT is presented as U/mg protein; where, 1 IU is defined as amount of enzyme that degrades 1 mol of H_2O_2/min per mg of protein.

Estimation of skin GST activity

The cytosolic GST activity was estimated by measuring the absorbance in UV spectrometer at room temperature.[27] To prepare the substrate for reaction, 1.7 mL of 100 mM phosphate buffer (pH 6.5) was added to 0.1 mL of 30 mM CDNB (1-chloro-2,4-dinitrobenzene) and incubated at 37 °C for 5 min. The reaction was induced by the adding 0.1 mL of diluted cytosol or supernatant to the CDNB and the absorbance was read at 340 nm. The reaction mixture devoid of the enzyme was used as a control. The specific activity of GST is presented as μM of GSH-CDNB conjugate generated/min per mg protein at an extinction coefficient of 9.6/mMcm.

Estimation of GR activity

The GR activity was measured as per protocol accounted earlier.[28] A reaction mixture consisting of 0.2 M sodium phosphate buffer of pH 7.0, 2 mM EDTA, 1 mM oxidized glutathione (GSSG), and 1.2 mM NADPH was added to 25 μL of supernatant (prepared from tissue homogenate as discussed above) and the enzyme activity was estimated by observing the oxidation of NADPH indicated by a decrease in absorbance/min for a minimum of 3 min at 340 nm. One unit of enzyme activity is described as nmol NADPH consumed/min per mg protein at an extinction coefficient of 6.22/mMcm.

Estimation of GPx activity

Coupled assay method as reported previously was used to measure the GPx activity.[29] In brief, reaction mixture consisting of 50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 0.24 U/mL yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 1.5 mM H_2O_2 was mixed with the supernatant. The reaction was induced by adding NADPH and its oxidation was observed at 340 nm by recording the decrease in absorbance/min for duration of 3

min. One unit of enzyme activity is known as nmol of NADPH consumed/min at an extinction coefficient of 6.2/mMcm.

PGE_2 immunoassay for quantitation of prostaglandin E_2

Samples of skin papillomas were homogenized in 100 mM phosphate buffer of pH 7.4 containing 1 mM EDTA and 10 μM indomethacin. The supernatant was collected and the PGE_2 in supernatants was quantified by using the Cayman PGE_2 Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions.

Assay for pro-inflammatory cytokines

Skin papilloma homogenates from each treatment group were used for the analysis of cytokines, such as TNF-α, IL-1β, and IL-6 using ELISA kits following the manufacturer's instructions.

Preparation of lysates and western blot analysis

The samples of skin papilloma tissues/skin tissues were collected from at least three animals in each group and lysates for western blot studies were prepared as described previously.[30] Proteins (25–50 μg) were resolved using 10% Tris-glycine gel and transferred onto nitrocellulose membrane. Membrane was soaked in blocking buffer for 1 h, followed by overnight incubation at 4 °C with the primary antibodies. The membrane was then washed with PBS and further incubated with HRP-conjugated secondary antibody, as reported earlier.[20] Chemiluminescence producing reagents were employed to visualize the protein bands. To check equal loading of proteins on the gel, the blots were stripped and re-probed for β-actin.

Histopathological studies

At the end of experiments, animals were sacrificed by cervical dislocation and skin or skin papillomas tissues of the control and treated groups were subjected to the histopathological studies. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned 4–5 µm thick, and stained with hematoxylin and eosin. The slides were investigated by pathologists at the Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Statistical analysis

The data are presented as mean ± S.D. Statistical analyses were performed by one-way analysis of variance followed by Tukey's multiple comparisons test using GraphPad Prism 6.0. Values of $P \leq 0.05$ were considered significant.

Results

In vitro permeation and pH determination of (+)-catechin gel

For an effective topical formulation, an optimum drug release is the essential condition. Therefore, it was essential to measure the rate and degree of drug release from the emulsified gel base. The samples were withdrawn from the Franz diffusion cell and subjected to HPLC studies for the estimation of drug release from the prepared formulation at a regular time interval from 1 h to 10 h. Results indicated that 5.843 µg/mL of (+)-catechin was liberated after 1 h, it reached to 22.103 µg/mL after 6 h and thereafter, the (+)-catechin discharge remained steady (Table 1). To achieve the desired pH, triethanolamine was added to the formulation. The pH of the entire emulsified gel was found to be in the range of 6.2 to 6.5, which falls well within the normal pH range of the skin.

Effect of topical (+)-catechin gel on DMBA/TPA-induced papillomas in BALB/c mice

The animal group treated with DMBA/TPA (group 2) displayed a 100% prevalence of the skin papillomas. The tumor incidence rates in DMBA/TPA-administered and topical (+)-catechin-treated groups at various weeks are shown in Fig. 2A & 2B. The onset of papillomas was observed at week 7 in the DMBA/TPA-administered mice (group 2); and thereupon, a continuous increase in the tumor growth was seen that reached 100% during week 12. Group 3 mice, those received topical (+)-catechin gel right through the experiment, displayed a 30% prevalence of tumor incidence at the end of the experiment. In the topical (+)-catechin-treated mice, the appearance of skin papillomas was deferred until week 13. On the whole, a remarkable reduction (30% *vs*. 100%, $P < 0.0001$) in the occurrence of skin papillomas/tumors was noticed in the topical (+)-catechin-administered animals at week 20 compared with the DMBA/TPA-treated group (Fig. 2A). At the end of the study, the average number of papillomas/mouse in the (+)-catechin-treated group were also considerably less ($P < 0.0001$) than the DMBA/TPA-treated group. The mean count of papillomas/mouse in the (+)-catechin-administered group was 3.0, whereas in the DMBA/TPA-treated animals, it was found to be 6.5 (Fig. 2B). When gel base alone (without (+)-catechin) was administered on the depilated backs of animals, it did not induce any growth of the tumors. Throughout the experiment, no significant difference in the mean body weight was observed among all the three animal groups excluding the end phase of the study; when, a minor decrease in the mean body weight was observed in the group of DBMA/TPA-treated animals (group 2) (Fig. 2C). The survival rate of the DBMA/TPA-administered animals was also declined radically in comparison with the (+)-catechin-treated group ($P < 0.0001$). Wherein, at the end of study, 70% animals of the (+)-catechin-treated group survived the skin carcinoma, while at the same time, only 20% animals of the DMBA/TPA-

treated group were able to survive the skin carcinogenesis (Fig. 2D). The histological examinations disclosed the presence of subcutaneous tissues and normal skin in the control animals (group 1, Fig. 3A). The detailed examination of the hematoxylin and eosin stained sections of the papillomas/tumors developed on the application of DMBA/TPA, revealed the presence of characteristic squamous pearls (group 2). The presence of islands of dysplastic squamous epithelial cells in the dermis layer of DMBA/TPA-treated animals clearly indicated the invasive or malignant nature of the carcinoma. Necrotic keratinocytes were also observed during the histopathological examination of the papilloma/tumor tissues of DMBA/TPA-treated animals (Fig. 3B and 3C). Contrastingly, skin papillomas/tumors of topical (+)-catechin-administered mice showed integral basement membrane along with hyperplasia of the overlying epidermis, which undoubtedly suggesting the benign nature of these skin papillomas/tumors (Fig. 3D).

Biochemical studies in mouse skin papillomas

Effect on lipid peroxidation

The animal group treated with DMBA/TPA displayed a 62.86% increase ($P < 0.01$) in the lipid peroxidation activity, while animals treated with (+)-catechin showed only a 10.95% raise ($P > 0.05$ or ns) in the lipid peroxidation levels compared with the control group. In general, the (+)-catechin-treated group showed a 31.87% decrease ($P < 0.05$) in MDA contents when compared with the DMBA/TPA-administered group (Table 2).

Effect on GSH level

Mice treated with DMBA/TPA showed a remarkable 31.41% decrease ($P < 0.001$) in the GSH level, while mice treated with topical (+)-catechin displayed a mere 6.75% reduction in the GSH

level ($P > 0.05$ or ns) compared with control animals. A significant 35.97% increase ($P < 0.01$) in the GSH level of DMBA/TPA/topical (+)-catechin-treated mice was observed in comparison with the GSH level of DMBA/TPA-treated mice (Table 2).

SOD activity

DMBA/TPA-treated mice displayed a 61.09% decrease ($P < 0.001$) in the SOD level, while mice treated with topical (+)-catechin showed a 31.95% decrease ($P < 0.01$) in the SOD level compared with control subjects. Animals treated with topical (+)-catechin exhibited a mean increase of 74.90% ($P < 0.05$) in the SOD level in comparison with DMBA/TPA-treated animals (Table 2).

CAT activity

Mice treated with DMBA/TPA exhibited a 62.35% decrease ($P < 0.001$) in the CAT activity, while mice treated with topical (+)-catechin showed a 36.31% decrease ($P < 0.01$) in the CAT activity compared with control subjects. In particular, a 36.31% increase ($P < 0.05$) in the CAT activity was observed in topical (+)-catechin-treated animals in comparison with the CAT activity of DMBA/TPA-treated animals (Table 2).

GST activity

Mice treated with DMBA/TPA exhibited a 48.97% decrease ($P < 0.001$) in the GST activity, while mice treated with topical (+)-catechin showed a paltry 21.08% decrease ($P < 0.05$) in the GST activity compared with control subjects. At large, a significant 54.67% increase ($P < 0.001$) in the GST activity was seen in topical (+)-catechin-treated animals in comparison with the GST level of DMBA/TPA-treated animals (Table 2).

GR activity

The GR activity in the DMBA/TPA-administered subjects was decreased by 31.93% ($P < 0.001$), whereas only a 22.95% decrease ($P < 0.01$) in the GR level was noticed in topical (+)-catechin-treated subjects compared with the control group. As a whole, an 11.64% increase in the GR activity was observed in topical (+)-catechin-treated subjects in comparison with the GR activity of DMBA/TPA-treated mice (Table 2).

GPx activity

The GPx activity in the DMBA/TPA-administered animals was decreased by 27.98% ($P < 0.01$), whereas only a 15.32% decrease ($P > 0.05$ or ns) in GPx level was seen in the topical (+)-catechin-treated animals compared with the control group. In general, a 14.95% increase in the GPx activity was recorded in topical (+)-catechin-treated subjects in comparison with the GPx activity of DMBA/TPA-treated mice (Table 2).

Topical (+)-catechin inhibits COX-2 expression and PGE_2 production in the skin papilloma

The western blot analysis indicated that exposure of the skin to DMBA/TPA resulted in the up-regulation of COX-2 as compared with the normal skin exposed to control treatment. On the contrary, mice treated with topical (+)-catechin showed a decrease in the COX-2 level as compared with the COX-2 level in the DMBA/TPA-treated group. The levels of PG metabolites in the skin papillomas, particularly PGE_2 was further estimated because, PGE_2 plays a critical role in cutaneous inflammation. In the skin papilomas of the DMBA/TPA-treated animal group, the level of PGE_2 was remarkably higher ($P < 0.0001$) as compared with the level of PGE_2 in the

control subjects. A significant reduction ($P < 0.0001$) in the PGE_2 level was observed in the topical (+)-catechin-treated group when compared with the DMBA/TPA-treated group (Fig. 4).

Topical (+)-catechin inhibits the levels of pro-inflammatory cytokines and inflammation biomarkers in the skin papillomas

Treatment of mice with DMBA/TPA resulted in the development of squamous cell papillomas/tumors, which on analysis showed up-regulation of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 as compared with control mice. Conversely, treatment of mice with (+)-catechin substantially decreased the expressions of TNF-α ($P < 0.0001$), IL-1β ($P < 0.001$), and IL-6 ($P < 0.001$) as compared with the DMBA/TPA-treated animals (Fig. 5). To further explore, whether the decline in the growth of DMBA/TPA-induced skin papillomas/tumors on simultaneous topical application of (+)-catechin emulsified gel was related to the inhibition of DMBA/TPA-induced inflammatory mediators, various biomarkers of the skin papillomas/tumors were analyzed in different treatment groups. The bands in the western blot analysis disclosed that treatment of mice with the (+)-catechin emulsified gel caused a noticeable fall in the expression levels of COX-2, NF-κB, and iNOS (Fig. 6A–6D).

Discussion

A number of recent studies have demonstrated that natural dietary antioxidants are capable of thwarting the growth of cancerous cells.[31] In addition, these natural antioxidants possess the ability to detoxify enzyme components, such as GST.[32] The naturally existing polyphenolic compounds bear the prospect to be developed as cancer chemopreventive agents, but the biggest hurdle related to these compounds, including (+)-catechin is that they have poor oral

bioavailability.[15] The current reports further verify that the bioavailability issue of these compounds can be overcome by delivering these compounds via a topical route of drug administration. In this paper, we report the development of topical emulsified gel of (+)-catechin as a chemopreventive formulation against the skin cancer. The use of (+)-catechin as an emulsified gel base topical formulation may be a better choice for human use. The topical (+)-catechin emulsified gel was found to accomplish all the criteria required for a desired topical formulation. There was no sign of phase separation in any form of the preparation over the test period of three months (Data not shown). The topical formulation of (+)-catechin showed marked prevention of % incidence of skin papillomas/tumors induced by DMBA and promoted by TPA. The histopathological observation of skin sections obtained from experimental mice also confirmed that there was a significant reduction of the formation of keratinocyte pearls and dyskeratosis. The overall survival rate of animals treated with topical formulation was much higher in comparison with DMBA/TPA-treated group. At the same time, a little improvement in the average weight was seen in the (+)-catechin-treated animals, which further indicates the recovery from the effect of DMBA/TPA or in other words it showed better papilloma control.

Polyphenolic compounds have been reported to prevent skin cancer by activating multiple biochemical mechanisms, including phase-II detoxification enzyme induction and the antioxidant defense mechanism.[31] The phase-II detoxification enzymes play a critical role in enhancing the hydrophilicity and helps in the excretion of xenobiotic substances. These enzymes inactivate and remove carcinogenic agents either by destroying their active centers or by conjugating them with endogenous ligands, thereby assisting in their excretion. Numerous studies have accounted that antioxidants scavenge the DNA damaging species.[32] The levels of these antioxidant enzymes, such as GST, GSH, GR, and GPx were decreased in DMBA/TPA animals compared with control

subjects. Topical administration of (+)-catechin increased the activity of phase-II detoxification enzymes in DMBA/TPA-treated subjects, which in turn promoted the excretion of dihydrodiol epoxide, an active metabolite of DMBA. The excessive production of free radicals in the cells accounts for oxidative damage to the cellular DNA; and thus, contributing to oncogenesis. Both the enzymatic and non-enzymatic antioxidants constitute the first line of defense against the highly unstable free radicals-regulated lipid peroxidation. The activity of antioxidative enzymes, such as SOD, CAT, and GST, was found to be decreased in the DMBA/TPA-treated animals. Earlier studies have described that the decrease in the levels of SOD and CAT in squamous cell carcinomas induce a pro-oxidant state of the cells, promoting tumorigenesis.[33] An enhanced level of antioxidative enzymes on administration of topical (+)-catechin suggests that the (+)-catechin suppressed the oxidative stress induced oncogenesis. Various studies have accounted that the GSH is a more potent antioxidant than SOD and CAT.[34] The GSH modulates the activity of lipoxygenase and cyclooxygenase that take part in tumorigenesis. Nevertheless, the role of GSH has been found to be highly ambiguous and depends on the cell type, the nature of the carcinogen and its regulatory pathways.[35,36] Previous report suggested that the chemopreventive action of flavonoids increases the level of GSH in the mouse skin.[37] In general, the combined effect of modulating antioxidative enzymes may cause a positive swing in the intracellular oxidation/reduction balance, resulting in the decreased activity of lipid peroxidation. The oxidative stress condition causes lipid peroxidation, which in consequence produces malonaldehyde and other aldehydes in the body. This can originate messy cross-linkages between the proteins and nucleic acids, causing altered replication and transcription leading to the tumor promotion.[38] Enhanced contents of MDA in skin papillomas of mice treated with DMBA/TPA, are indicative of oxidative stress. An appreciably decreased level of MDA in the

(+)-catechin-administered mice is indicative of its contribution in alleviating oxidative stress and thereby signifying its combating potential against the skin carcinoma. This positive outcome of topical (+)-catechin with respect to reduced lipid peroxide levels might have been orchestrated by augmented GSH and the antioxidative enzymes, such as CAT, SOD, GR, and GPx. The down-regulated levels of lipid peroxides in the (+)-catechin-treated subjects further anchor the reason that is responsible for the poor metastasis of these papillomas as shown in the histopathological studies.

In order to find out the possible mechanism of chemopreventive (+)-catechin, we determined its effect on multiple targets that includes DMBA/TPA-induced inflammatory mediators and cell cycle regulators in skin papillomas. The chemical carcinogens, like DMBA and TPA induces chronic inflammation, which has been implicated in the skin tumor initiation, promotion, and progression. One of the most important enzymes in the process of inflammation and tumor development is inducible COX-2. The COX-2 is a rate-limiting enzyme for the production of PG metabolites of arachidonic acid[39] and its up-regulation has been associated with the pathophysiology of inflammation and cancer;[40] because, the augmented production of PG metabolites have been indicated as a potential causative aspect in the growth of nonmelanoma skin cancers.[41, 42] In this study, we have found that topical application of (+)-catechin appreciably suppressed the inflammatory reactions in terms of inhibiting COX-2 and iNOS expressions, and PGE_2 production. The long term exposure to high levels of pro-inflammatory cytokines has been linked to skin cancer threat.[43,44] As a reason, the elevated levels of pro-inflammatory cytokines can be projected as contributor factors towards tumor initiation, promotion, and progression processes. Our results showed that treatment of mice with topical (+)-catechin emulsified gel significantly inhibited the pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in the

skin papillomas/tumors that in turn suppressed the tumorigenesis. The roles of the transcription factor NF-κB, predominantly its association with inflammation and cancer, have aroused great curiosity in the recent past. The presence of NF-κB in the skin is critical for morphogenesis and homeostasis. Any alteration in its role may produce skin defects, inflammatory skin ailments, and skin carcinogenesis.[45] The transcription factor NF-κB governs the expressions of TNF-α, COX-2, and various chemokines. The western blot analysis showed that the topical application of (+)-catechin down-regulated the level of NF-κB in the mice skin papillomas/tumors.

Conclusions

Our findings suggest that topical (+)-catechin emulsified gel possesses the chemopreventive potential and suppressed the DMBA/TPA-induced squamous cell carcinogenesis of the skin in a mouse model via modulating the antioxidant system and pro-inflammatory biomarkers in the skin papillomas. Currently, we have undertaken advanced studies in our laboratory to further elucidate the anticancer mechanism of (+)-catechin.

Conflict of interest

The author(s) confirm that this article content has no conflict of interest.

Abbreviations

DMBA, 7,12-dimethylbenz(a)anthracene; TPA; 12-O-tetradecanoylphorbol-13-acetate; GSH, glutathione; SOD, superoxide dismutase; CAT; catalase; GST, glutathione S-transferase; MDA, malondialdehyde; GR; glutathione reductase; GPx, glutathione peroxidase; TNF-α, tumor

necrosis factor-alpha; IL, interleukin; iNOS, inducible nitric-oxide synthase; NF-κB, nuclear factor-kappa B; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; HPLC, high-performance liquid chromatography; CDNP, 1-chloro-2,4-dinitrobenzene.

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Supplementary data

FT-IR, ^{1}H NMR, ^{13}C NMR, and Mass spectra, and HPLC purity chromatogram of (+)-catechin are provided.

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Table 1. *In vitro* estimation of the amount of (+)-catechin released from emulsified gel formulation

S. No.	Time interval	Peak area	Conc. (µg/mL)
1	0 h	0	0
2	1 h	613268	5.843
3	2 h	1954399	18.612
4	3 h	1998881	19.036
5	4 h	2259657	21.512
6	5 h	2320940	22.103
7	6 h	1955418	18.623
8	7 h	1953978	18.604
9	8 h	1953877	18.600
10	9 h	1951151	18.586
11	10 h	1851101	17.628

Table 2. Effect of the topical (+)-catechin on various oxidative stress biomarkers in mouse skin papillomas

S. No.	Parameter	Control	DMBA/TPA	Topical (+)-catechin
1	MDA (nmol/mg protein)	2.10 ± 0.41	3.42 ± 0.31**	2.33 ± 0.29*,^{ns}
2	GSH (nmol/mg protein)	30.24 ± 1.41	20.74±1.30***	28.20 ± 1.18**,^{ns}
3	SOD (U/mg protein)	12.80 ± 1.48	4.98 ± 0.50***	8.71 ± 1.08**,*
4	CAT (U/mg protein)	6.72 ± 0.90	2.53 ± 0.31***	4.28 ± 0.51**,*
5	GST (CDNB-GSH conjugate/min per mg protein)	30.22 ± 2.60	15.42 ± 2.09***	23.85 ± 1.69*
6	GR (nmol NADPH oxidized/min per mg protein)	3.79 ± 0.36	2.58 ± 0.17***	2.92 ± 0.48**
7	GPx (nmol NADPH oxidized/min per mg protein)	12.40 ± 1.67	8.93 ± 2.14**	10.50 ± 3.19^{ns}

The results presented are mean ± S.D. of three readings. Group 1: Control; Group 2: DMBA/TPA; Group 3: DMBA/TPA/ Topical (+)-catechin. **1. MDA**: $^{**}P < 0.01$; DMBA-TPA-treated group *vs*. control group, $^{*}P < 0.05$; (+)-catechin-treated group *vs*. DMBA-TPA-treated group, $^{ns}P > 0.05$ or not significant; (+)-catechin-treated group *vs*. control group. **2. GSH**: $^{***}P < 0.001$; DMBA-TPA-treated group *vs*. control group, $^{**}P < 0.01$; (+)-catechin-treated group *vs*. DMBA-TPA-treated group, $^{ns}P > 0.05$ or not significant; (+)-catechin-treated group *vs*. control group. **3. SOD**: $^{***}P < 0.001$; DMBA-TPA-treated group *vs*. control group, $^{**}P < 0.01$; (+)-catechin-treated group *vs*. control group, $^{*}P < 0.05$; (+)-catechin-treated group *vs*. DMBA/TPA-treated group. **4. CAT**: $^{***}P < 0.001$; DMBA-TPA-treated group *vs*. control group, $^{**}P < 0.01$; (+)-catechin-treated group *vs*. control group, $^{*}P < 0.05$; (+)-catechin-treated group *vs*. DMBA/TPA-treated group. **5. GST**: $^{***}P < 0.001$; DMBA-TPA-treated group *vs*. (+)-catechin-treated and control groups, $^{*}P < 0.05$; (+)-catechin-treated group *vs*. control group. **6. GR**: $^{***}P < 0.001$; DMBA-TPA-treated group *vs*. control group, $^{**}P < 0.01$; (+)-catechin-treated group *vs*. control group. **7. GPx**: $^{**}P < 0.01$; DMBA-TPA-treated group *vs*. control group, $^{ns}P > 0.05$ or not significant; (+)-catechin-treated group *vs*. control group.

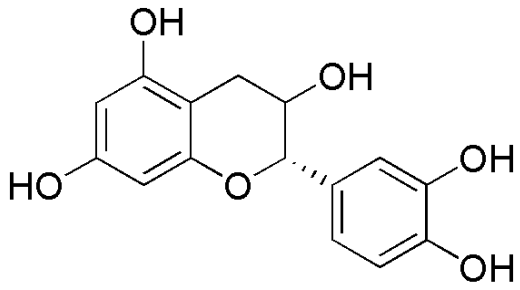

Fig. 1. Chemical structure of (+)-catechin

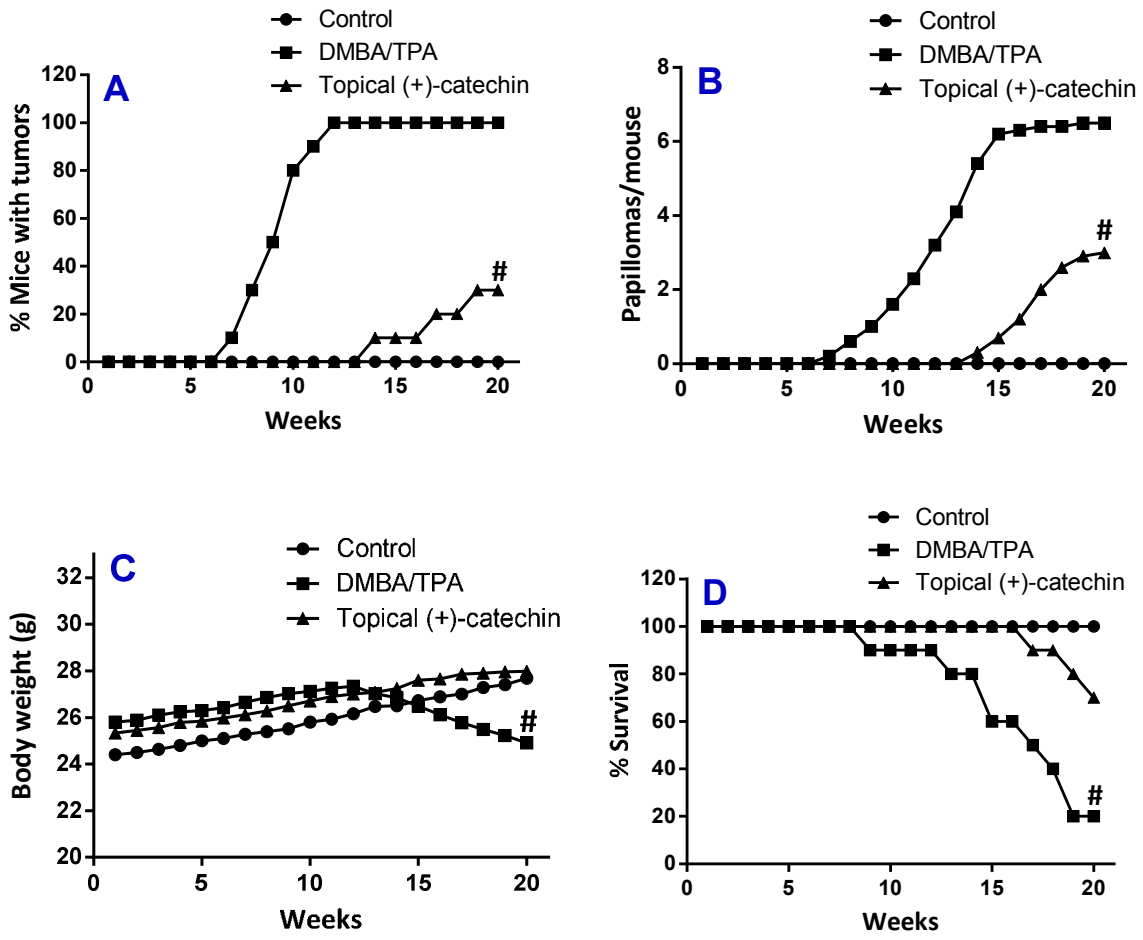

Fig. 2. The inhibitory effects of the (+)-catechin on DMBA-initiated and TPA-promoted squamous cell carcinoma of mouse skin. **A)** Effect of the (+)-catechin on the tumor incidence rate in mice (percentage of tumor bearing animals). **B)** Effect of the (+)-catechin on the average number of papillomas per mouse (papilloma yield). **C)** Effect of the (+)-catechin on the average body weight of mice. **D)** Effect of the (+)-catechin on the survival rate of mice. $^{\#}P < 0.0001$; topical (+)-catechin-treated group *vs*. DMBA/TPA-treated group at week 20, in all four graphs.

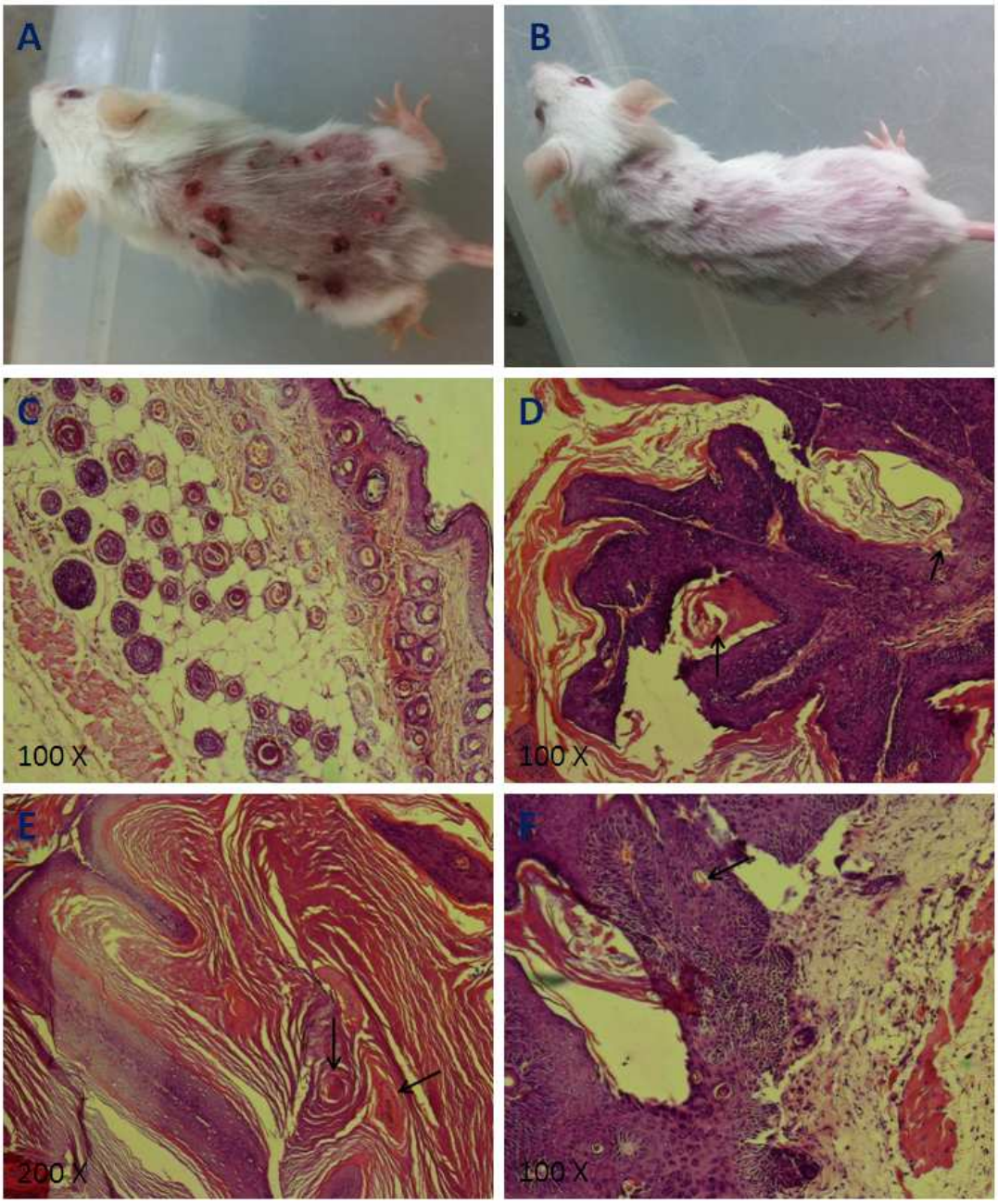

Fig. 3. Histopathological studies of control, DMBA/TPA-treated, and (+)-catechin emulsified gel-treated mouse skin. **A)** Mouse treated with DMBA/TPA. **B)** Mouse treated with (+)-catechin emulsified gel. **C)** Histological section of the normal mouse skin of control group at 100 X. **D)** Histological section of the DMBA/TPA-induced skin tumor in mouse at 100 X. **D)** Characteristic squamous epithelial pearls and necrotic keratinocytes in the DMBA/TPA-induced mouse skin tumors at 200 X. **D)** Histological section of the (+)-catechin emulsified gel-treated mouse skin at 100 X. The arrows represent presence of tumor in the mouse skin.

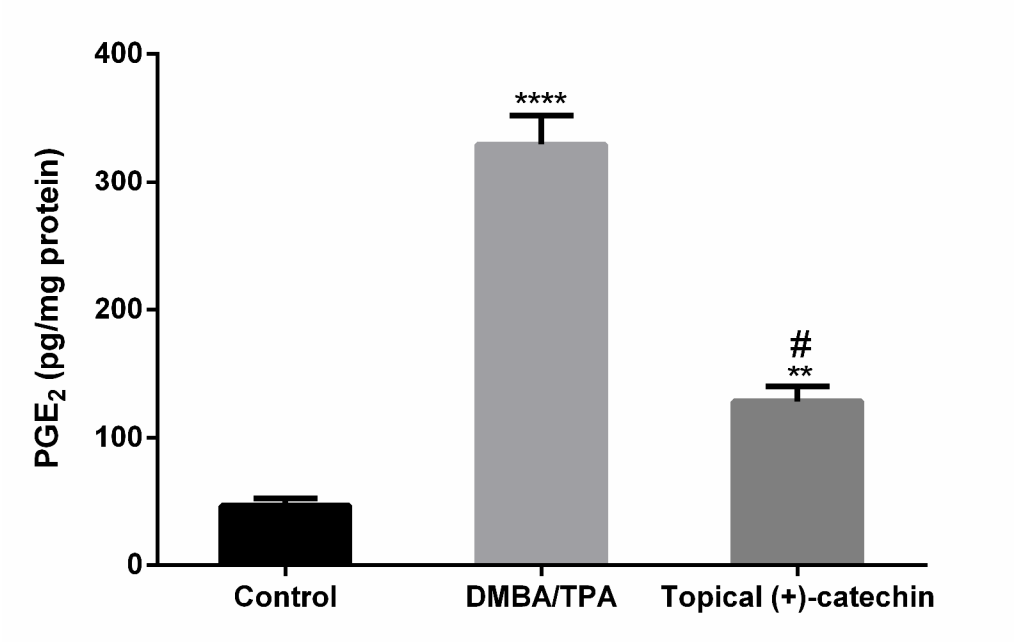

Fig. 4. The level of PGE_2 in the DMBA-induced and TPA-promoted mice skin squamous cell carcinoma, determined by using a PGE_2 immunoassay kit. The concentration of PGE_2 is expressed in terms of pg/mg protein as a mean ± S.D. of three readings; where, n = 10. ****P < 0.0001; DMBA/TPA-treated group *vs*. control, **P < 0.01; (+)-catechin-treated group *vs*. control, $^{\#}P$ < 0.0001; (+)-catechin-treated group *vs*. DMBA/TPA-treated group.

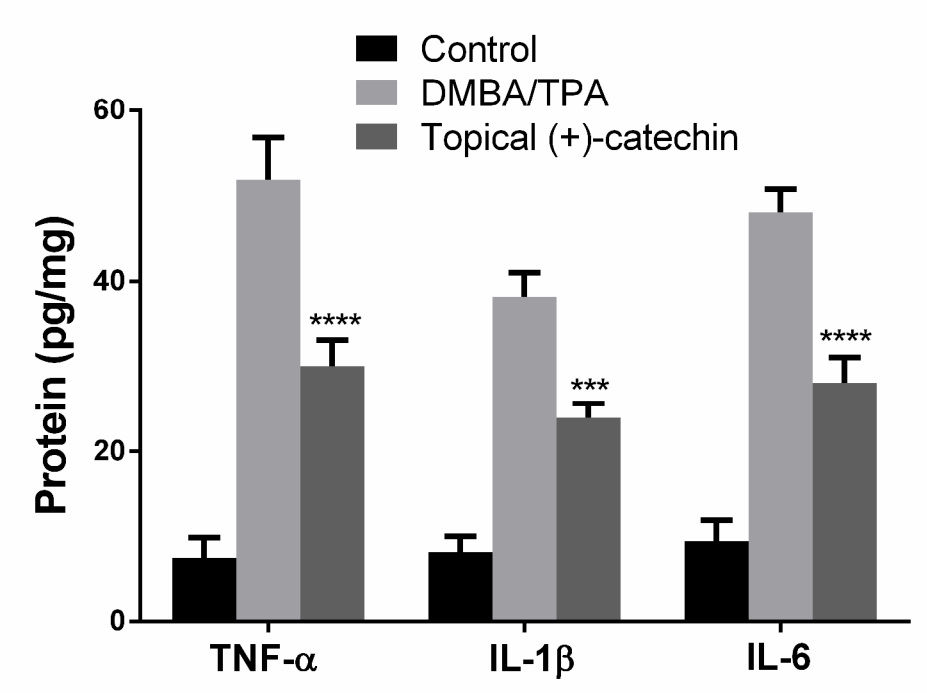

Fig. 5. The expression levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the DMBA-induced and TPA-promoted mice skin squamous cell carcinoma. The levels of proteins were determined by using ELISA kits and data are presented as the mean ± S.D. of three readings in terms of pg/mg protein; where, n = 10. ****$P < 0.0001$; (+)-catechin-treated group *vs*. DMBA/TPA-treated group, ***$P < 0.001$; (+)-catechin-treated group *vs*. DMBA/TPA-treated group.

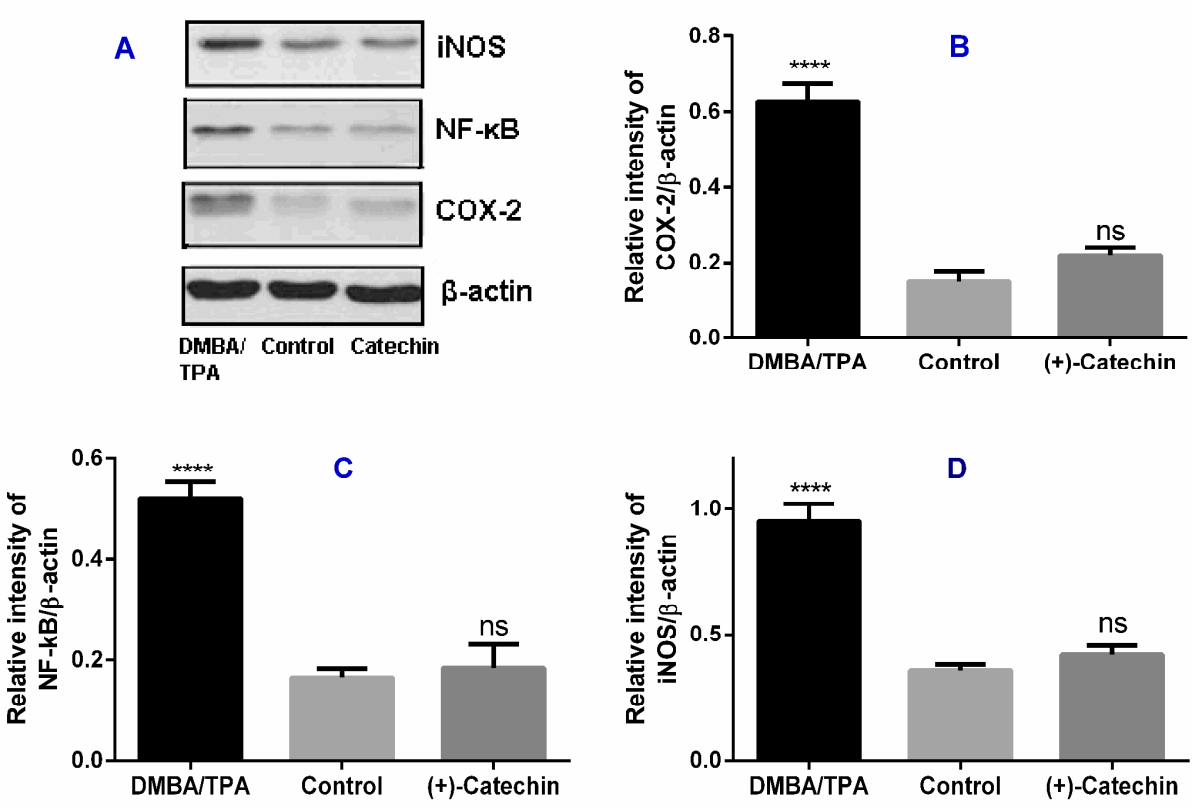

Fig. 6. A) The western blot analysis of COX-2, NF-κB, and iNOS proteins expressed in the DMBA-induced and TPA-promoted mice skin squamous cell carcinoma. **B)** Densitometric analysis of the COX-2 level in the western blot. **C)** Densitometric analysis of NF-κB level in the western blot. **D)** Densitometry analysis of iNOS level in the western blot. The results shown are the mean ± S.D. of three separate experiments. ****$P < 0.0001$; DMBA/TPA-treated group *vs*. (+)-catechin-treated and control groups, $^{ns}P > 0.05$ or not significant; (+)-catechin-treated group *vs*. control group.