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Research Article

Chemomodulatory Potential of n-Hexane Extract of *Conyza bonariensis* Against Skin Carcinogenesis in Mice Induced by Carcinogens: An *in Vitro* and *in Vivo* Study

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Abstract

Conyza bonariensis (CB) is a cosmopolitan herb, family Asteraceae (Compositeae), commonly known as mrich booti or gulava previously used to cure cancer in traditional medicine. In the present study, n-hexane fraction by CB's triterpenoid constituents was assessed for their cytotoxic property using the human skin carcinoma cell line (SCC-13) and human peripheral blood mononuclear cells (PBMC). The chemo modulatory potential of CB n-hexane was evaluated against skin carcinogenesis in mice induced by (7-12-dimethylbenz(a)antheracene) DMBA and Croton oil. CB n-hexane extract was orally administered at the doses of 3, 6, and 9 mg/kg/day, and 9 mg/kg was also applied locally on mice skin for 16 weeks. The results from cell lines revealed that CB induces cytotoxicity against the SCC-13 cells (IC50=7.5 μM). It was found to be 4.05 times more selective in exerting cytotoxicity against SCC-13 than the PBMC (IC50=30.4 μM). The results from the mice model of skin carcinogenesis have shown that whole plant n-hexane extract administered via oral and topical routes, both reduced the number of precancerous skin papillomas and their incidence. At these concentrations, CB n-hexane also augmented the actions of catalase and superoxide dismutase, glutathione content and reduced the skin lipid peroxidation levels. These outcomes presented the cytotoxic and antioxidative effects of CB n-hexane whole plant extract and also authenticates the traditional assertions and strengthen the technical base of its chemopreventive activity.

Keywords: *Conyza bonariensis,* n-hexane, skin carcinogenesis, papillomas; DMBA, croton oil, quercetin

1. Introduction:

Parallel to the rising pandemics worldwide, cancer is one of the deadliest diseases due to its fast metastatic nature from organs to blood vessels and lymph nodes. The major etiological factors include genetic predisposition and exposure to environmental pollutants like harmful chemicals and radiation, which cause either internal factors such as spontaneous mutations, endocrinological and nutritional imbalances in the body, or act as external stimulators (Zamora et al. 2019). Phytotherapy is grabbing interest in conventional therapy daily because medicines originate from natural sources and help stimulate the body's immune system with minimal or acute adverse effects by providing essential phytochemicals and

nutrients for optimizing the therapy and boosting its effectiveness (Josephine Ozioma and Antoinette Nwamaka Chinwe 2019). Among all life-threatening types of cancers, skin cancer is characterized by menaces on the outermost layer of the skin (Stratmann 2016). Various carcinogens like sun rays, artificial light sources, chemicals, and polycyclic aromatic hydrocarbons are the reason for carcinogenesis. They initially cause mutations at the genetic level and alter the mechanism of normal protein functionality, resultantly enhancing the production of abnormal cell growth in different layers of the skin. This will then start the promotion and progression stage of cancer, and after metabolism, it increases the rate of reactive oxygen species (ROS) in the skin tissues (Goyal et al. 2022). Conventional herbs can protect the skin from various carcinogens due to active constituents, including polyphenols, flavonoids, steroids, quinones, coumarins, essential oils, terpenoids, anthocyanidins, saponins, alkaloids, and nitrogenous compounds (R. Abid et al. 2022). *Conyza bonariensisis* (CB), a cosmopolitan herb, family Asteraceae (Compositae), commonly known as mrich booti or gulava is one of the plants having various active anticancer constituents and is traditionally used against microbial infections, anticoagulation, hepatic, and gastroenteritis toxicities, diarrhea, leucorrhoea, menorrhagia, antioxidant and anticancer in breast (MCF7), colorectal (HCT116), cervical (HELA) cell lines (Wu 2007). The cytotoxic effect of methanol extract on skin carcinogenesis in mice has already been reported (Rezadoost, Kumleh, and Ghasempour 2019). The current study was conducted to determine the chemo-modulatory potential of n-hexane extract of CB in cell lines and against two-stage DMBA and Croton oil-induced skin carcinogenesis in Swiss albino mice.

2. Materials & Methods

2.1. Plant Assembly

Whole plants of CB were collected during May and June 2018 at 35 to 42 ℃ from the backyard of Government College, University Faisalabad, Pakistan, and identified by a plant taxonomist Dr. Mansoor Hameed Head of Botany Department, Agriculture University, Faisalabad, Pakistan.

2.2. Extract Preparation

The aerial parts were washed, chopped, and dried under shade at room temperature for many days until fully dried, ground by the electric grinder, powdered, and sieved. This material was macerated in n-hexane for 7 days with frequent shaking every day, filtered out by using Whatman filter paper, separated solvent from a solid material by using a rotary evaporator at 45-55 °C and residues obtained were stored in small amber jars at 4°C (Muhammad and Naveed 2011).

2.3. Materials and Chemicals

Carcinogen 7-12-dimethylbenz(a)anthracene (DMBA), Croton oil, 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), NADPH (nicotinamide adenine dinucleotide phosphate, pyrogallol, thiobarbituric acid (TBA), bovine serum albumin (BSA) were procured from Sigma Aldrich Chemical Company USA. All the other chemicals used were of analytical grade. Acetone was used as a vehicle for all topically applied carcinogens, and dilution of plant extract and n-hexane (analytical grade) were purchased from Asian Scientific Store, Jinnah Colony, Faisalabad, Pakistan.

2.4. Experimental Animals

A total of 54 male mice 6-8 weeks old, weighing 20-30g, were obtained from the National Institute of Health Islamabad and kept in the animal house of the Department of Pharmacology, Government College, University Faisalabad, Pakistan, under controlled conditions of temperature (25±2°C) and humidity (52±5°C) with a photoperiod of 12 h light and 12 h dark cycle. They were given a standard diet and water. Mice were acclimatized to the environment for one week before the commencement of the experiment. They were provided water ad libitum and all-time access to the commercially available pellet diet from Animal feed, Faisalabad, Pakistan (S. Abid et al. 2022).

The experiments were performed by the experimental protocol approved by the Institutional Animal Ethical Committee that confirms the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Government of Pakistan.

2.5. *Experimental Protocol*

The dorsal skin of albino mice was shaved with an electric clipper for approximately 2 x 2 cm area and marked with a permanent marker. Moreover, a total of 9 animals were present in each group. Group I (Control): This group of animals served as vehicle control and received a topical application of acetone (100 µL/mouse) on the shaved area of the skin and 0.5% Tween 80 (10 ml/kg) by intragastric gavage for 16 weeks. Group II (DMBA/Croton oil applied): The animals in this group received topical applications of DMBA at 72 hours at a dose of 0.05 g/kg in acetone (100 µL/mouse). Starting from eight days after the first DMBA application, croton oil (1% w/v) in acetone (100 µL/mouse) was applied twice a week for a total of 16 weeks. Group III, IV, V (Treated with CB orally): The animals in this group were applied with DMBA/Croton oil similar to group II following concomitant treatment with CB n-hexane extract administered orally using intragastric gavage at three doses; 3 mg/kg, (Group III), 6 mg/kg (Group IV) and 9 mg/kg (Group V) respectively for 15 days before the first DMBA application and continued up to 16 weeks after DMBA application. Group VI (Treated with CB n-hexane extract topically): The animals in this group were applied with DMBA/Croton oil similar to the DMBA/Croton oil-treated group along with the topical application of CB n-hexane extract at a dose of 9 mg/kg prepared with 100 µL of 0.05% Tween-80 for 15 days before the first DMBA application and continued for 16 weeks after DMBA application.

During the 16 weeks of the experimental period, the animals were observed daily for the appearance of skin papillomas, and the tumor volume was recorded. At the end of 16 weeks, all the mice were sacrificed, and the skin samples were collected.

2.6. *In vitro Cytotoxic Efficacy of CB n-hexane Extract*

The SCC13 cells were procured from ATCC (Manassas, VA), and human PBMC was isolated by density centrifugation of heparinized blood of healthy donors using the dextran T-500 sedimentation method (Patil et al. 2016). The cells were grown adherently in RPMI-1640 media supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO2 with 95% air (Saleem et al. 2015).

2.7. *Cell Viability Assay*

The viability of the cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The SCC13 and PBMC cells were seeded at 5×10^3 cells/well in 5% CO^2 at 37°C in RPMI medium (containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL of streptomycin) in a 96-well plate. After overnight incubation to allow cell attachment, the RPMI medium in each well was replaced by media containing various concentrations of CB n-hexane and incubated for 48 hours. Further, 20µl of MTT (5 mg/mL in PBS) was added to each well, and the cells were incubated for another 4 hours at 37°C. The supernatants were then aspirated carefully, and 200µl of dimethyl sulfoxide was added to each well of the microplate. The plates were shaken for an additional 10 min, and the absorbance values were read by the Microplate reader (BioTek, USA) at 570 nm. Cell viability was calculated as a percentage using the formula: (mean OD of treated cells/mean OD of control cells) × 100. The results were expressed as a percent of control or normal cells (Mohammad et al. 2015).

2.8. *In vivo Chemomodulatory Efficacy of CB n-hexane Extract*

The following parameters were used to determine the *in-vivo* chemo-modulatory efficacy of CB n-hexane on DMBA/Croton oil-induced skin carcinogenesis.

- ***Morphological Studies*:** The skin of each mouse was observed weekly for loss of hair, redness, ulceration, and outgrowths.

These were counted and measured by digital Vernier Caliper till the end of the 16th week. Recorded morphological parameters are Tumor incidence: number of tumor-bearing mice, Cumulative number of Papilloma, Tumor Yield: average number of tumors per mouse, and Tumor Burden: number of tumors per tumor-bearing mice (Gupta and Trivedi 2018).

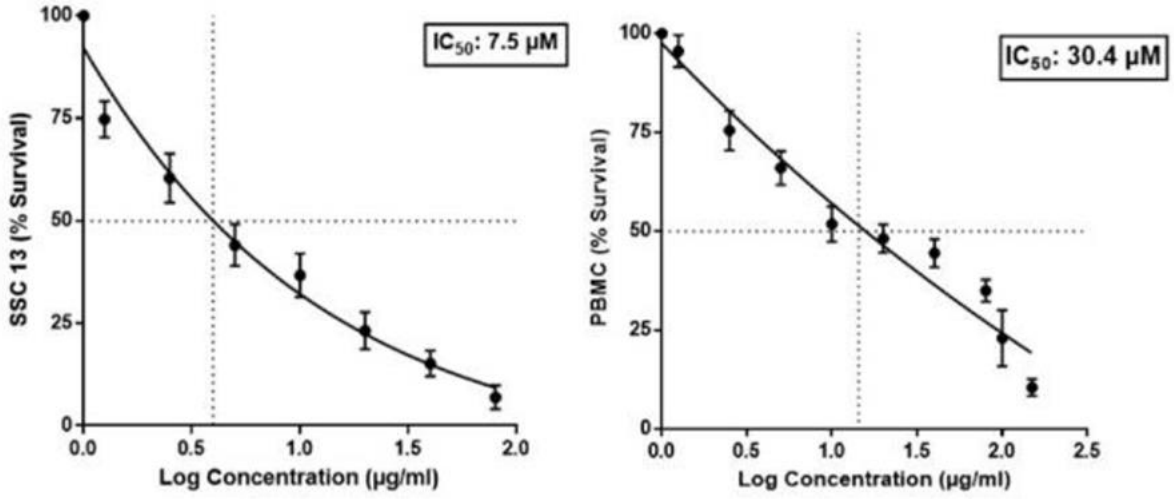

Figure 01: Effect of CB n-hexane extract on SCC13 and PBMC viability in MTT assay.

- ***Measurement of body weight:*** The body weight of the individual mouse was recorded initially and at one-week intervals till the end of the experiment or the death of the mouse.
- ***Tumor incidence:*** The number of mice carrying at least one tumor was expressed as a percentage incidence.
- ***Tumor burden:*** The average number of tumors per tumor-bearing mouse was assessed.
- ***The cumulative number of tumors:*** The total number of tumors observed till the end of the experimental protocol was estimated.
- ***Tumor diameter and tumor volume:*** The tumor diameters across three dimensions were measured using a Mitutoyo Digimatic caliper (model 500-196-20). The tumor volume was calculated by the formula Volume = (4/3) π [D1/2][D2/2][D3/2], where D1, D2, and D3 are the three diameters (mm) of the tumors.
- ***Tumor weight:*** The weight of each tumor was measured at the termination of the study protocol. At the end of 16 weeks post-DMBA application, the mice were sacrificed. The skin samples from each mouse were processed. Estimation of oxidative injury using skin tissue homogenate.

2.9. Biochemical Parameters

The skin tissue was separated and washed several times in ice-cold saline. The skin tissue (100 mg) was carefully chopped into fine pieces using the surgical blade. The minced skin tissue was homogenized in an ice-cold phosphate buffer (pH 7.4) with the help of a homogenizer (RQ127A/D, Remi, Maharashtra, India). Typically, the homogenizer was set at a speed of 1000 rpm (rotation per minute), and 10 strokes for 30 sec 2-3 times at 4°C were applied to get a complete homogenate of the skin tissue. The resultant homogenate was centrifuged at 20,000 rpm for 5 min at 4°C. The tissue debris was separated as a pellet, and the supernatant was pipetted out. The skin tissue homogenate was stored at -20°C until

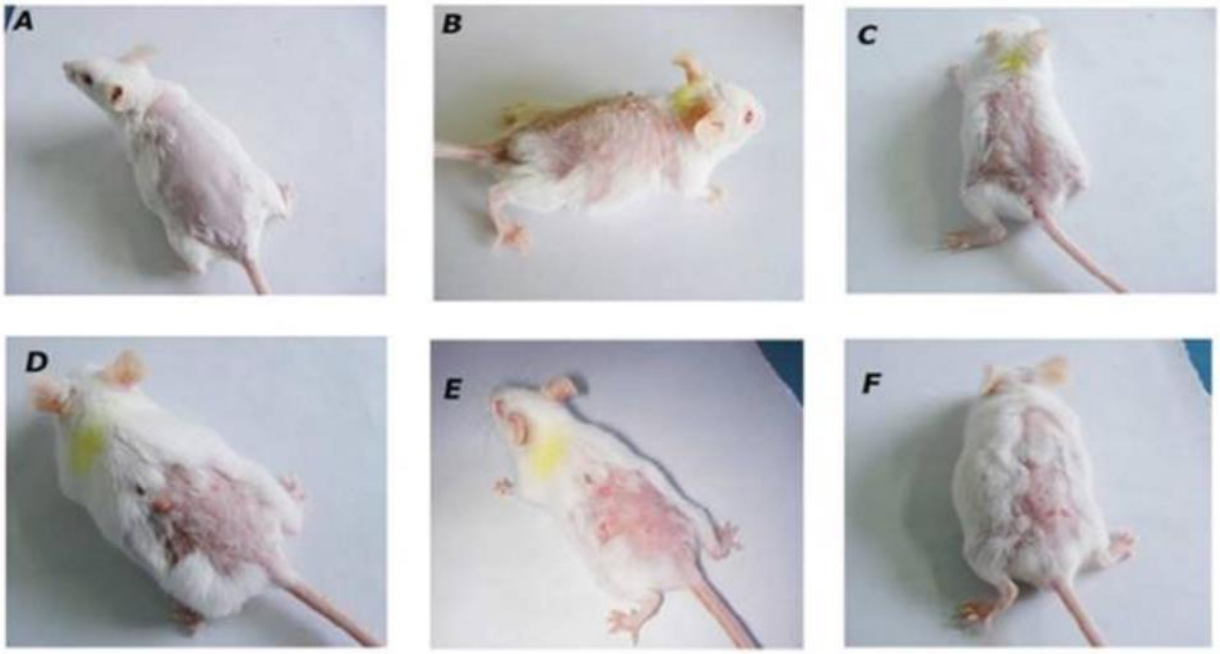

Figure 02: Effect of CB n-hexane on morphological features of the tumor. A: Normal control or vehicle-treated mice; B: DMBA/Coroton oil-treated mice; C: CB (3 mg/k p.o.); D: CB (6 mg/kg p.o.); E: CB (9 mg/kg p.o.); F: Topical CB (9 mg/kg topical).

further use. The aliquot of the homogenate prepared in chilled conditions was used to estimate the biochemical parameters.

2.9.1. Estimation of Glutathione (GSH)

The GSH contents in the skin tissues were estimated by the method of (Majed et al. 2015). Briefly, 100 µl of tissue homogenate was mixed with 100 µl of 10% trichloroacetic acid and vortexed. The contents were then centrifuged at 5000 rpm for 10 min. Subsequently, 0.05 ml of supernatant was mixed with a reaction mixture containing 3.0 ml of phosphate buffer (pH 8.4, 0.3 M) and 0.5 ml of DTNB [5, 5′ dithiobis (2-nitrobenzoic acid)]. Within 10 min, the absorbance was measured at 412 nm using a spectrophotometer. The GSH content was determined from a standard curve produced using commercially available standard GSH (Sigma Chemicals, MO, USA). The amount was expressed as µg of GSH/mg of protein. In samples, the GSHPx activity was measured by quantifying the rate of H2O2-induced oxidation of GSH to oxidized glutathione catalyzed by glutathione peroxidase (GSHPx) (Miloš et al. 2020).

2.9.2. Estimation of Lipid Peroxidation

The level of lipid peroxidation in the skin tissue was determined by measuring the malondialdehyde (MDA) content using the method described by (Patil et al. 2016). Briefly, 0.2 ml of homogenate was mixed with 0.2 ml of sodium dodecyl sulfate (8.1%), 1.5 ml of acetic acid (pH 3.5, 30%), and 1.5 ml of thiobarbituric acid (0.8%). The reaction mixture was heated for 60 min at 95°C and then cooled in an ice bath. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol: pyridine (15:1 v/v) solution were added and centrifuged at 5000 rpm for 20 min. The absorbance of the pink color in the organic layer was measured at 532 nm. In the spectrophotometric measurement of lipid peroxidation, 1,1,3,3 tetra ethoxy propane (Sigma Chemicals, MO, USA) was used as the standard MDA, and levels were expressed as µg/g of protein. Estimation of catalase activity The catalase activity was determined by the method described by (Safdar et al. 2015). Briefly, to the tissue supernatant (50 µl), 1.0 ml of 50 mM phosphate buffer (pH 7) and 0.1 ml of 30 mM

Table 01: The data were expressed as mean ± SEM (n=12 mice). ***p<0.001, **p<0.01 compared to DMBA/Croton oil applied on mice skin. $^{###}$p<0.001 compared to the normal control group.

Experimental groups	Tumor incidence (%)	Number of tumors/mice	Tumor volume (mm3)	Tumor weight (gm)	Mortality
Normal	0	0	0	0	0
DMBA control	100	12.06+2.39$^{###}$	147.5+23.5$^{###}$	1.92+0.03$^{###}$	6
CB (3mg/kg) p.o	84.5	9.92+2.9	136.9+13.8	1.08+0.01	4
CB (6mg/kg) p.o	63.2	6.89+1.4***	93.7+9.2***	0.92+0.01**	3
CB (9mg/kg) p.o	34.8	4.57+0.9***	59.6+9.8***	0.39+0.01***	0
CB (9mg/kg) topical	51.6	5.83+3.01**	78.8+18.8***	0.51+0.01***	1

hydrogen peroxide were added. The absorbance was read at 240 nm every 5 sec for 30 sec on the spectrophotometer. The activity of catalase was expressed as U/mg of protein.

2.9.3. Estimation of Superoxide Dismutase Activity

The SOD activity was determined by the method of (Gusain, Singh, and Sharma 2015) with slight modifications. Briefly, to the tissue supernatant (100 µl), 2.85 ml of phosphate buffer (pH 8.4, 0.1 M) and 50 µl of 7.5 mM pyrogallol were added. The absorbance of the resultant mixture was measured on the spectrophotometer at 420 nm for 3 min at 30-second intervals. The enzyme levels were expressed as U/mg of protein.

2.10. Histopathological Study

The skin tissues were fixed in the buffered formalin (10%), cut into four segments, and embedded in the paraffin wax. These formalin-fixed paraffin-embedded sections were cut to get the thin serial sections of 4 µm thickness and subjected to the hematoxylin and eosin (H & E) staining protocol. These sections after H&E staining were examined under the light microscope (Nikon, Tokyo, Japan), and digital images were captured. The slides were evaluated by a qualified, experienced pathologist masked to the experimental groups and treatments. A minimum of 10 fields for each slide were examined and scored (Saleem et al. 2015).

2.11. Chromatogram by HPLC for Identification of Active Constituent

High-performance liquid chromatography (HPLC) was performed to confirm the presence of quercetin in CB n-hexane extract (Saleem et al. 2022). The sample was dissolved in 5 ml distilled water and 12 ml n-hexane, kept for 5 minutes, again added 6 ml distilled water, stayed for 5 minutes, and added 10 ml 5M HCl in this solution. Placed in the oven for 2 hours and filtered the solution by a syringe filter. Isocratic: dichloromethane: methanol (60:20:20) was used as the mobile phase with a flow rate of 1 ml/min. The column was ODS (octadecyl-silica) 250mm x 4.6 mm, and a UV detector was used to obtain a chromatogram at 280 nm at room temperature (Qi et al. 2006).

2.12. Statistical Analysis

The data were expressed as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test or Dunnett's post hoc test using the Graph Pad Prism software version 6.0. The criterion of statistical significance was set at P<0.05.

3. Results

3.1. In vitro Cytotoxic Efficacy of CB n-hexane on SCC13 and PBMC Cell Lines

The cytotoxic efficacy of CB n-hexane was evaluated in SCC13 and human PBMC cells using an MTT assay. As depicted in figure 1, the IC50 of CB for the SCC13 cell line and PBMC were observed to be 7.5 µM and 30.4 µM, respectively. The selectivity index for CB n-hexane against the SCC13 cells was 4.05.

- ***Effect of CB on changes in body weight:*** A continuing rise in body weight after the beginning of treatment was observed in all the groups, and such weight was found to

be near normal at the end of the experimental period. The normal mice and the mice treated with CB n-hexane do not show significant changes in body weight through the experimental protocol.

- ***Effect of CB on morphological examination of the tumor:*** The incidence of tumors starting from the 6th week in DMBA/Croton oil-treated animals was considered 100%, and all mice in the carcinogen-treated group developed papillomas (100% incidence) at the end of the 16th week. The oral treatment with CB n-hexane at doses of 3, 6, and 9 mg/kg reduced the incidence of papilloma in a dose-dependent manner, and incidence in these groups was calculated at 84.5%, 63.2%, and 34.8%, respectively. The group that received CB n-hexane topically at 9 mg/kg dose showed 51.6% tumor incidence (figure 02; Table 01).

Table 2: Effect of CB on malondialdehyde content as lipid peroxidation. The data were expressed as mean ±SEM. Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. ###p<0.001 as compared to normal group and ***p<0.001 as compared to DMBA treated mice.

Experimental groups	MDA (µg/mg of protein)	GSH content	GSHPx	SOD	Catalase
Normal	150.6±17.32	30.03±17.90	8.59±2.19	10.41±2.60	6.30±1.30
DMBA control	225.4±26.61	3.01±0.90	13.40±1.10	11.22±3.64	8.93±1.19###
CB (3mg/kg) p.o	213.2±20.47	9.81±1.90	15.49±2.19	12.67±3.18*	9.37±1.86
CB (6mg/kg) p.o	153.7±19.85	15.74±1.3	18.63±1.17###	13.16±2.10	11.98±1.21*
CB (9mg/kg) p.o	124.5±15.4	23.08±1.2	21.72±2.10***	14.44±1.73*	13.47±1.78
CB (9mg/kg) topical	149.6±19.32	25.01±0.90	25.85±1.10	15.10±1.56	15.40±0.10

The cumulative number of tumors in tumor-bearing mice treated with CB n-hexane showed a significant decrease ($p<0.01$) than the mice treated with DMBA/Croton oil (Table 1). The tumor volume was increased significantly ($p<0.01$) in the group that applied DMBA/Croton oil than the normal group. The groups treated with CB showed a significant ($p<0.01$) reduction in tumor volume as compared to DMBA/Croton oil-applied mice (Table 1). The mice who applied DMBA/Croton oil showed a significant ($p<0.001$) increase in tumor weight compared to the normal mice. Treatment with CB n-hexane showed a dose-dependent decrease in the weight of the tumor as compared to DMBA/Croton oil-applied mice (Figure 01).

- ***Effect of CB n-hexane on malondialdehyde content:*** The level of MDA in the skin tissue homogenate of animals applied DMBA/Croton oil showed a significant ($P<0.01$) increase in the level as 225.4±26.61 concerning vehicle control animals. The animals treated with CB at the dose of 3 mg/kg have shown a negligible reduction in MDA level. CB at the dose of 6 and 9 mg/kg showed a significant ($P<0.01$) decrease (153.7±19.85 and 124.5±15.4, respectively) in the level of MDA as compared to the mice applied with DMBA/Croton oil. The mice treated topically with CB showed a mild reduction in MDA level of 149.6±19.32 (Table 2).
- ***Effect of CB on oxidative stress markers:*** Oxidative stress was assessed by the GSH content, and activities of GSHPx, SOD, and catalase were represented as markers of enzymatic and nonenzymatic antioxidant defense systems. DMBA/Croton oil induced a state of evident oxidative stress as

demonstrated by a significant (P<0.01) decrease in GSH content (3.01±0.90) and reduced activities of GSHPx (13.40±1.10), SOD (11.22±3.64) and catalase (8.93±1.10) in a dose-dependent manner in skin tissue homogenates. However, treatment with CB n-hexane dose-dependently ameliorated oxidative stress, as evidenced by reduced depletion of GSH content and restoration of GSHPx, SOD, and catalase activities.

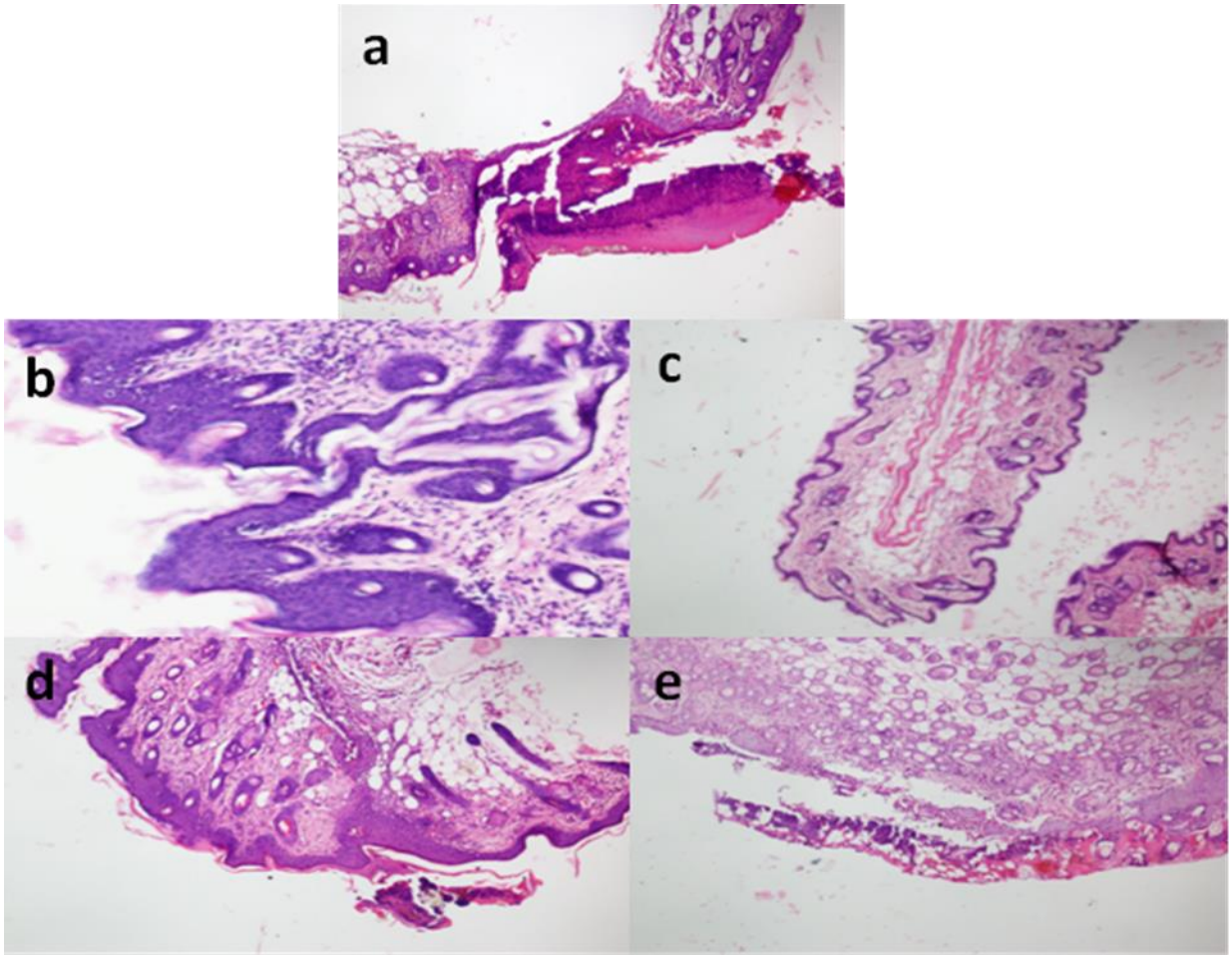

Figure 03: Histopathology of *Conyza bonariensis* n-hexane extract. Carcinogen Control (a): ulceration and inflammatory slough on the epidermis. CB 3 mg/kg p.o (b): mild acanthosis, hyperkeratosis, and mild papillomatosis with normal cytological features on the epidermis. C.B 6 mg/kg p.o (c): mild papillomatous changes on epidermis. CB 9 mg/kg p.o (d): epidermis showed mild acanthosis with inflammation. CB 9 mg/kg topical (e): mild degree of acanthosis with normal cytological features on the epidermis.

3.2. Effect of CB on histopathology of the skin

The animals in the vehicle-treated normal group showed normal skin layers, architecture, and structure, such as the epidermis, dermis, and basal layer (Figure 03). On the other hand, carcinogen and promoter DMBA/Croton oil-induced skin cancer in mice was evidenced by the occurrence of squamous cell carcinoma, which exhibited thickening of the epidermis, scratching in the layers of skin, and proliferative growth of the cell. The treatment of mice with CB at the dose of 3 mg/kg showed mild loss to the epidermis and the layers of skin. CB treatment at doses; 6 and 9 mg/kg showed the normal structure and architecture of skin layers and prevented further impairment to skin tissues, thus reconfirming the

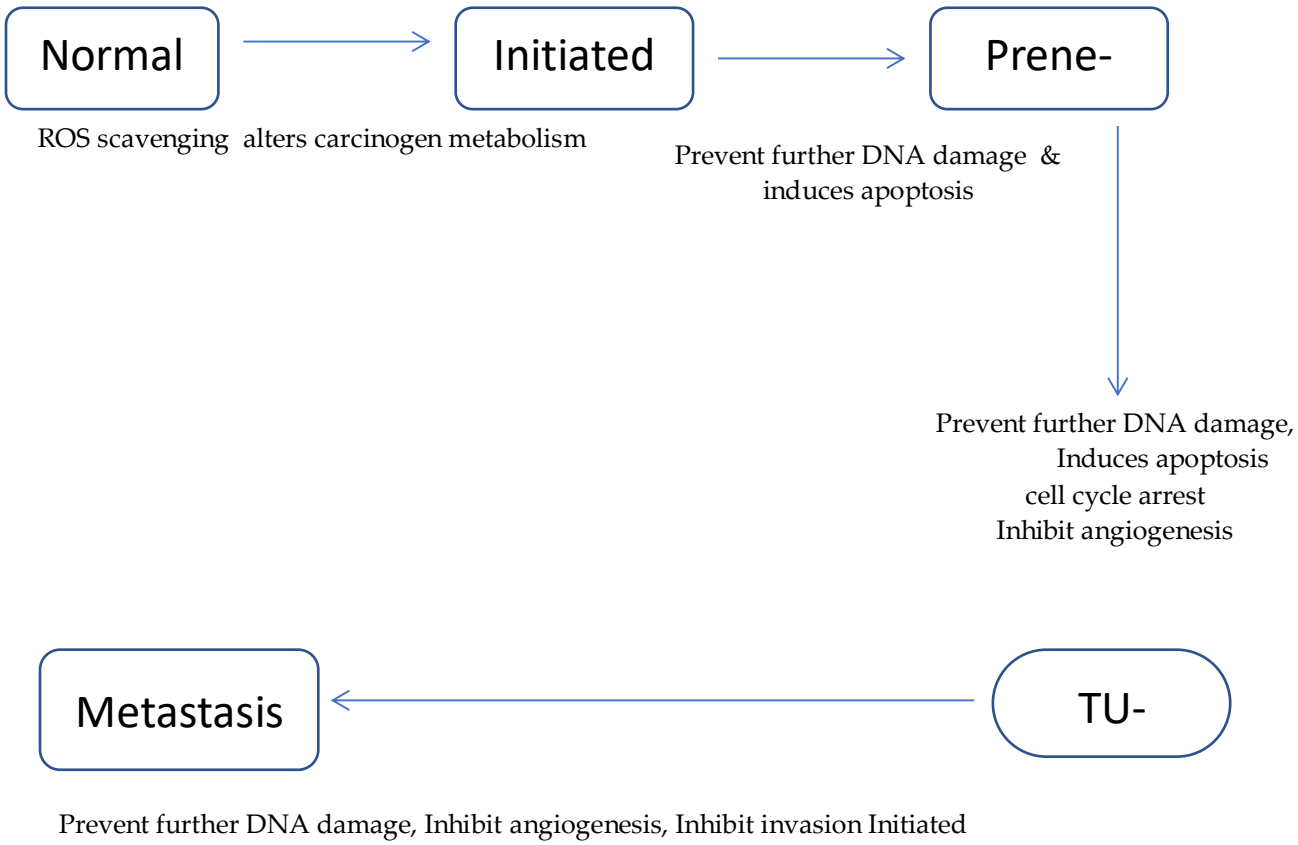

Figure 04: Multistage of carcinogenesis and potential effects of polyphenols on cancer progression.

biochemical correlates of the chemopreventive efficacy of CB. The animals treated topically with CB at the dose of 9 mg/kg showed mild to moderate inhibition of scratched-to-skin layers and invasion of epidermal cells in the dermis and formation of keratin pearls as compared to the DMBA/Croton oil-induced skin cancer in mice (Table 2).

The present study findings reveal that CB n-hexane extract elicits selective cytotoxicity against the cutaneous squamous cell carcinoma cell line and markedly suppresses the initiation and progression phases of DMBA/Croton oil-induced two-stage skin carcinogenesis by modulating the PhaseII detoxification enzyme and improving antioxidant defense**.** The preclinical model of skin carcinogenesis induced in mice by application of DMBA/Croton oil involves sequential steps of tumor initiation (conversion/neoplasia), promotion (propagation/ dysplasia or hyperplasia), and progression (metastatic).

4. Discussion

The present study's application of DMBA/Croton oil-induced tumor incidence and treatment with CB n-hexane showed dose-dependent regression of two-stage carcinogenesis. The effects observed in our study are in agreement with a published article showing the cytotoxic effect of methanol extract of CB on DMBA-induced skin carcinogenesis: An in vivo study (Omara et al. 2020).

The initiation and progression of tumor development and uncontrolled cell proliferation result from damaged or exhausted cellular apoptotic mechanisms (Klaunig and Kamendulis 2004). The skin of the mice subjected to DMBA/Croton oil shows an increase in the level of lipid peroxidation that is indicative of an

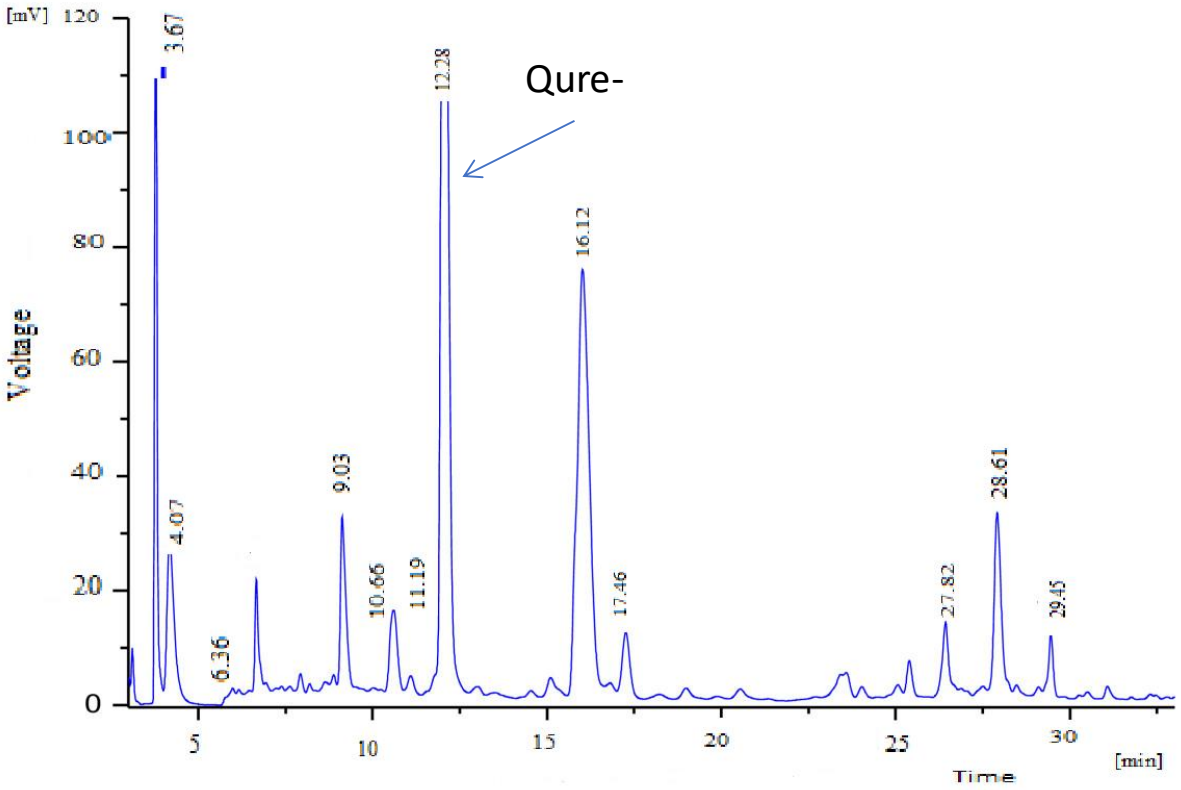

Figure 05: HPLC Chromatogram for analysis of *Conyza bonariensis* n-hexane extract.

overwhelming rise in the oxidative stress level. The CB n-hexane treatment markedly attenuated this raised lipid peroxidation (LPO) product formation in a dose-dependent manner. Cancer chemoprevention by phytochemicals or herbal medicines is grabbing high interest now a day. These phytochemicals exert their anticancer potential due to chemical constituents such as flavonoids, polyphenols, carotenoids, terpenoids, and tannins obtained from our daily dietary agents (Behl et al. 2021). Flavonoids are potent anti-inflammatory, antioxidant, and cytotoxic anti-tumor agents. They can reverse the process of carcinogenesis and inhibit the development of persistent tumors (Figure 04).

When tumor initiator, 7-12-dimethyl benz(a)antheracene (DMBA), and tumor promoter, Croton oil (active constituent: 12-O-tetradecanoylphorbol-13-acetate) applies on the mice skin, they were produced inflammation and reactive oxygen species (ROS) (Mohammad et al. 2015). These ROS, including O_2-, OH-, and H_2O_2, can move from the site of formation to the other healthy cells. DMBA, with its active metabolites, causes a mutation in healthy cells via diol epoxide induction. Increased ROS disturbs the balance of oxidation/reduction reaction and oxidative stress parameters and takes part in chemical carcinogenesis by changing the gene expression and destroying the cellular components. TPA and ROS increase the epidermal ornithine decarboxylase, COX-2, and nitric oxide synthase levels.

Enzymatic oxidative stress parameters, including SOD and CAT, and nonenzymatic GSH help to play an important role in the enzymatic defense system, and their lower level promote the tumor in healthy cells. Reduced glutathione content helps to protect the body from xenobiotics, toxic metabolites, and ROS (Pieme et al. 2017). SOD and CAT capture the reactive oxygen species and minimize their carcinogenic and mutagenic potential, balancing the hydrogen/oxygen peroxide level by causing alteration in O_2 and

H_2O_2 radicals. In the present study, in the carcinogen control group, the level of GSH, SOD, and CAT were significantly decreased, and LPO increased along with the tumor incidence, yield, and burden due to increased ROS.

The whole plant of CB n-hexane extract decreased the tumor incidence, tumor yield, tumor burden, cumulative number of papilloma, and lipid peroxidation level compared to the carcinogen control group (Talib et al. 2020). This plant extract resulted in two folds of increased GSH, SOD, and CAT levels in a continuous group in which plant extract was applied throughout the experimental period (16 weeks) with higher effects at 9 mg/kg/b.wt as compared to 3 and 6 mg/kg. The phytochemical analysis showed the presence of flavonoids, saponins, tannins, and terpenoids, and HPLC confirmed the kaempferol and quercetin, i.e., flavonoids (Figure 05). These potent bioactive molecules possess anticarcinogenic potential since they can interfere with cancer initiation, development, and progression by modulating cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis (Shanmugam et al. 2016), as shown in Figure 03. Flavonoids have the potential as a chemopreventive agent for cancer treatment due to their ability to induce apoptosis (Rahmani et al. 2022).

It has been reported and proved that the major constituent of a tumor suppressor is flavonoids. Its cytotoxic activity is due to its unique structure. The activity of flavonoids is monitored by quantity, location, and substitution of OH groups or groups on A and B rings and a double bond at the C2-C3 level (De, Jo, and Kim 2022). Its activity is also connected with DNA topoisomerase II, which induces the destruction between DNA double stands and helps to reconnect these, which perform various important functions in the cellular processes. Studies depicted that quercetin, an important flavonoid having significant anticarcinogenic activity by inhibiting the overexpression of tumor-causing genes, functions of topoisomerase II, arrest the uncontrolled cell growth at G1, S, G2, and M all phases of cell cycle and maintains its balance (Eisvand et al. 2022). Along with these, it indirectly increases the level of tumor suppressor genes and their related genes and protein expression.

Previous works of literature have proved that quercetin can capture the ROS, superoxide anions, hydroxyl and lipid peroxy radicals, inhibit cyclooxygenase, lipooxygenase, monooxygenase, phospholipase A2, protein kinase and NADH-oxidative pathways and also inhibits the CYP3A4, which is the most abundant P450 enzyme in the liver and beneficial in metabolizing a significant number of carcinogens and their metabolism (Rivas 2022). Furthermore, quercetin is abundantly found in the human diet and extensively metabolized during absorption in the small intestine and liver, thus exerting a dose-dependent inhibitory effect on cell proliferation (Alam et al. 2022). In addition, animal and in vitro studies have shown that quercetin increases the activity of several detoxifying and antioxidant enzymes, such as glutathione reductase, glutathione peroxidase, glutathione S-reductase, and catalase, and inhibits the protein tyrosine kinase which is also involved in cell proliferation (Varghese et al. 2022).

We concluded that all those agents which can capture the free radicals *in vivo* might have cytotoxic potential against cancer, and 9 mg/kg dose of CB n-hexane had shown more positive results as a cytotoxic agent against carcinogen-induced skin papillomas.

5. Conclusion

This study concluded that mice having skin carcinoma treated with CB n-hexane showed a dose-dependent decrease in the weight of the tumor. Moreover, CB at the dose of 6 and 9 mg/kg showed a significant ($P<0.01$) decrease in the level of MDA as compared to DMBA/Croton oil-applied mice. It also revealed the normal structure and architecture of skin layers and prevented further impairment of skin tissues, thus reconfirming the biochemical correlates of the chemopreventive efficacy of CB. This

investigation has introduced new phytochemicals for cancer prevention and will open new ways in this research era.

Conflict of Interest

The authors declare that they have no competing interests.

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Consent Forms

Not applicable

Authors Contribution

FN Conceptualized the study, AA and WQ wrote the final manuscript, RA helped in the analysis and writing the first draft, did the experimental analysis, and IA supervised the whole project and wrote the final manuscript.

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