

RESEARCH ARTICLE

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Chemopreventive Effects of Resveratrol and Curcumin on Melanoma Growth in C57BL/6 Mice

Salman Ul Islam¹, Abdullah Al Saidi², Shehla Akbar³, Amina Akhlaq¹, Adeeb Shehzad^{4*}, Mazhar Ul-Islam^{2*}

Abstract

This study investigates the chemopreventive potential of two phytochemicals, resveratrol (RV) and curcumin (CUR), in a C57BL/6 mouse model of melanoma. Utilizing a xenograft approach, we implanted B16F10 cells subcutaneously into mice and treated them with RV and CUR at a dose of 50 mg/kg via intraperitoneal injection. Our findings reveal that both compounds significantly inhibit tumor growth, as evidenced by reduced tumor volumes and enhanced apoptotic markers. Western blot analyses revealed activation of the intrinsic (mitochondrial) apoptotic pathway, as indicated by increased expression of pro-apoptotic proteins such as cleaved caspase-3 and Bax, alongside reduced levels of the anti-apoptotic protein Bcl-2 in the RV- and CUR-treated groups. Furthermore, both compounds modulated key signaling pathways associated with apoptosis, suggesting their role in enhancing programmed cell death in melanoma cells. Histopathological examination confirmed reduced tumor cell proliferation and increased apoptosis in treated tissues. Additionally, the treatments influenced oxidative stress markers, indicating a potential role in redox balance regulation. These findings highlight the therapeutic potential of RV and CUR in skin cancer prevention and treatment. Given their efficacy in suppressing melanoma progression, further studies are warranted to elucidate their precise molecular mechanisms and evaluate their clinical applicability in combination with standard melanoma therapies.

Keywords: Skin carcinogenesis- Melanoma- Resveratrol- Curcumin; B16F10 cells- Dietary phytochemicals

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Introduction

The skin is the largest organ in the human body and acts as the primary protective barrier against environmental threats. The skin barrier is especially vulnerable to damage from several sources, including harmful chemicals, ultraviolet (UV) radiation from the sun, and exposure to microorganisms [1]. The accumulation of these stresses can lead to skin carcinogenesis, a multistage process that includes cancer initiation, promotion, and progression [2, 3]. The initiation stage occurs after exposure to a carcinogen, such as ultraviolet radiation, which results in cellular DNA damage. UV radiation can induce carcinogenesis both directly, through photon-induced DNA damage, and indirectly, by affecting DNA, membranes, and proteins through reactive oxidative stress [4]. If the DNA damage is not repaired, the cell may undergo irreversible genetic mutations, allowing it to grow autonomously [5]. Following the initiation stage, the promotion stage occurs when these initiated cells are repeatedly exposed to compounds that encourage

selective clonal proliferation, eventually forming a benign tumor over time. Regenerative proliferation, which is associated with repeated injuries, UV radiation, chronic inflammation, and oxidative stress, has been linked to skin tumor promotion [6]. Finally, in the progression stage, the benign tumor experiences further genetic mutations and becomes increasingly invasive, evolving into a malignant neoplasm capable of metastasizing [2]. The rapid tumor growth during this progression stage relies on the recruitment of nutrients and oxygen through angiogenesis, a process in which new blood vessels develop from existing vascular structures [7].

Phytochemicals may play a distinctive role in the context of skin cancer. Firstly, pre-cancerous and cancerous skin lesions are easily accessible for both patients and physicians [8]. This accessibility is beneficial for developing topical treatments that can be directly applied to the affected areas, minimizing harm to surrounding healthy skin. In contrast, using phytochemicals for tumors in internal organs often necessitates oral ingestion, leading to systemic effects. Secondly, the evaluation of skin lesions

¹Department of Pharmacy, International Institute of Science, Arts, and Technology (IISAT), Gujranwala 52250, Punjab, Pakistan.

²Department of Chemical Engineering, College of Engineering, Dhofar University, Salalah, Oman. ³Institute of Pharmaceutical Sciences, Khyber Medical University, Hayatabad, Peshawar 25000, Pakistan. ⁴Biodiversity Unit, Research Center, Dhofar University, Salalah 211, Oman. *For Correspondence: ashehzad@du.edu.om, mulislam@du.edu.om

and treatment outcomes can be straightforward for both medical professionals and patients. While pathological confirmation is often invasive for many cancers, skin biopsies are generally less intrusive [9]. Consequently, future studies assessing the efficacy of phytochemicals in treating skin cancer may be more practical. Lastly, any local adverse reactions from topical applications can be quickly identified by patients, which helps minimize discomfort and reduces the risk of long-term or severe side effects.

RV (trans-3,4',5-trihydroxystilbene) is present in a variety of vegetables, such as peanuts, Japanese knotweed, blueberries, Scots pine, and *Reynoutria japonica* [10]. Currently, red grapes are the primary source of RV. Commercial grape juice contains approximately 4 mg/L of RV [11]. RV naturally exists in both cis and trans stereoisomeric forms, and it is predominantly found in dietary sources as a glycosylated compound known as piceid (resveratrol-3-O- β -D-glucoside). While both isoforms exhibit similar effects, the trans form has been the focus of more extensive research [12].

RV has been found to possess significant anticancer properties, functioning as a reactive oxygen species scavenger and effectively lowering ROS levels in human skin fibroblasts [13, 14]. Research indicates that RV, in combination with other phytochemicals, inhibited tumor formation and decreased epidermal hyperplasia in mice, while also reducing the expression levels of COX-2, Bcl-2, and p21 [15]. Kowalczyk et al. reported that RV decreased the viability of skin cancer cells during melanoma treatment and enhanced the cytotoxic effects of temozolomide [16]. Additionally, RV has been shown to inhibit the activity of redox factor-1, which increases the sensitivity of skin cancer cells to dacarbazine [17].

Fuggetta et al. showed that RV has anti-melanoma properties both in vitro and in vivo. In their study, a concentration of 7 μ g/ml of RV was able to prevent the proliferation of melanoma cells, achieving a 50% reduction in cell growth [18]. Furthermore, RV also inhibited the growth of A431 cells (squamous cell carcinoma cells) [19]. Cell death and reduced proliferation due to RV were observed in FaDu, Cal27 (human head), and Det562 (human neck) SCC cell lines as well [20]. Kim et al. found that a 72-hour treatment with 25 μ M RV led to decreased DNA synthesis [21]. An in vivo study indicated that administering 50 mg/kg body weight of oral RV inhibited carcinoma cell growth, resulting in over a 50% decrease in both tumor volume and murine weight [20]. Overall, these results suggest that RV may possess significant anticancer activity in both in vitro and in vivo settings.

CUR, a polyphenolic phytochemical, is derived from the rhizome of turmeric (*Curcuma longa*). Numerous studies have demonstrated that CUR possesses impressive antioxidant and anti-inflammatory properties, providing significant therapeutic benefits in conditions such as atherosclerosis, ulcerative colitis, psoriasis, and Crohn's disease [22]. Additionally, CUR exhibits anticancer effects by interacting with and modulating various protein targets [23]. Dahmke et al. [24] showed the oncostatic effects of CUR in a mouse model of melanoma, where it upregulated

miRNA-205-5p, a crucial regulator of cell proliferation and apoptosis. Another study reported that CUR induced cell death while inhibiting both proliferation and invasion through the upregulation of mmu-miR-205-5p and the downregulation of proliferating cell nuclear antigen, Bcl-2, and JAK/STAT signaling pathways [25]. Kim et al. found that CUR decreased the phosphorylation of Akt, S6K, ILGF-1 receptor, IRS-1, and 4EBP in mouse keratinocytes, significantly blocking DMBA-induced skin cancer in mice [26]. Zhao et al. noted that CUR halted the progression of A375 and C8161 human melanoma cells at the G2/M phase of the cell cycle and triggered autophagy. Furthermore, CUR inhibited the activation of P70S6K and reduced the expression of AKT and mTOR, suggesting potential targets for melanoma treatment [27]. In research by Wu et al., CUR was shown to suppress the STAT3 signaling pathway in A431 cells, leading to a marked decrease in cell invasion [28]. In WM-115 melanoma cells, CUR facilitated the opening of the mitochondrial permeability transition pore, resulting in cell death [29]. Chinembiri et al. reported that CUR inhibited the NF- κ B pro-survival pathway, downregulated Bcl-2 expression, and activated the p53 tumor suppressor protein, ultimately causing cell death and suppressing skin cancer [30].

Gupta et al. [31] investigated the effects of CUR on the SRB12-p9 skin cancer cell line using a mouse model. Their findings showed that oral administration of CUR effectively suppressed the growth of squamous cell carcinoma and reduced levels of pS6, a well-established downstream marker of the mTOR and MEK/ERK signaling pathways. Additionally, the study revealed that CUR at concentrations of 20 μ M or greater completely halted the proliferation of SRB12-p9 cells. The good tolerability and safety profile of CUR highlight its potential as a valuable phytochemistry for the treatment of skin cancer.

Materials and Methods

Chemicals and reagents

Cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco (Carlsbad, CA, USA). RV and CUR were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342 dye was purchased from Calbiochem (USA). Antibodies were obtained from Santa Cruz Biotech [Cleaved caspase-3 (sc-22171-R), cleaved PAPR (sc-23461-R), Bax1 (sc-20067), Bcl2 (sc-7382), p53 (sc-126), p21 (sc-6246), and β -actin (Cat# sc-47778)]. All the chemicals and reagents were used as directed by the manufacturers.

Cell culture

B16F10 (ATCC CRL-6475) cells were cultured in DMEM, supplemented with 10% FBS and 2% penicillin-streptomycin, and were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Animal study protocol

Twenty-four male C57BL/6 mice were housed at a density of 6 mice per cage, under conditions of constant temperature (22°C) and a 12 h light/dark cycle. Our experimental protocol complied with animal maintenance and use guidelines of Kyungpook National University. When the mice were six weeks old, 1×10^6 B16F10 cells were suspended in 150 μ l PBS and implanted subcutaneously. At one-week post-implantation, the mice were divided into two groups: (A) control (vehicle) and (B) RV/CUR (50 mg/kg i.p. injection). Tumor volumes were measured weekly using Vernier calipers in both treated and control groups and calculated using the formula: $V = (4/3)\pi W^2L$, where W is the width (short diameter) and L is the length (long diameter) of the tumor, both in millimeters (mm^3). Tumors were harvested after 30–45 days post-implantation using scissors.

Paraffin processing of tissues

Tumor specimens were incubated for approximately 24 h in 10% neutral buffered formalin immediately after collection to protect cells from necrotic and degenerative changes. To embed the tissues in paraffin, they were cleared with hydrocarbon. For gradual dehydration of tissues, they were immersed in ethanol solutions of increasing concentration until pure, water-free alcohol was reached. The ethanol content within the tissue was gradually replaced with a series of xylene washes (10%, 25%, 50%, 75%, 80%, 90%, 95%, and anhydrous 99.8%). Tissues were then maintained in a mixture of paraffin with xylene, followed by liquid paraffin at 55°C, which remained in place for several hours. Tissues paraffin blocks were prepared by pouring liquified paraffin into a mold and inserting thin pieces of tissue with the appropriate spatial orientation. Paraffin-embedded tissues were then cut into 2–5 μ m thick sections using a microtome. Each section was then flattened on a hot water surface. Afterward, paraffin sections were placed in calcium fluoride (CaF_2) and incubated for 3–6 h at 50°C to achieve permanent adherence to the slide surface.

Hoechst 33342 staining assay

Nuclear fragmentation was analyzed by staining apoptotic nuclei with Hoechst 33342. Tissues on CaF_2 slides were stained with 5 μ g/ml Hoechst 33342 dye for 15 min. Digital images were captured using a fluorescence microscope (DM/R-TCS, Leica).

Western blot analysis

Tissues were homogenized with a Polytron PT-10-35-GT homogenizer (Thomas Scientific, Swedesboro, NJ, USA) in an ice bath. The tissue homogenates were then quantified using the Bio-Rad Protein Assay according to the manufacturer's protocol. Samples (20–40 μ g) were prepared in SDS sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% β -mercaptoethanol, separated on a 10%–12% SDS-PAGE gel, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham, Piscataway, NJ, USA). The membranes were blocked with 3% albumin (Gendepot, USA) solution for 2 h at 4°C. After incubation with the

appropriate antibodies, chemiluminescent signals were developed using a Clarity ECL Western Blotting Substrate (Bio-Rad) according to the manufacturer's instructions.

Statistical analysis

All samples were prepared in triplicate and all experiments were repeated at least three times. Data are presented as mean $\pm$ standard deviation (SD). Differences between groups were evaluated by the Student's t-test and p-values < 0.05 were considered statistically significant.

Results

We evaluated the effects of RV against skin cancer (melanoma). For this study, we established xenograft tumors in C57BL/6 mice by injecting them with 1×10^6 B16F10 cells. After one-week post-implantation, the mice were divided into two groups: (A) control (vehicle) and (B) RV (50 mg/kg i.p. injection). The detailed experimental approach is described in the materials and methods section. We observed that in the vehicle-treated group, B16F10 cells show enhanced tumor growth. However, RV effectively reduced tumors generated from B16F10 cells. Body weight remained stable throughout the experiment in both groups (Figure 1A).

In order to analyze the underlying mechanism through which RV decreased tumor volume, we performed Hoechst staining. As shown in Figure 1B, RV treatment induced shrinkage and fragmentation of tumor cells. This appearance, typical of apoptotic cells, was not observed in the control group. To further analyze whether intrinsic pathways of cell death were activated upon RV treatment, a western blot analysis was done to detect the expression levels of cleaved caspase 3, cleaved PARP, Bax, and Bcl-2 in tumor tissue. The results indicated that cleaved caspase 3 and PARP were significantly increased in the RV-treated group compared with the control group. Additionally, an increased level of pro-apoptotic Bax and a decreased level of anti-apoptotic Bcl-2 protein were clearly observed in tumor tissue lysates from RV-treated mice (Figure 1C). Furthermore, we observed that RV treatment enhanced the expression levels of p53 and p21 (Figure 1C). Additionally, we evaluated the effects of CUR on skin melanoma in our laboratory and obtained results consistent with those seen with RV (Figure 2A–C). Our findings indicate that treatment with both RV and CUR induces cell death and cell cycle arrest in xenograft tissues by modulating signaling proteins involved in cell apoptosis.

Discussion

The skin serves as the body's primary barrier against environmental threats, making it particularly vulnerable to damage from various harmful agents, including ultraviolet radiation and microorganisms. The multistage process of skin carcinogenesis comprising initiation, promotion, and progression highlights the complexity of skin cancer development. UV radiation can directly damage cellular DNA, leading to irreversible mutations if not repaired, while also inducing reactive oxidative stress that exacerbates this damage. As these initiated cells proliferate

under conditions of repeated injury and inflammation, benign tumors can form, which may eventually progress to malignant neoplasms. This underscores the need for effective preventive and therapeutic strategies.

In this context, the concept of chemoprevention has emerged as a promising approach, particularly through the use of natural phytochemicals. RV and CUR are two notable examples that have shown significant anticancer properties in both *in vitro* and *in vivo* studies. Our research highlights the effectiveness of these compounds in combating skin cancer. RV, derived primarily from red grapes, has demonstrated the ability to reduce tumor growth and induce apoptosis in skin cancer cells. Specifically, our findings indicate that RV treatment leads to increased expression of pro-apoptotic proteins while decreasing anti-apoptotic signals, thereby promoting cell death.

Similarly, CUR, extracted from turmeric, exhibited remarkable antioxidant and anti-inflammatory properties, along with direct anticancer effects. Our laboratory studies confirmed that CUR can induce apoptosis and halt the proliferation of melanoma cells, further supporting its role as a potential therapeutic agent. Both compounds demonstrate a capacity to influence key signaling pathways associated with DNA damage response, highlighting their potential to modulate cancer progression effectively.

The accessibility of skin lesions for topical treatment presents a unique opportunity to utilize these phytochemicals more effectively compared to systemic approaches often required for internal cancers. This allows for targeted therapy with minimal side effects, making them valuable candidates for further clinical evaluation.

It is important to highlight that we have delivered RV and CUR via the parental route. Nevertheless, the outcomes might differ when both compounds are taken orally. Bioavailability refers to the speed and degree of drug absorption at the action site. When CUR and RV are administered orally, they demonstrate low bioavailability due to their short half-life and quick elimination. To enhance intracellular uptake, substantial doses must be used, which limits their practicality as supplements [32-34].

Most polyphenols are not absorbed in their native form [35]. Instead, these compounds are hydrolyzed either by colonic microflora or intestinal enzymes prior to absorption. During the absorption process, polyphenols undergo significant modifications, including conjugation in intestinal cells, followed by glucuronidation, methylation, and/or sulfation in the liver [36, 37]. The bioavailability and the types of metabolites present in plasma are determined more by the chemical structure of the polyphenols than by their concentration. The therapeutic efficacy of polyphenols varies among different types. Following metabolism, polyphenols yield various metabolites that differ from their original forms, circulating in the bloodstream and being absorbed by tissues. Assessing the potency of each metabolite poses challenges [38].

The first biodistribution study indicated that a significant portion of CUR is metabolized in rats

when administered orally [39]. It was found that CUR metabolism primarily occurs in the liver [39-41]. Holder et al. identified glucuronides of tetrahydrocurcumin and hexahydrocurcumin as the principal metabolites of CUR in rats, while dihydroferulic acid and trace amounts of ferulic acid were noted as minor metabolites [42]. Additionally, the urine of rats treated with CUR exhibited sulfate conjugates alongside glucuronides [43]. Research by Pan et al. revealed that 99% of the CUR conjugates detected in plasma were glucuronides due to glucuronidase-catalyzed hydrolysis. The same study ultimately concluded that the primary metabolites produced by CUR *in vivo* are tetrahydrocurcumin-glucuronoside and dihydrocurcumin-glucuronoside [44].

In a separate study involving healthy human participants, the pharmacokinetics of a CUR preparation were assessed over a period of 0.25 to 72 hours following a single oral dose. The administered doses of CUR were 10 g (n = 6) and 12 g (n = 6). Analysis of serum samples from the subjects using HPLC (with a limit of detection of 50 ng/mL) revealed that free CUR was identified in only one subject at any of the 14 time points. In contrast, CUR glucuronides and sulfates were present in samples from all subjects. No plasma samples from any other participant showed the presence of free CUR [45].

The rapid and extensive metabolism of RV, leading to the formation of various metabolites such as RV glucuronides and RV sulfates, ultimately results in zero bioavailability when taken orally [46]. *In vitro* studies included the treatment and incubation of human hepatocytes, human liver microsomes, and rat hepatocytes. *In vivo* studies involved administering RV to rats and mice through both oral and intraperitoneal routes. Analysis of rat urine, mouse serum, human hepatocytes, rat hepatocytes, and human liver microsomes via HPLC using methanolic extracts revealed that trans-resveratrol-3-O-glucuronide and trans-resveratrol-3-sulfate were present in high amounts across all samples. The structures of these conjugates were confirmed by incubation with beta-glucuronidase and sulfatase, which released free RV [47].

In a different study, ten subjects were administered 500 mg of RV in immediate-release uncoated caplets. The initial dose was set at 1 g, which was subsequently increased to 2.5 g and then to 5.0 g. After pharmacokinetic data was collected at the 5 g dosage, the subjects received a dose of 0.5 g. HPLC analysis of plasma and urine samples revealed the presence of two monosulfates, one disulfate, two monoglucuronides, and one glucuronide-sulfate. These results further confirm the extensive metabolism of RV in humans. The short half-life, inability to retain, rapid elimination, and unfavorable degradation/biotransformation contribute to the low bioavailability of the parent compounds of both CUR and RV at their site of action [48].

In conclusion, our findings collectively emphasize the potential of phytochemicals, particularly RV and CUR, as effective agents in the prevention and treatment of skin cancer. Their ability to induce apoptosis, inhibit tumor growth, and modulate critical signaling pathways involved in the DNA damage response positions them as promising

therapeutic options. As research continues to elucidate their mechanisms of action, these natural compounds could significantly contribute to the development of innovative strategies for skin cancer management. Future studies should focus on optimizing delivery methods and further evaluating the clinical efficacy of these phytochemicals to ensure their successful integration into therapeutic protocols.

Author Contribution Statement

Investigation and experimentation: S.U.I.; formal analysis: A.A.; Conceptualization: S.A.; data curation: A.A.S.; writing-original draft preparation: S.U.I.; writing-review and editing, A.A.S., A.S., M.U-I; supervision: A.S., M.U-I. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to animal maintenance and use guidelines of Kyungpook National University (nos. KNU 2012-37 and 2016-42).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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