Original Paper

Medical Principles and Practice

Med Princ Pract 2013;22:357–361
DOI: 10.1159/000346622

Received: September 12, 2012
Accepted: December 13, 2012
Published online: February 7, 2013

# Chemopreventive Effect of *Ardisia crispa* Hexane Fraction on the Peri-Initiation Phase of Mouse Skin Tumorigenesis

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**Key Words**

*Ardisia crispa* · 7,12-Dimethylbenz(α)-anthracene · Tumor burden · Tumor volume

**Abstract**

***Objective:*** To investigate the chemopreventive effect of the hexane extract of *Ardisia crispa* during the peri-initiation phase of mouse skin tumorigenesis. ***Materials and Methods:*** This study was conducted for 12 weeks on two-stage 7,12-dimethylbenz(α)-anthracene (DMBA)-induced tumor initiation followed by croton-oil-induced tumor promotion in mice. *A. crispa* root hexane extract (ACRH) was applied at various doses (30, 100, 300 mg/kg) 7 days prior to and after DMBA treatment. Throughout the study, morphological observations, i.e., tumor incidence, tumor volume and tumor burden were measured for each of the treated groups. At the end of the experiment, the mice were sacrificed and their skin tissues were examined histopathologically. ***Results:*** The highest dose of ACRH (300 mg/kg) significantly delayed tumor formation (week 9, $p < 0.05$) and exhibited the lowest tumor volume ($0.71 \pm 0.00$ mm$^3$, $p < 0.05$), tumor burden ($2.00 \pm 0.00$, $p < 0.05$), and tumor incidence (16.67%, $p < 0.05$) compared to other doses of ACRH. A 100-mg/kg dose produced tumor latency at week 7, tumor volume of $2.44 \pm 0.88$ mm$^3$ ($p < 0.05$), tumor burden of $1.60 \pm 0.60$ ($p < 0.05$), and tumor incidence of 50%; 30 mg/kg produced tumor latency at week 8, tumor volume of $2.04 \pm 0.45$ mm$^3$ ($p < 0.05$), tumor burden of $2.17 \pm 0.54$, tumor incidence of 60% and carcinogen control (tumor latency at week 7; tumor volume, 3.56 mm$^3$; tumor incidence of 66.67%). ***Conclusion:*** The highest dose of *A. crispa* hexane extract delayed tumor development, thus showing a chemopreventive effect on mouse skin tumorigenesis.



## Introduction

Cancer is a major disease at a worldwide level, accounting for more than 7 million deaths per annum [1]. Progress made in cancer therapy has not been sufficient to significantly decrease annual death rates from most epithelial tumor types, and there is an urgent need for new strategies in cancer control [2]. Skin cancer is the most common form of cancer in the United States. Over the years, studies have shown that among all cancers, skin cancer is the most preventable [3]. Increasing incidence of skin cancer due to constant exposure of skin to environmental carcinogens, including both chemical agents and ultraviolet radiation, provides a strong basis for chemoprevention with synthetic, natural, and both internal

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and topical remedies [4]. The two-stage skin tumorigenesis protocol in the mouse model has advanced our understanding of the stages and mechanisms of carcinogenesis. Most tumor-initiating agents either generate or are metabolically converted to electrophilic reactants that bind covalently to cellular DNA [5].

One approach to cancer chemoprevention involves the administration of natural and/or synthetic nutrient or non-nutrient compounds to examine their potential role in the prevention of initiation and/or promotional stages of carcinogenesis [6]. Chemoprevention aims at directly modulating specific steps in the carcinogenic process, i.e., blocking mutagenic carcinogens, preventing DNA damage by free radicals, and suppressing the differentiation of epithelial cells and apoptosis. Chemoprevention with phytochemicals is currently regarded as one of the most important strategies for cancer control [7].

Initiation involves mutation of cellular DNA resulting in the activation of oncogenes and the inactivation of tumor suppressor genes. Initiation is thought to be irreversible and consists of a single-gene mutation that in most cases is caused by environmental genotoxic agents such as chemicals, radiation and viruses [8].

*Ardisia crispa*, from the family Myrsinaceae, is found throughout subtropical and tropical regions. The root and leaves of this plant have long been used by local villagers as folk medicine to treat various ailments such as dysmenorrhea, throat and chest pain, cough, skin disorder, fever, diarrhea, broken bones and sprains, rheumatism, and as an antidote and diuretic [9–11]. Scientifically, *A. crispa* has been reported to exhibit antihypertensive, anti-platelet-aggregating properties, anti-inflammatory, antihyperalgesic, antifungal, antimetastatic, antipyretic and antiulcer effects [12–18]. As inflammation basically contributes to tumor development [19], it is therefore postulated that root extract of *A. crispa* may possess significant antitumor properties, hence the decision to study the chemopreventive effect of the hexane fraction of *A. crispa* (ACRH).

## Materials and Methods

*Plant Material*

*A. crispa* roots were collected in April, 2010 from Machang, Kelantan, Malaysia and deposited (voucher specimen No. 20841) in the herbarium of Universiti Kebangsaan Malaysia, Bangi for future reference.

*Preparation of Plant Extract*

Roots of *A. crispa* (1 kg) were cut into smaller pieces and dried at 40°C for 3 days. Dried roots were then ground using Wiley's laboratory mill. Later, the ground materials (400 g) were macerated in 90% aqueous ethanol (5,000 ml) 3 times for 48 h. The extract was concentrated in a rotary evaporator under reduced pressure to give crude aqueous ethanolic extract (29.8 g), which was subjected to subsequent n-hexane fractionation. The solvent was then removed in a rotary evaporator at 40°C. ACRH (6.1 g) was obtained after the concentrate was dried at room temperature. The extract was prepared into the desired doses (30, 100 and 300 mg/kg) by dissolving in acetone.

*Experimental Animals*

Six- to eight-week-old ICR female mice, weighing 20–30 g, were obtained and kept at the animal house of the Faculty of Medicine and Health Sciences, University Putra Malaysia with ethical approval from the Animal Care and Use Committee (UPM/FPSK/PADS/BR-UUH/00315). The mice were housed 10 per cage and stabilized for 1 week prior to the commencement of experiments. They were fed on a standard laboratory diet with free access to water. Three days before treatment, the mice were dorsally shaved with an electric hair clipper (approximately 2 cm × 2 cm area, about 1 cm off the tail).

*Drugs and Chemicals*

7,12-Dimethylbenz(α)anthracene (DMBA), acetone and curcumin were purchased from Sigma-Aldrich Co. (United States). Croton oil was purchased from TCI Chemicals (Japan). As a tumor initiator, DMBA was dissolved at a concentration of 100 μg/100 μl in acetone. Croton oil, which served as a tumor promoter, was dissolved in acetone to give 1% croton oil solution. Curcumin, a positive control, was dissolved in acetone at a dose of 10 mg/kg.

*In vivo Two-Stage Skin Tumorigenesis Study*

The experiment was conducted for 12 weeks. A modified version of the methodology previously described by Garima et al. [7] was used. The animals were divided into six groups; each group had 10 ICR female mice. Group I received a single topical application of 50 μg/100 μl/mouse of DMBA in acetone, followed by the application of 100 μl/mouse of 1% croton oil in acetone twice a week for 10 weeks with the addition of a topical application of ACRH at a dose of 30 mg/kg, 7 days prior to and after the DMBA treatment.

Groups II and III were treated similar to group I, with topical application of ACRH at 100 and 300 mg/kg, respectively, 7 days prior to and after DMBA treatment. Group IV served as the positive control where mice received the same treatment as group I with the modification that this group received a topical application of 10 mg/kg of the known chemopreventive agent, curcumin, for 7 days prior to and after DMBA treatment. Group V served as the carcinogen control where mice received a single topical application of 50 μg/100 μl/mouse of DMBA in acetone followed by the application of 100 μl/mouse of 1% croton oil in acetone twice a week for 10 weeks. Group VI served as the vehicle control where mice received a topical application of 100 μl/mouse of acetone on the shaved dorsal skin throughout the entire experiment.

*Morphological Assessment*

Body weight, the latency period of tumor formation, percentage of tumor incidence, tumor burden and tumor volume were observed and measured at a weekly interval. Only tumors that persisted for more than 1 week with a diameter greater than 1 mm were taken into consideration for data analysis. The latency pe-

**Table 1.** Chemopreventive effect of ACRH on two-stage mouse skin tumorigenesis

| Groups | | Body weight, g | | Cumulative number of tumors | Tumor burden | Tumor volume, $mm^3$ | Tumor incidence, % |
|---|---|---|---|---|---|---|---|
| | | initial | final | | | | |
| I | (30 mg/kg ACRH) | 33.4 | 43.0 | 13 | 2.17±0.54 | 2.04±0.45* | 60.00 |
| II | (100 mg/kg ACRH) | 27.0 | 42.6 | 8 | 1.60±0.60* | 2.44±0.88* | 50.00 |
| III | (300 mg/kg ACRH) | 24.3 | 37.2 | 2 | 2.00±0.00* | 0.71±0.00* | 16.67* |
| IV | (curcumin) | 27.0 | 37.0 | 2 | 1.00±0.00* | 0.66±0.14* | 20.00* |
| V | (carcinogen) | 33.5 | 44.4 | 15 | 2.50±1.31 | 3.56±2.13 | 66.67 |

Values are expressed as mean ± SEM and all values at the end of the experiment are compared.
* Significance level between treated groups (groups I–IV) and carcinogen control (group V) at $p < 0.05$.

riod of tumor formation was determined by the appearance of the first tumor. Percentage of tumor incidence was calculated by dividing the number of tumor-bearing mice with the total number of mice in a particular group multiplied by 100%. Tumor burden was obtained by dividing the total number of tumors with the number of tumor-bearing mice in a group. Tumor volume was measured by multiplying Π/6 by the length, width and height of the tumor [17].

*Histopathological Analysis*

The experiment was terminated at the end of week 10 of tumor promotion, and the mice were sacrificed for histopathological analysis. Skin samples obtained from dissection were fixed in 10% formalin before being processed in an automatic tissue processor by standard protocols. Processed tissues were embedded in paraffin wax, sectioned with a microtome at a thickness of 4 μm and stained with hematoxylin and eosin stain using a routine protocol. Stained slides were observed under a light microscope and digital micrographs of the slides were taken.

*Statistical Analysis*

All data were statistically analyzed by one-way analysis of variance with covariance followed by LSD multiple comparison test to assess the significant differences of mean between groups. SPSS 16.0 software was used for the calculations, and all values were expressed as mean ± SEM. Where necessary, $p < 0.05$ was considered as statistically significant.

## Results

The findings of the present study are shown in table 1 and figure 1. The administration of ACRH during the peri-initiation phase did not affect the body weight of the animals throughout the experiment (table 1). Papillomas started to appear on the mouse skin from week 6–9 during the promotion period (fig. 1).

In the carcinogen control, the cumulative number of papillomas was recorded as 15. The average number of papillomas per mouse (tumor burden) as well as the papillomas per papilloma-bearing mouse (tumor volume) were found to be 3.56 ± 2.13 and 2.50 ± 1.31, respectively. The group developed skin papillomas at week 6 (66.67% tumor incidence).

Interestingly, at the highest dose of ACRH, 300 mg/kg (group III), the development of tumors was greatly delayed until week 9 (16.67% tumor incidence) compared to the carcinogen control. Furthermore, in terms of the cumulative number of papillomas, it was greatly reduced to 2 compared to the carcinogen control, while at the other dosages, i.e., 30 and 100 mg/kg (groups I and II), both recorded a cumulative number of papillomas, 13 and 8, respectively.

For tumor burden and tumor volume, group III (ACRH 300 mg/kg) also showed a significant reduction of both parameters, i.e., 2.0 and 0.71 $mm^3$, respectively, compared to group V (carcinogen control). ACRH only significantly reduced the tumor volume to 2.04 ± 0.45 $mm^3$ at 30 mg/kg, yet it significantly reduced both tumor burden and tumor volume to 1.60 ± 0.60 and 2.44 ± 0.88 $mm^3$, respectively, at 100 mg/kg. Nevertheless, ACRH did not show any significant difference of tumor incidence at either 30 or 100 mg/kg (table 1).

Curcumin, as positive control (group IV), displayed its chemopreventive effect by delaying tumor appearance until week 9 and showed a similar tumor latency effect as group III. Moreover, the cumulative number of tumors, tumor burden and tumor volume showed a significant reduction of 2, 0.66, 1.00 $mm^3$ and 0.66, respectively, compared to carcinogen control.

Overall, groups I, II and III showed a dose-dependent effect compared to the carcinogen control group (group V) in terms of tumor burden, tumor volume and tumor incidence, respectively. Group III (300 mg/kg) showed a significant effect equipotent to the positive control group, curcumin.

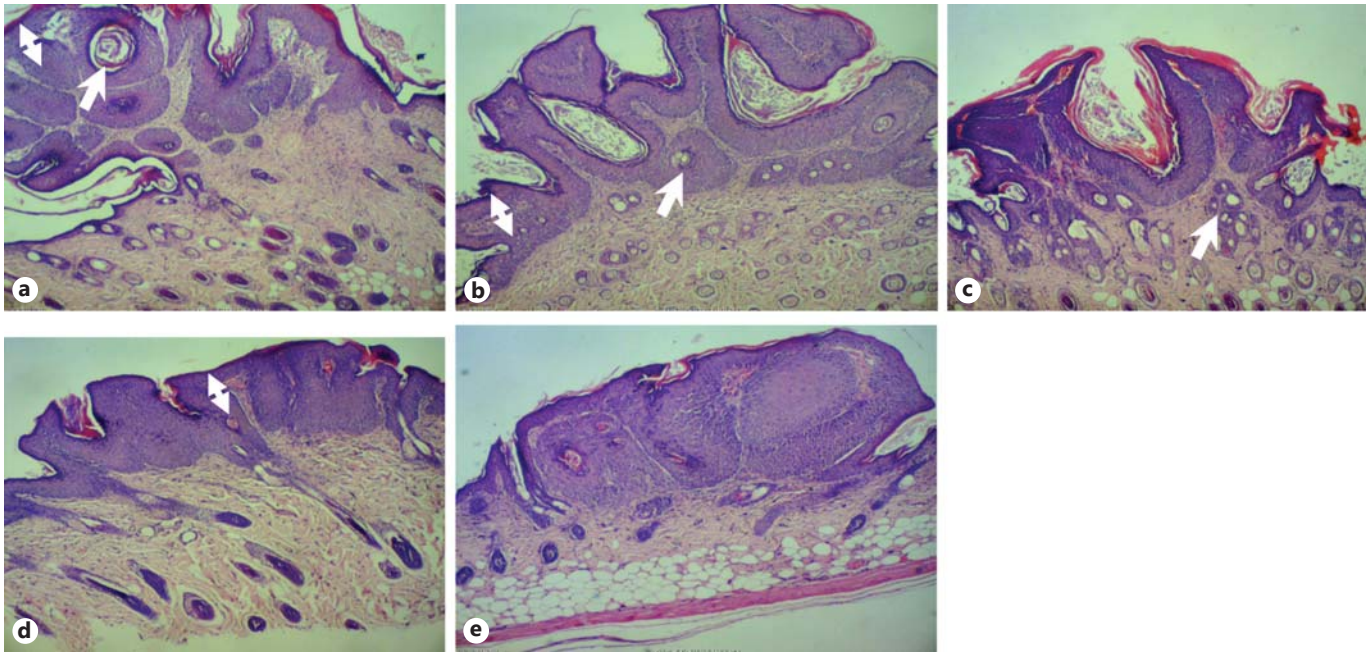


**Fig. 1.** Representative section of cutaneous tissue from group I (**a**), group II (**b**), group III (**c**), group IV (**d**), and group V (**e**) at the end of the experiment. The single-head arrow indicates a keratin pearl, the two-head arrow indicates hyperplasia. ×4.

Further histopathological analysis showed that group V (carcinogen control) had the greatest number of keratin pearls and rete ridges. Histopathological examination on all tissue sections of the skin tumor from the carcinogen control group demonstrated extensive hyperplasia, rete ridges and keratin pearls. In some of the tissue sections examined, parts of the basement membrane were found to be disrupted (fig. 1e). These lesions indicate that the tumors had progressed to the premalignant state. Among the ACRH-treated groups, group III did not show any presence of keratin pearls or rete ridges and there was also a reduction of hyperplasia compared to the carcinogen control. Furthermore, the basement membrane was undisrupted (fig. 1c).

In contrast, in group II (100 mg/kg), the tumors showed that they were all benign papillomas because no parts of the basement membrane were disrupted. Keratin pearls and rete ridges were less in groups I and II compared to group V (carcinogen), but were more in group I compared to group II. In group IV (curcumin), the degree of hyperplasia was similar to group III (300 mg/kg) but showed a lesser degree of hyperplasia compared to group I (30 mg/kg), group II (100 mg/kg) and group V (carcinogen control).

## Discussion

Together with our previous study, it was shown that ACRH acts on both initiation and promotion of two-stage carcinogenesis, so that it may play a different role during each stage. In this study, ACRH at the highest dose showed significant anti-tumor-initiating activity due to DMBA. However, ACRH had been reported to suppress tumor promotion at lower doses [20]. Interestingly, in the current study, at the highest dosage (300 mg/kg), ACRH exhibited anti-tumor-initiating activity and a promoting effect comparable to curcumin at a similar dose [21]. Thus, this could indicate that ACRH might be a tumor-initiating inhibitor at a higher dosage, while at the studied dose it appeared to possess an anti-tumor promotion effect.

Curcumin, a yellow-coloring ingredient derived from *Curcuma longa* L. (Zingiberaceae), is one of the most extensively investigated and well-defined chemopreventive phytochemicals. Curcumin had been shown to protect against skin, oral, intestinal, and colon carcinogenesis and also to suppress angiogenesis and metastasis in a variety of animal tumor models. It also acts in all stages of multistep carcinogenesis [21].

In the present study, ACRH delayed tumor development for an equivalent period as curcumin, and it also gave

a similar cumulative number of papillomas as curcumin at the end of the experiment. Curcumin inhibits tumor initiation by blocking the metabolic activation of carcinogens or by stimulating their detoxification. It also exerts antitumor-promoting effects by suppressing inflammatory signaling mainly mediated by cyclooxygenase-2 and inducible nitric oxide synthase that are under the control of nuclear factor-κB and other transcription factors [22]. Hence, hypothetically, ACRH could display similar tumor initiation inhibitory effects as curcumin via the aforementioned mechanisms [13, 21]. Furthermore, ACRH's anti-tumor-initiating activity could also be due to either modulation of carcinogen metabolism or antioxidant capacity, as suggested by Gerhauser et al. [1]. Moreover, there is an increasing body of evidence reporting antioxidative compounds that act both as chemotherapeutic and chemopreventive agents [22]. ACRH has also been shown to have antioxidant properties [personal commun.] and an anti-inflammatory effect [15]. Thus, these data indicate that ACRH may intercept and neutralize potent chemical carcinogens, such as reactive oxygen species (superoxide, peroxyl and OH radicals) and nitric oxide donors. It has also been reported that there are certain antioxidants that either inhibit skin tumor initiation or skin tumor promotion, and some even inhibit both stages [23]. It is therefore essential to perform assays of biochemical enzyme activity on its tumor tissue, to measure its lipid peroxidation level which contributes to its chemopreventive effect.

## Conclusion

These findings showed that ACRH at high doses may be a useful agent for cancer chemoprevention. However, more comprehensive studies need to be done on the isolation and identification of the phytochemicals of ACRH as well as to elucidate the exact anti-tumor-initiating mechanism underlying this suppressing effect.

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