RESEARCH COMMUNICATION

# Suppression of DMBA/Croton Oil-induced Mouse Skin Tumor Promotion by *Ardisia Crispa* Root Hexane Extract

**AH Roslida*, O Fezah, LT Yeong**

## Abstract

***Ardisia crispa*** **(Family: Myrsinaceae) has been used as a traditional medicine for various ailments. Previous studies showed that *Ardisia crispa* possesses antimetastatic and anti-inflammatory properties. Nevertheless, research done on the plant is still limited. Therefore, the present study was designed to evaluate the suppression effect of *Ardisia crispa* root hexane (ACRH) extract on 7, 12-dimethylbenz (α) anthracene (DMBA)-induced mice skin tumor promotion in ICR mice with topical application twice weekly for 10 weeks. Results showed significant difference between treatment groups (mice treated with 30 mg/kg, 100 mg/kg and 300 mg/kg of ACRH extract; denoted as group I, II and III respectively) for tumor incidence and tumor burden (*P*<0.05). Significant reduction in tumor incidence (20%), tumor burden (1.5 ± 0.50), tumor volume (2.49 ± 1.70) and delayed latency period of tumor formation was observed in group I (30 mg/kg) in comparison to carcinogen control. This study indicates that ACRH extract could be a promising skin tumor promotion suppressing agent at a lower dosage (30 mg/kg). Further studies are required to elucidate the underlying mechanism(s) leading to this effect.**

**Keywords:** DMBA skin carcinogenesis - tumor incidence/burden - cancer chemoprevention - *Ardisia crispa*

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## Introduction

Defined as "the uncontrolled growth and spread of cells that often invade surrounding tissue and metastasize", cancer has for all time been a major public health problem (World Health Organization). For the past few years, skin cancer prevalence has been increasing drastically (Cancer Research UK, 2008). According to World Health Organization (WHO), the global occurrence of skin cancer per annum appears to be between 2 and 3 million cases for non-melanoma and 132,000 cases for melanoma. However, at least one-third of all cancers are preventable and this renders cancer prevention an essential approach for all cancer control programs (Stewart and Kleihues, 2003).

Cancer chemoprevention can be understood as 'the prevention or delay of carcinogenesis in humans by ingestion of dietary or pharmaceutical agents (Sporn et al., 1976). Chemopreventive study with mouse skin model can be dated back over the last 60 years to study done by Deelman (1927), who found that wounding led to skin tumors after carcinogenic tar treatment. The discovery of croton oil as a potent promoting agent by Berenblum (1941) eventually led to the development of two-stage protocol of mouse skin tumorigenesis which is generally used nowadays. A two-stage carcinogenesis is based on the concept that initiation with a single subchronic dose of carcinogen, such as DMBA and followed by repeated promotion by a tumor promoter like croton oil will lead to the development of skin tumors (Boutwell, 1974; 1964). Among the two stages (initiation and promotion), animals studies show that the promotion stage takes longer period to occur and it may be reversible, probably at an early stage. Therefore, cancer prevention by suppression of tumor promotion is expected to be an efficient approach.

Of all currently proven and identified anticancer agents, 62% are derived from natural products (Newman et al., 2003). These include Catharanthus roseus alkaloids (vinblastine and vincristine), epipodophyllotoxins from Podophyllum species, taxanes from Taxus spp., and camptothecins from Camptotheca (Mann, 2002; Butler, 2005; Newman and Cragg, 2005; Ramawat, 2007). Along with side effects reported from conventional chemoprevention, research has now directed towards identifying chemopreventive compounds from natural products, especially plants. In this current project, *Ardisia crispa* was selected to evaluate its n-hexane root extract effect on DMBA-induced and croton oil-promoted skin tumor at the promotion stage by using two-stage carcinogenesis model in mice.

*Ardisia crispa*, which belongs to the family of Myrsinaceae, has been locally known as "Mata Ayam", "Mata Itik", "Mata Pelanduk", "Akar Beluloh" or Village Ardisia. It is an evergreen, fruiting shrub with white flowers and red berries. The plant can grow between 0.6-2 m with moist soils in woodland garden, shady edges, hillsides or forests as its common habitat (Chen and Pipoly, 1996). In traditional medicine, *Ardisia crispa* has

*Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Kuala Lumpur, Malaysia*
**For correspondence : roslida@medic.upm.edu.my*

been used as skin liniment, antidote and diuretic (Duke and Ayensu, 1985; Muhamad and Mustafa, 1994). In Thailand, the roots are used in combination with some other plants to "wash out dirty blood" in women suffering from menstrual pain (Jansakul, 1995). The root juice is also useful as treatment for throat and chest pain, cough, fever, diarrhea, as well as rheumatism. In Canton, it has been marketed as "sin-lo-san", a herbal decoction for sprains and broken bones (Burkill, 1966).

Previous scientific reports revealed that *Ardisia crispa* possesses antimetastatic, cytostatic, anti-inflammatory, anti-hyperalgesic, antiplasmodial, anti-pyretic and utero-contracting effects (Jansakul, 1995; Kang et al., 2001; Noor Rain et al., 2007; Roslida and Kim, 2008; Lau et al., 2009). In 1987, Jansakul had successfully isolated two utero-contracting saponins, termed ardisiacrispin A and B, from the plant and it was concluded that ardisiacrispin B may exert a $PGE_2$-like effect which may be developed into an abortifacient drug (Jansakul, 1995). Apart from that, an antimetastatic and cytostatic benzoquinonoid compound AC7-1 (2-methoxy-6-tridecyl-1,4-benzo-quinone), was also isolated. The compound has been shown to serve as potent PAF antagonist and it was suggested that AC7-1 may be a potential antimetastatic agents for tumor metastasis prevention although its antimetastatic mechanism is yet to be identified (Kang et al., 2001).

## Materials and Methods

*Preparation of extract from plant*

*Ardisia crispa* plants were collected from Kelantan, Malaysia and deposited (voucher specimen no: 20841) in the herbarium of University Kebangsaan Malaysia (UKM), Bangi (Malaysia). Roots of *Ardisia crispa* were cut into smaller pieces and dried at 40°C for three days. Dried roots were then grounded by using Wiley laboratory mill. Thereafter, the grounded materials were macerated in 90% aqueous ethanol for 48 hours. The extract was concentrated in a rotary evaporator under reduced pressure to give crude aqueous ethanol extract which will be subjected to successive fractionation with n-hexane. Anhydrous sodium sulphate was added and the solution was filtered. The solvents were then removed in a rotary evaporator at 40°C. Hexane fraction of *Ardisia crispa* root extract (ACRH) was obtained after the concentrates dried at room temperature (Roslida and Kim, 2008). The extract was prepared into desired dosage (30 mg/kg, 100 mg/kg and 300 mg/kg) by dissolving with acetone.

*Animals*

6-8 weeks old ICR male mice, weighing 20-30 g, were obtained and kept at the animal house of Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM) with ethical approval from the Animal Care and Use Committee (ACUC) of UPM (UPM/FPSK/PADS/BR-UUH/00315). The mice were housed ten per cage and stabilized for one week prior to the commencement of experiment. They were fed on standard laboratory diet with free access to water. Three days before treatment, the mice were dorsally shaved with an electric hair clipper, for an approximately 2 cm x 2 cm area (about 1 cm off tail).

*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). All other reagents were commercially available. DMBA, as tumor initiator, was dissolved at a concentration of 100 μg/100μl in acetone. Croton oil which served as tumor promoter was dissolved in acetone to give 1% croton oil solution. Curcumin, used as positive control, was dissolved in acetone at a dose of 10 mg/kg.

*In vivo two-stage skin tumorigenesis study*

All mice were divided into six groups (n=10). In group I-V, mice were initiated with a single topical application of DMBA one week prior to the promotion period. During the promotion period, mice in group I, II and III were treated with *Ardisia crispa* root hexane extract at a dosage of 30 mg/kg, 100 mg/kg and 300 mg/kg respectively, 30 minutes before croton oil application; while mice in group IV (positive control) were treated with 10 mg/kg of curcumin, 30 minutes before croton oil application. As for group V (carcinogen control), only croton oil was applied along the promotion period. Group VI (vehicle control) received only acetone throughout the experiment. All treatments were applied topically, twice a week for 10 weeks of promotion period.

*Morphological assessment*

Body weight, latency period of tumor formation, percentage of tumor incidence, tumor burden and tumor volume was observed and measured at weekly interval. Only tumors that persisted more than one week with diameter greater than 1 mm were taken into consideration for data analysis. Latency period of tumor formation was determined when the first tumor appeared. Percentage of tumor incidence was calculated by dividing the number of tumor-bearing mice with the total number of mice in a particular group and multiplied with 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 to the length, width and height of tumor (Girit et al., 2008).

*Histopathological analysis*

The experiment was terminated at the end of the 10th week of tumor promotion and 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 microtome at a thickness of 4μm and stained with Haematoxylin and Eosin (H&E) stain using routine protocol. Stained slides were observed under light microscope and digital micrographs of the slides were taken.

*Statistical analysis*

All data were statistically analyzed by one-way analysis of variance (ANOVA) 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 ± S.E.M (standard error of mean) at 5% significant level. Values with $P<0.05$ were considered statistically significant.

## Results

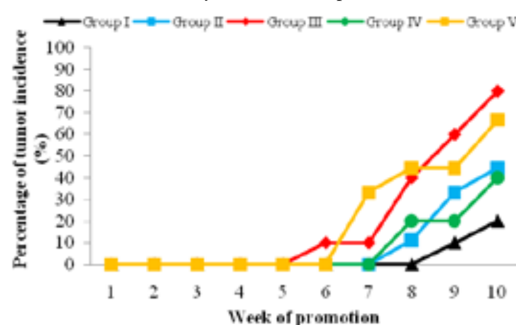
Findings from the present study are depicted in Table 1 and Figure 1. During ACRH treatment, no toxic effect had been seen with the selected dose of ACRH, as evident by body weight, skin texture and overall morphological appearance of the mice (data not shown). Significant rises of the average body weight of all groups were observed at the termination of the experiment (Table 1). Tumors begin to appear on the skin from week 6 to 9 during the promotion period. The latency period of tumor formation was greatly delayed to the ninth week in group I (30 mg/kg) in contrast to group V (carcinogen control), which started to develop tumor in the seventh week. Tumor formation was prompted one week earlier to the sixth week in group III (300 mg/kg) in comparison to group V. Both group II (100 mg/kg) and group IV (positive control) had a latency period of eight weeks. Tumor incidence (Figure 4.3) were significantly different between treatment groups at $P<0.05$. Group V recorded 66.7% of tumor incidence at the end of the study whereas group III had a higher tumor incidence of 80% in comparison to group V. Both the groups, group I (20%) and group II (44%), were found to have a highly significance difference level of tumor incidence in contrast to group IV (80%) and group V.

Statistical analysis also showed that there is significance difference between groups for tumor burden ($P<0.05$). Tumor burden is highest in group V with a value of 2.50 ± 1.31 whilst those in group III and group IV were recorded as 2.25 ± 0.56 and 2.25 ± 0.48, respectively. As in tumor incidence, tumor burden of group I (1.50 ± 0.50) and group II (1.25 ± 0.25) were also found to be significantly different from group V at $P<0.05$.

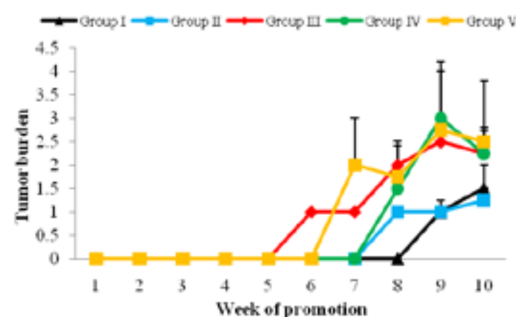
There was no significance difference between groups in tumor volume. However, group II (2.36 ± 0.50) and group IV (2.01 ± 0.96) showed significance difference in tumor volume when compared to group V ($P<0.05$). Tumor volume in group I (2.49 ± 1.70) was lower than that in group V, though there was no statistically significant difference between these two groups. A higher value of tumor volume was also observed in group III (3.83 ± 0.68) in comparison to group V (3.56 ± 2.13).

In overall, group I (30 mg/kg) and group II (100 mg/kg) showed better effects than the carcinogen control group (g roup V), in terms of tumor incidence, tumor volume and tumor burden. Group II showed comparable effect to the positive control group (group IV) whilst for group I (30 mg/kg), the suppressing effect was even greater than group IV, especially in tumor incidence and tumor burden. Group III (300 mg/kg), nevertheless, turned out to be poorer in tumor incidence and tumor volume in comparison to the carcinogen control group.

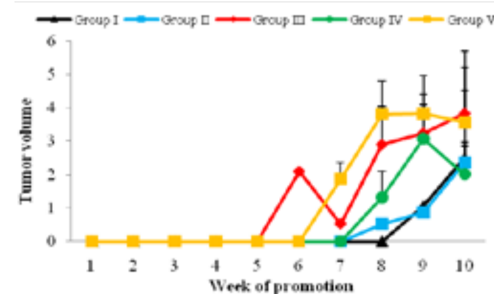
Further histopathological analysis showed varying degree of hyperplasia and keratin pearls in all treated groups (Figure 2). The epidermis is less hyperplastic in group I and group II in contrast to carcinogen control (group V), whereas group III has more keratin pearls with comparable hyperplastic epidermis to the carcinogen control. Tumors formed are benign papillomas which are still confined within an intact basement membrane without sign of penetration into the dermis. However, premalignant lesions were seen in carcinogen control group.




(a) Percentage tumor incidence




(b) Tumor burden




(c) Tumor volume

**Figure 1. (a) Percentage of Tumor Incidence; (b) Tumor Burden; (c) Tumor Volume of Mice in Control and Treatment Groups during the Observation Period**

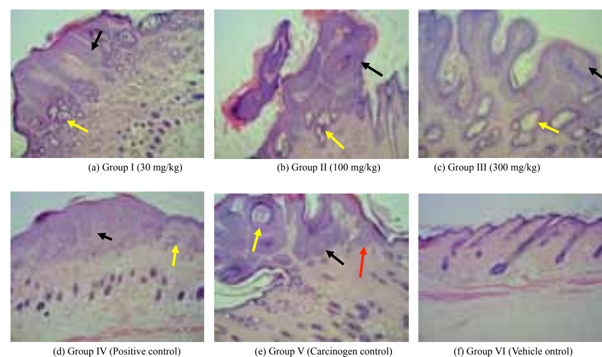



**Figure 2. Representative Figures Obtained from Control and Treatment Groups at the End of the Study** Arrows showed hyperplasia of epidermis (black), keratin pearls (yellow) and premalignant lesions where basement membrane was almost breached (red). (H&E stained micrographs taken at 40x magnifications)

100.0
75.0
50.0
25.0
0

**Table 1. Effect of Ardisia Crispa Root Hexane Extract on DMBA-induced Mouse Skin Tumor Promotion After 10 Weeks**

| Groups | Number of animals | | Body weight (g) | | Tumor | Tumor burden | Tumor volume |
|---|---|---|---|---|---|---|---|
| | Initial | Effective | Initial | Final | incidence (%) | | |
| I ( ACRH 30 mg/kg) | 10 | 10 | 27.3 ± 0.96 | 39.8 ± 1.47 | 20[a,b] | 1.50 ± 0.50[a] | 2.49 ± 1.70 |
| II (ACRH 100 mg/kg) | 10 | 9 | 25.0 ± 0.84 | 40.0 ± 2.05 | 44[a,b] | 1.25 ± 0.25[a] | 2.36 ± 0.50[a] |
| III (ACRH 300 mg/kg) | 10 | 10 | 27.8 ± 0.93 | 40.5 ± 1.41 | 80 | 2.25 ± 0.56 | 3.83 ± 0.68 |
| IV (curcumin ) | 10 | 10 | 29.9 ± 1.21 | 42.3 ± 0.99 | 40 | 2.25 ± 0.48 | 2.01 ± 0.96[a] |
| V (carcinogen control) | 10 | 9 | 25.1 ± 1.14 | 44.4 ± 2.11 | 67 | 2.50 ± 1.31 | 3.56 ± 2.13 |

Values expressed as mean ± S.E.M; [a] Significance levels between treated groups and carcinogen control group (group V) at P<0.05; [b] Significance levels between treated groups and positive control group (group IV) at P<0.05; *Treatment groups refer to group I (30 mg/kg), group II (100 mg/kg) and group III (300 mg/kg)

## Discussion

Chemoprevention by phytochemicals appeared to be one of the most practicable means in controlling cancer and various phytochemicals such as carotenoids, phenolic compounds and terpenoids isolated from vegetables, fruits, spices, teas, herbs and medicinal plants have been reported to suppress carcinogenesis (Sengupta et al., 2004). Molecules that reverse or stop progression of premalignant cells in which damage has already occurred are referred to as chemopreventive agents. Many laboratories have been investigating chemopreventive effects of natural substances against skin cancer development (Hong and Sporn, 1997; Kelloff, 2000). It is therefore becoming progressively important in searching natural products that can either block or preferably, reverse the process of carcinogenesis and can be developed as a promising anti-cancer agent (Craig, 1997; Kellen, 1999; Nishino et al., 2005).

It is widely believed that inhibition of tumor promotion is a better strategy in cancer chemoprevention than inhibition of tumor initiation because initiation is a short irreversible event whereas promotion is a long cumulative process that is reversible during the initial stage (Agarwal and Mukhtar, 1991; DiGiovanni, 1992). The present study showed tumor suppressing effect in mice treated with *Ardisia crispa* root hexane extract at low dosages (30 mg/kg and 100 mg/kg). This is postulated to be due to presence of phytochemicals, such as saponin, triterpenoid, flavonoid and tannins in the hexane fraction of extract at low but sufficient dose to express their effect (Roslida and Kim, 2008). Chemopreventive properties of the mentioned phytochemicals have been extensively reported in other plant extract (Nishino et al., 1986; Ito et al., 1999; Lee et al., 1999; Chung et al., 2003). There are also studies that evidenced the ability of certain phytochemicals to exert tumor suppressive effect at low dose (Rao and Shen, 2002; Russo, 2007).

Literatures suggest that closely related species in the same family might be expected to share identical or similar properties, as inherited with very little change from their ancestor (Soltis and Soltis, 2001). Thus, it is postulated that *Ardisia crispa* display similar anticancer properties as in other species from the Myrsinaceae family, such as Ardisia compressa, which had inhibited liver carcinogenesis in Wistar rats (de Mejía et al., 2004). Apart from that it was also hypothesized that the suppression effect could be due to closely resemblance of *Ardisia crispa* chemical structure with polyphenols and benzene rings substituted compounds (Bariwal et al., 2008; Shankar et al., 2009).

The mechanism for tumor suppression was suggested to be closely linked to curcumin's since comparable effect was observed in low dosage (30 mg/kg and 100 mg/kg). It has been published that curcumin is able to inhibit inflammatory enzymes, cyclooxygenase-2 (COX-2) and 5-lipoxygenase (LOX), in which their inhibition effects on tumor growth and promotion have been widely reported (Huang et al., 1991; Ara and Teicher, 1996).

*Ardisia crispa* is also proposed to exhibit anti-cancer mechanism by targeting cell DNA, either as an alkylating agent, inhibitor of topoisomerase, antimitotic agents and so on. "Antioxidant hypothesis" is also often related to cancer prevention. It is thus suggested that *Ardisia crispa*, possessing antioxidants phytochemicals, could possibly exert its chemoprevention through antioxidation mechanism. For example, curcumin from turmeric has been shown to possess strong proapoptotic activity against cancerous cells (Aggarwal, 2004).

Despite all, high dosage of the extract (300 mg/kg) demonstrates tumor promoting effect. This could be due to chronic excess of sublethally toxic dosage which potentiates tumors development that would otherwise not formed at subtoxic doses (Marks, 1976; Schulte-Herman et al., 1983). Flavin (1984) found that excessive dosage of compounds leading to toxicity cause cells to response by developing hyperplasia whereas subtoxic doses are less likely to induce hyperplasia, tumor promotion and express carcinogenic effect, as shown in this study.

This current study revealed that *Ardisia crispa* root hexane extract did show effectiveness in suppressing skin tumor growth in DMBA-induced and croton-oil promoted mice during the promotion period, particularly at low dosage (30 mg/kg). Though, additional study is required to elucidate the exact anti-cancer promoting mechanism underlying this suppressing effect.

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