

RESEARCH ARTICLE

Isolation of a Quinone-rich Fraction from *Ardisia crispa* Roots and its Attenuating Effects on Murine Skin Tumorigenesis

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Abstract

Ardisia crispa (Family: Myrsinaceae) is an evergreen, fruiting shrub that has been traditionally used as folklore medicine. Despite a scarcity of research publications, we have succeeded in showing suppressive effects on murine skin papillomagenesis. In extension, the present research was aimed at determining the effect of a quinone-rich fraction (QRF) isolated from the same root hexane extract on both initiation and promotion stages of carcinogenesis, at the selected dose of 30 mg/kg. Mice (groups I-IV) were initiated with a single dose of 7,12-dimethylbenz[α]anthracene (DMBA, 100 μ g/100 μ l) followed by repeated promotion of croton oil (1%) twice weekly for 20 weeks. In addition, group I (anti-initiation) received QRF 7 days before and after DMBA; group II (anti-promotion) received QRF 30 minutes before each croton oil application; group III (anti-initiation/promotion) was treated with QRF as a combination of group I and II. A further two groups served as vehicle control (group V) and treated control (group VI). As carcinogen control, group IV showed the highest tumor volume (8.79 $\pm$ 5.44) and tumor burden (3.60 $\pm$ 1.17). Comparatively, group III revealed only 20% of tumor incidence, tumor burden (3.00 $\pm$ 1.00) and tumor volume (2.40 $\pm$ 1.12), which were significantly different from group IV. Group II also showed significant reduction of tumor volume (3.11), tumor burden (3.00) and tumor incidence (11.11%), along with prominent increase of latency period of tumor formation (week 12). Group I, nonetheless, demonstrated marked increment of tumor incidence by 40% with prompted latency period of tumor formation (week 7). No tumor formation was observed in groups V and VI. This study provided clear evidence of inhibitory effects of QRF during promotion period which was in agreement with our previous findings. The mechanism(s) underlying such effects have yet to be elucidated.

Keywords: Skin cancer - murine carcinogenesis model - tumor initiation - tumor promotion - *Ardisia crispa*

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Introduction

Despite advances in contemporary medicine, cancer incidence continues to upsurge. An approximate of 2-3 million cases of non-melanoma skin cancer and 132,000 cases of melanoma skin cancer occurred annually worldwide (World Health Organization). Amongst others, fair skin, extensive exposure to sunlight, skin disease, genetics and chemicals are risk factors attributable for causing skin cancer. Up to date, there is still absence of an effective chemopreventive agent against the development and/or progression of skin cancer, though treatments such as surgery, radiation therapy and topical chemotherapy are available. In view of that, researchers have been striving hard in the exploration of potential chemopreventive agent from natural products which is conceivably far less invasive than contemporary treatments.

Recognized as a well-established *in vivo* model, two-stage murine skin carcinogenesis has been widely applied in research of chemopreventive agents for it comprises of two distinctly separated stages (initiation and promotion)

with ease identification of anti-initiation, anti-promotion or anti-initiation/promotion agents. As a leading step, 'initiation' causes gene mutations (predominantly *Hras1* gene) in epidermal keratinocytes in response to chemical mutagen exposure, which is often polycyclic aromatic hydrocarbon such as 7,12-dimethylbenz[α]anthracene (DMBA) (Ise et al., 2000; Abel et al., 2009). This step is irreversible and produces no visible tumor until repeated application of tumor promoting agent such as 12-O-tetradecanoylphorbol-13-acetate (TPA) takes place. Apart from clonal outgrowths of papilloma, tumor promoters are also accounted for stimulating cell signaling, increasing production of growth factors, generating oxidative stress, as well as causing tissue inflammation (DiGiovanni, 1992).

Ardisia crispa (Family: Myrsinaceae), or locally known as "mata ayam" or "mata itik", is an evergreen shrub with white flowers and red berries. It can be found in woodland garden, shady edges, hillsides or forests and is widely distributed in Asia stretching from Japan and the Himalayas to Java and the Philippines (Chen and Pipoly,

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1996). The plant has been traditionally used to “wash out dirty blood” in women suffering from menstrual pain, to treat rheumatism, to serve as skin liniment, as well as to reduce pain and swellings (Perry and Metzger, 1980; Muhammad and Mustafa, 1994; Jansakul, 1995).

Our previous study showed that *Ardisia crispa* root hexane (ACRH) extract suppresses mouse skin papillomagenesis (Roslida et al., 2011; Sulaiman et al., 2012; Hamid et al., 2013). As an extension to that, this research is aimed at determining effect of quinone-rich fraction isolated from the same root hexane extract on both initiation and promotion stages of carcinogenesis. The compound, 2-methoxy-6-undecyl-1,4-benzoquinone (quinone), had been formerly isolated by Roslida (2004) from the root hexane fraction and was documented as anti-inflammatory and analgesic agents. According to Hussain and Harris (2007), 1 out of 4 cancers are believably arise from chronic inflammation. Thus, it is hypothetical that quinone-rich fraction (QRF) which contains anti-inflammatory compounds may perhaps effective in suppressing carcinogenesis.

Materials and Methods

Plant extraction and isolation of quinone-rich fraction *Ardisia crispa* plants were collected from Machang, Kelantan, Malaysia and a voucher specimen (no: 20841) was deposited in the herbarium of Universiti Kebangsaan Malaysia (UKM), Selangor, Malaysia. Plant extraction was done according to method by Roslida and Kim (2008), with slight modification. Roots of *Ardisia crispa* were cut into smaller pieces and dried at 60°C for three days. Dried roots were grinded by using Wiley laboratory mill and extracted with 80% aqueous ethanol by using Soxhlet apparatus. The extract was filtered and evaporated in a rotary evaporator at 40°C, under reduced pressure to give crude aqueous ethanol extract. Crude aqueous ethanol extract was fractionated with n-hexane, filtered and evaporated in a rotary evaporator to yield *Ardisia crispa* root hexane extract (ACRH).

ACRH was chromatographed on a silica gel column eluted with n-hexane:ethyl acetate at increasing polarity. Fractions were collected and thin-layer chromatography (TLC) was carried out on aluminium plates precoated with silica gel 60 F254 (Merck, Germany) as adsorbent. Chloroform was used as developing solvent. Fractions with retention factor (R_f) similar to compound of interest (0.69) were pooled as quinone-rich fraction (QRF) (Roslida, 2004).

Quinone composition analysis and identification by UPLC and GC-MS

UPLC analysis was done according to method by Shelar et al. (2009), with slight modification. The system consisted of an Agilent 1290 series Ultra Performance Liquid Chromatography (UPLC) equipped with an autosampler, a diode array detector (DAD) and a reverse phase analytical column (HyperClone 3u ODS C18, 3 µm, 4.0×125 mm, Phenomenex, USA). Mobile phase consisted of Methanol (A) and 0.1% TFA (B) (in proportion of 88:12 v/v) was degassed before used. The

flow-rate was kept at 1.0 ml/min, temperature of column was set at 29.99°C±1°C and the injection volume was 20 µl. Detection was carried out at 288 nm. Peaks in chromatograms were identified by comparing retention time and UV spectra of samples with reference standard isolated and identified previously by Roslida (2004).

Further confirmation of quinone compound in QRF was carried by using GC-MS analyses. The system consisted of an Agilent model 5973 MSD gas chromatograph equipped with a HP-5 MS column (30m x0.25mm x0.25µm). Sample (1 µl) was injected in splitless mode at an injector temperature of 250°C. Helium, at a flow rate of (1 ml/min), was used as the carrier gas. The oven temperature was programmed from 70°C with gradual increase to 300°C in 6 min and held for 29 min. The mass spectrometer was electron impact (EI) ionization mode. Library mass spectra searches were performed via National Institute of Standard and Technology (NIST) library and compound of interest (quinone) was confirmed with comparison of data from the literature (Roslida, 2004).

Animals

6-8 weeks old female, ICR mice were obtained and kept at the animal house of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) with ethical approval from the Animal Care and Use Committee (ACUC) of UPM (UPM/FPSK/PADS/BR-UUH/00398). The mice were housed ten per cage and acclimatized for one week prior to the commencement of experiment. All mice were fed on standard laboratory diet and water *ad libitum*. Three days before treatment, the mice were dorsally shaved with an electric hair clipper, for an approximately 2cm x2cm area (about 1cm off tail).

Chemicals

7,12-dimethylbenz(α)anthracene (DMBA) and croton oil were purchased from Sigma-Aldrich Co., USA. DMBA was dissolved at a concentration of 100 µg/100 µl in acetone. Croton oil was dissolved in acetone to give 1% croton oil solution.

Experimental design

A total of 55 mice were randomly divided into the following six groups and treated as described below: Group I: Peri-initiation period treated group (n=10) – Mice were treated with a single dose of DMBA (100 µg/100 µl) applied topically over the shaven skin area, and promoted by repeated application of croton oil (1%, 100 µl/twice weekly) a week later for 20 weeks. These animals received topical application of QRF (30 mg/kg, 100 µl in acetone) 7 days before and 7 days after DMBA application. Group II: Promotion period treated group (n=10) – Mice were treated with DMBA and croton oil, as in Group I. Starting from the promotion period (i.e. time of croton oil treatment began), mice received QRF (30 mg/kg, 100 µl in acetone) 30 minutes before each croton oil treatment, twice weekly for 20 weeks. Group III: Peri-initiation + promotion period treated group (n=10) – Mice were treated with QRF (30 mg/kg, 100 µl in acetone) 7 days before and 7 days after DMBA application (as in group I), and also during promotion period (as in group

II) for 20 weeks. Group IV: Carcinogen control group (n=10) – Animals in this group received only a single dose of DMBA and croton oil (twice weekly for 20 weeks), without application of extract. Group V: Vehicle control (n=10) – Animals received only acetone throughout the experiment. Group VI: Treated control (n=5) – Mice were treated with 30 mg/kg of QRF throughout the experiment. Body weight of mice and tumor sizes (length, width and height) were observed and measured at weekly interval. Only tumors that persisted for more than one week with diameter greater than 1 mm were taken into consideration for data analysis. The following parameters were taken into consideration: 1) 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%. 2) Tumor burden was obtained by dividing the total number of tumors with the number of tumor-bearing mice in a group. 3) Tumor volume was measured by multiplying $\Pi/6$ to the length, width and height of tumor (Girit et al., 2004). 4) Latency period of tumor formation was determined when the first tumor appeared.

Results

Isolated QRF was subjected to quinone composition analysis by using UPLC, prior to commencement of *in vivo* study. The UPLC spectra of QRF showed three major peaks at the retention time (R_t) of 3.4, 4.694 and 5.422 minutes; whilst quinone standard showed only one major peak at R_t =2.519 minutes (Figure 1). The major peak of QRF at R_t =3.4 min (60.9%, peak-area-percent) was believed to be quinone compound, though slight difference in R_t was observed when comparing to standard. Due to this difference in retention time, we further confirm the results by performing GC-MS on QRF and the standard compound. GC analysis showed similar retention time of quinone compound found in QRF (18.905) and standard (18.897 minutes) (data not shown). Mass spectrometry analysis on the standard quinone compound reveals m/z 292.1, corresponding to the molecular weight of AC-2 (2-methoxy-6-undecyl-1,4-benzoquinone) (Figure 2), identified previously by Roslida (2004).

After 20 weeks of experimental and observation period, macroscopic pictures of mouse skin tumors were taken from all groups before mice were sacrificed (Figure 3). Table 1 summarizes findings of anti-initiation (group I), anti-promotion (group II) and anti-initiation/promotion

(group III) effects of QRF on DMBA-croton oil induced mouse skin carcinogenesis. It is noteworthy that topical application of QRF did not result in extensive differences in body weight of mice among treated and control groups (data not shown).

Significant difference was found between groups ($P<0.05$) with group I reporting the highest percentage

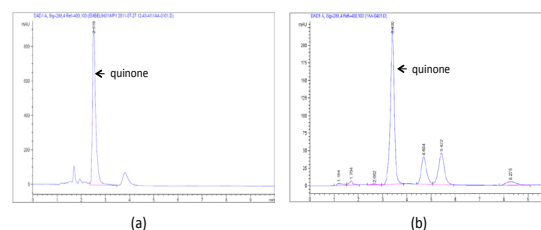


Figure 1. UPLC Chromatograms of (a) Standard (2-methoxy-6-undecyl-1,4-benzoquinone), and (b) QRF

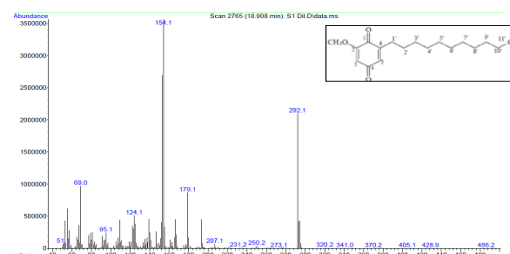


Figure 2. Mass Spectrum and Chemical Structure of Standard Quinone (2-methoxy-6-undecyl-1,4-benzoquinone), with m/z value of 292.1

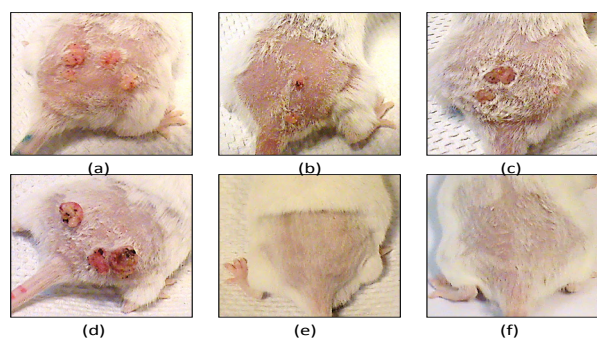


Figure 3. Representative Morphological Pictures of Mouse Skin Tumors. (a) group I (anti-initiation); (b) group II (anti-promotion); (c) group III (anti-initiation/promotion); (d) group IV (carcinogen control); (e) group V (vehicle control); (f) group VI (treated control)

Table 1. Effects of QRF on DMBA-croton Oil Induced Mouse Skin Tumorigenesis after 20 Weeks

	No. of animals		Body weight (g)		Tumor Incidence (%)	Tumor burden	Tumor volume (mm ³)
	Initial	Effective	Initial	Final			
Group I	10	10	21.5±0.72	29.2±1.69	70 ^a	2.29±0.47 ^a	6.75±2.13
II	10	9	20.6±0.90	26.3±0.85	11.11 ^a	3.00±0.00 ^a	3.11±0.00 ^a
III	10	10	21.6±0.69	28.5±1.11	20 ^a	3.00±1.00 ^a	2.40±1.12 ^a
IV	10	10	21.2±0.55	28.4±0.79	50	3.60±1.17	8.79±5.44
V**	10	10	19.3±0.83	29.5±1.28	-	-	-
VI**	5	5	15.2±0.20	32.8±0.49	-	-	-

*Treated groups refer to group I (anti-initiation), group II (anti-promotion) and group III (anti-initiation/promotion); at 30 mg/kg. **Group V and group VI refers to vehicle control and treated control (30 mg/kg QRF), respectively. ^aSignificance levels between treated groups and carcinogen control (group IV) at $P<0.05$. Values expressed as mean±S.E.M

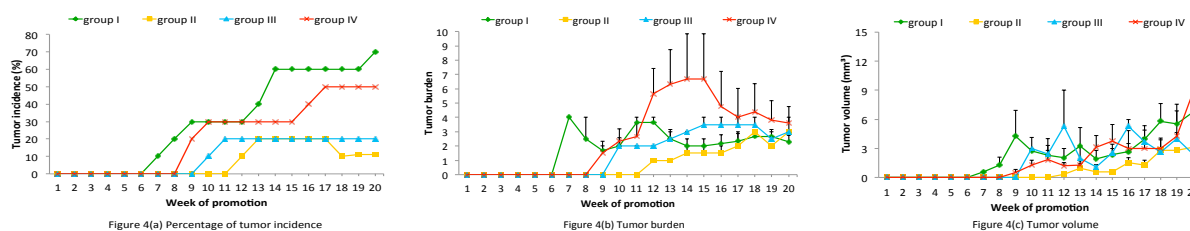


Figure 4. Topical Application of QRF. (a) percentage of tumor formation; (b) tumor burden; (c) tumor volume; in group I (anti-initiation), group II (anti-promotion), group III (anti-initiation/promotion) and group IV (carcinogen control)

of tumor incidence of 70% whereas mice in carcinogen control (group IV) revealed only 50% of tumor incidence. Lower tumor incidence was noted in group II (11.11%) and group III (20%), with no significance difference found between the groups. In terms of latency period of tumor formation, group I developed tumors in a slightly shorter period (week 7) as compared to group IV (week 9) whereas the onset of tumor was noticeably delayed in group II (week 12) and group III (week 10) (Figure 4a).

As carcinogen control, group IV marked the highest values for both tumor burden (Figure 4b) and tumor volume (Figure 4c), reporting 3.60 ± 1.17 and 8.79 ± 5.44 respectively. Both parameters were significantly different between all treated groups and carcinogen control, except for tumor volume between groups I (6.75 ± 0.47) and IV. Despite its high tumor volume, group I displayed a low tumor burden of 2.29 ± 0.47 , which was significantly different from all the other treated groups. Relatively, tumor volumes of both groups II (3.11) and III (2.40 ± 1.12) were much lower, along with a slightly lower but significantly different from carcinogen control tumor burden (3.00). Though group III displayed lower tumor volume than group II, no significant difference was found between both groups.

Discussion

Carcinogenesis is a complex, multi-step process comprising of initiation, promotion and progression stages whose hallmarks include gene mutations and cellular proliferation that eventually leads to neoplastic transformation and loss of heterozygosity (Zoumpourlis et al., 2003). This multi-stage nature of carcinogenesis allows naturally occurring phytochemicals to intervene at either one of the stages to inhibit, delay or reverse the process of tumor development. Abundance of phytochemicals have been investigated and evidenced to be a promising chemopreventive agent. Eugenol, resveratrol, zerumbone and zapotin are among reported phytochemicals which warrants further investigation as chemopreventive agents attributable to their anti-tumor effects during initiation, promotion, or a combination of both stages (Murakami et al., 2004; Cuendet et al., 2008; Kalra et al., 2008; Pal et al., 2010).

Literatures suggest that quinones are widely acceptable for their anti-tumor effects, either *in vitro* or *in vivo*. A structurally similar benzoquinone, 2-methoxy-6-tridecyl-1,4-benzoquinone (AC7-1), had been shown to strongly blocked B16-F10 melanoma cell adhesion to extracellular matrix (ECM) and cell invasion. It had also remarkably

inhibited pulmonary metastasis and tumor growth *in vivo* (Kang et al., 2001). Accordingly, as well as in sequel to our previous study, quinone-rich fraction (QRF) was isolated from *Ardisia crispa* root hexane extract and its chemopreventive potential was determined. Although a slight difference in retention time (UPLC) was observed for quinone found in QRF as compared to the standard, both peaks indeed refer to the same quinone compound, as validated by GC-MS where the same retention time and m/z value were noted.

In terms of tumor incidence, only 50% of mice develop tumors in carcinogen control group, despite their high tumor burden and volume. We postulated that this may be due to varying basal expression level of Nrf2 among wild type ICR mice which lead to production of variable levels of phase II detoxication enzymes such as glutathione-S-transferase (GST) and glutathione, thus rendering some mice very sensitive to carcinogenesis whereas some are less susceptible. This assumption was based on findings by Ramos-Gomez et al. (2001) where enhanced susceptibility to chemical carcinogenesis was observed in Nrf2 deficient mice, most plausibly due to its genetic polymorphisms.

Highest tumor incidence was noted in mice receiving QRF during peri-initiation period (group I, 40%); yet, similar observations were not found in group III, possibly because of the prolonged treatment with QRF during both initiation and promotion period. Brookes and Lawley (1964) proved that prolonged binding of DMBA to cellular DNA can persist for as long as 42 days after DMBA administration, which we presume that short-term treatment of QRF (group I) was insufficient to exert its protective effects against tumor initiation thus leading to the difference seen in group I and group III.

Conversely, we found that QRF significantly inhibit tumor promotion in two-stage mouse skin carcinogenesis where a marked reduction of 77.8% tumor incidence was noted in group II (anti-promotion), with prominent delay in latency period of tumor formation (week 12) and no observable toxic effects. This is in consistence with our previous finding where *Ardisia crispa* root hexane extract showed significant tumor suppression effect during promotion stage at a dosage as low as 30 mg/kg (Roslida et al., 2011). This finding could possibly a result of intervention by QRF on multiple molecular mechanisms and signaling pathways such as epidermal growth factor (EGF), proinflammatory cytokines and prostaglandins, whereby sustaining activation of these molecular targets have been closely associated with promotion of skin tumorigenesis (Rundhaug and Fischer, 2010).

Through this study, it was made clear that both anti-

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promotion and anti-initiation/promotion protocol showed indisputable inhibition effect against tumor development as compared to anti-initiation group, where low tumor incidences and tumor volumes were observed. Though tumor burdens of both groups were higher than that of group I, a prominent delayed of tumor formation made promotion stage, especially, a favored inhibiting target of QRF. In view of that, we suggest that further studies to be focused on anti-promotion stage to elucidate mechanism(s) underlying such effects, specifically at Nrf2-related pathway(s) and its inducible detoxication enzymes.

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