Chemopreventive Potential of *Annona Muricata* L Leaves on Chemically-Induced Skin Papillomagenesis in Mice

Sulaiman Hamizah, AH Roslida*, O Fezah, KL Tan, YS Tor, CI Tan

Abstract

*Annona muricata* L (Annonaceae), commonly known as soursop has a long, rich history in herbal medicine with a lengthy recorded indigenous use. It had also been found to be a promising new anti-tumor agent in numerous *in vitro* studies. The present investigation concerns chemopreventive effects in a two-stage model of skin papillomagenesis. Chemopreventive effects of an ethanolic extract of *A. muricata* leaves (AME) was evaluated in 6-7 week old ICR mice given a single topical application of 7,12-dimethylbenz(a)anthracene (DMBA 100ng/100ul acetone) and promotion by repeated application of croton oil (1% in acetone/ twice a week) for 10 weeks. Morphological tumor incidence, burden and volume were measured, with histological evaluation of skin tissue. Topical application of AMLE at 30, 100 and 300mg/kg significantly reduced DMBA/croton oil induced skin papillomagenesis in (i) peri-initiation protocol (AME from 7 days prior to 7 days after DMBA), (ii) promotion protocol (AME 30 minutes after croton oil), or (iii) both peri-initiation and promotion protocol (AME 7 days prior to 7 day after DMBA and AMLE 30 minutes after croton oil throughout the experimental period), in a dose dependent manner (p<0.05) as compared to carcinogen–treated control. Furthermore, the average latent period was significantly increased in the AMLE-treated group. Interestingly, At 100 and 300 mg/kg, AMLE completely inhibited the tumor development in all stages. Histopathological study revealed that tumor growth from the AMLE-treated groups showed only slight hyperplasia and absence of keratin pearls and rete ridges. The results, thus suggest that the *A. muricata* leaves extract was able to suppress tumor initiation as well as tumor promotion even at lower dosage.

Keywords: *Annona muricata* - chemopreventive - 7,12-dimethylbenz(a)anthracene - croton oil - papillomagenesis

Introduction

Cancer mortality rates have increased in the developed countries throughout this century and already as the cause death in some Western countries (Jemal et al., 2003; Karim-Kos et al., 2008). Skin cancer is one of the most common cancers and accounts for 30% of all new diagnosed cancers worldwide (Diepjen and Mahler, 2002; Deo et al., 2005). Cancer is the disease that commonly believed to be preventable (Anand et al., 2008). Chemoprevention of cancer can be defined as the use of natural, synthetic or biological substances that intervene in the early precancerous stages, therefore reverse and suppress the formation of tumor (Sporn, 1999). Chemopreventive agents can be targeted for intervention at either the stage of initiation, promotion or progression of carcinogenesis (Wattenberg, 1990). The use of natural products as anti-cancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine (Sharma et al., 2009). Many cancer patients use herbal medicine as alternative medicines including phytochemicals in addition to, or following the failure of standard cancer therapy (Eisenberg et al., 1998). Currently, several plant-derived compounds have been successfully employed in cancer treatment, for example, vincristine and visblastine isolated from periwinkle, *Catharanthus roseus*. According to Cragg and Newman (2000), over 50% of the drugs in clinical trials for anti-cancer activity were isolated from natural sources or are related to them. Experimental studies indicate that phytochemicals with anti-oxidative and anti-inflammatory properties can inhibit tumor initiation, promotion and progression (Sharma et al., 2009). Therefore, the scientific validation of traditional medicine should be done for its possible use in the prevention and treatment of cancer.

*Annona muricata* L commonly known as graviola or soursop, belongs to the family of Annonaceae. It is a typical tropical tree with heart shaped edible fruits and widely distributed in most of tropical countries (De Feo, 1992). The leaves are lanceolate with glossy and dark green in color had been traditionally used to treat headaches, hypertension, cough, asthma and used as antispasmodic, sedative and nervine for heart condition (Taylor, 2002; Lans, 2006). Previous reports over the years have demonstrated that the leaf, bark, root, stem, and fruit seed extracts of *Annona muricata* are anti-bacterial...
Annonaceous acetogenins, from *Annona muricata* L were found to be a promising new anti-tumor and anticancer agent in numerous studies. These acetogenins demonstrated to be selectively toxic against various types of the cancerous cells without harming healthy cells (Rieser et al., 1993; Wu et al., 1995a; Zeng et al., 1996). Due to the presence of various medicinal properties, the present study was an endeavour to investigate the chemopreventive effect of *A. muricata* L leaves, if any on two-stage mouse skin papillomagenesis model.

**Materials and Methods**

**Plant Material**

*Annona muricata* L leaves were collected from Raub, Pahang, Malaysia and deposited in Forest Research Institute Malaysia (FRIM), Kepong, Malaysia (Voucher Specimen no: FR1 57966). The leaves were cleaned before dried at 40°C for three consecutive days in the oven. Dried leaves were ground into powder form. The dried powder of plant was then subjected to extraction with 80% ethanol by using a soxhlet apparatus. The crude extract obtained was concentrated using rotary evaporator under reduced pressure and dissolved in 1% acetone into three different doses (30, 100 and 300 mg/kg) prior to pharmacological testing.

**Experimental Animals**

180 ICR male mice (6 to 7 weeks old), weighing from 20g to 30g were used. The animals were kept in Animal House of Faculty of Medicine and Health Sciences FMHS, Universiti Putra Malaysia (UPM). They were housed in cages (with 10 mice per cage) under normal laboratory conditions of humidity, temperature (25±4°C) and light (12/12 hour light dark cycle). The mice were allowed for 1 week adaptation period with free access of water and food ad libitum. The ethical clearance had been approved by Animal Care and Use Committee (ACUC), FMHS, UPM (Reference number: UPM/FPSK/PADS/ UUH/F01).

**Drugs and Chemicals**

7,12-dimethylbenz(a)anthracene (DMBA) and curcumin were obtained from Sigma-Aldrich Co (United States). Croton oil (CO) was purchased from TCI chemicals (Japan) and acetone was purchased from Mallinckrodt Baker (Mexico).

**In vivo two stage skin papillomagenesis**

All the animals were randomly divided into 18 groups with 10 mice per group. Three days before the treatment, the dorsal hair of all the animals was shaved by using electrical hair clipper (2x2cm). Initiation of tumor was done by application of single dose of DMBA on the dorsal, shaved surface and promotion by croton oil started a week after. This study is divided into three protocols where the animals received the treatment at different stages of study; (1) anti-initiation, (2) anti-promotion, and (3) anti-initiation/anti-promotion. The experimental design was as follows:

(i) Peri-initiation Study

Group I: All animals were received topical application of acetone (100µl/mouse) on the shaved dorsal skin area throughout the experiment.

Group II: All animals were received single topical application of DMBA in acetone (100µg/100µl/mouse), followed by topical application of croton oil in acetone (100µl of 1% of croton oil/mouse) twice a week for 10 weeks.

Group III: All animals received the same treatment as Group II, but curcumin (10mg/kg bwt) was topically applied for 7 days prior to 7 days after DMBA application. This group served as positive control.

Group IV, V, VI: All animals received the same treatment as Group III except the animals received the topical application of AMLE either 30, or 100 or 300mg/kg bwt respectively) instead of curcumin.

(ii) Promotion Study

Group I and II received same treatment as in peri-initiation study

Group III: All animals received the same treatment as Group II, but curcumin (10mg/kg bwt) was topically applied 30 minutes prior to croton oil treatment (twice weekly) until the end of 10 weeks of promotion period. This group served as positive control.

Group IV, V, VI: All animals received the same treatment as Group III except the animals received the topical application of AMLE either 30,100 or 300mg/kg bwt respectively instead of curcumin.

(iii) Peri-initiation and Promotion Study

Group I and II received same treatment as in anti-initiation study

Group III: All animal received the same treatment as Group II, but curcumin (10mg/kg bwt) was topically applied for 7 days prior to 7 days after DMBA application, and further applied 30 minutes prior to croton oil treatment (twice weekly) until the end of 10 weeks of promotion period. This group served as positive control.

Group IV, V, VI: All animals received the same treatment as Group III except the animals received the topical application of AMLE either 30 or100 or 300mg/kg bwt respectively) instead of curcumin.

**Morphological Observations of Papilloma Development**

During the study, each animal in all groups of all stages were weighed and shaved weekly for an easy application of the carcinogens/tested extracts and skin lesion observation. Body weight, latency of tumor formation, percentage of tumor incidence, tumor burden and tumor...
volume were observed, measured and recorded at weekly interval. Tumors with a diameter greater than 1 mm that persists for at least 2 consecutive observations were included in the cumulative counts. The data expressed as the percentage of tumor incidence (the number of tumor-bearing mice), tumor burden (the average number of tumors per tumor-bearing mouse) (Das et al., 2005) and the tumor volume (mm$^3$) = $\pi$/6 x length x width x height (Girit et al., 2008).

**Histopathological analysis**
All animals were sacrificed at the end of 10 weeks promotion period. The treated area of the skin from each mouse was sampled and fixed in 10% formalin before being processed. All skin tissue was processed with standard protocol in an automated tissue processor. Processed tissue were then embedded in the paraffin wax before sectioned into 4µm thick and stained with Haematoxylin and Eosin (H&E) stain. The slides were examined under the light microscope.

**Statistical analysis**
The data were expressed as mean value ± S.E.M (standard error mean). Analysis of variance (ANOVA) with covariate followed by LSD multiple comparison was used to compare the significant differences between treated and controlled groups. All the value of p<0.05 was considered as statistically significant.

**Results**
Approximately 1.5 kg of dried leaves powder of *A. muricata* was extracted with 80% aqueous ethanol by cold maceration for two days. The crude ethanolic extract of 158.18 g (10.55%, w/w) was obtained by using a rotary evaporator at a reduced pressure.

During 10 weeks of promotion period, the tumor latency, tumor incidence, tumor burden as well as tumor volume were measured weekly and then further statistically analyzed. Generally, topical application of AMLE in all of three protocols did not alter the average weight gain during the experimental period. The data in all three protocols were compared to both the carcinogen control and curcumin (a natural antitumor compound from *Curcuma longa*) treated group.

In peri-initiation study the effect of anti-initiation protocol was depicted in Table 1 and Figure 1. As illustrated, carcinogen control in the two-stage DMBA/croton oil generated the highest percentage of tumor incidence (44.4%) The administration of lowest dose of AMLE (30 mg/kg) in peri-initiation protocol significantly reduced the tumor incidence (33.33%) and tumor burden (3.00 ± 1.00; p<0.05) compared to carcinogen treated-control (Group II) and curcumin-treated (Group III).

Tumor development in AMLE 30 mg/kg was developed at week 9 which was a week later than tumor development in both carcinogen control and curcumin (Figure 1a). Interestingly, at 100 and 300 mg/kg, AMLE showed complete inhibition of tumor growth (Figure 1a, 1b, 1c). In promotion study, application of all the three doses of AMLE during the promotion phase caused significant decrease in all the percentage of tumor incidence, values of tumor burden and tumor volume compared to carcinogen treated-control (Group II). In carcinogen control, the first tumor appeared at week 6, and in curcumin-treated (Group III), at week 8. The latency period of tumor formation in Group IV (AMLE 30 mg/kg) was delayed to week 9 and it also displayed lowest percentage of tumor incidence (28.57%). In contrast, Group II (carcinogen control) and III (curcumin-treated) exhibited 60.00 and 40.00% of tumor incidence respectively. At the end of the tenth week promotion period, Group IV (AMLE 30 mg/kg) exhibited 1.5 ± 0.5 (p<0.05) of tumor burden and 0.52 (p<0.05) of tumor volume, which are significantly lower compared to both carcinogen and curcumin-treated. Similar to the peri-initiation study, application of 100 and 300 mg/kg AMLE completely inhibited the growth of tumors.

At the end of peri-initiation and promotion study, there was 55.55% of tumor incidence in carcinogen control-treated (Group II), compared to 14.29% in curcumin-treated (Group III) (Table 3) Tumor latency of Group II start at week 7 and the values of tumor burden and tumor volume of Group II were 4.40 ± 1.72 and 3.59 ± 1.34 respectively. In comparison, Group III

**Table 1. Effect of Annona Muricata Extract on Mouse Skin Papillomagenesis During Peri-Initiation Phase and Promotion Phase**

<table>
<thead>
<tr>
<th>Group</th>
<th>Peri-Initiation Phase</th>
<th>Promotion Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>Tumor</td>
</tr>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>I (Vehicle)</td>
<td>25.7 ± 1.14</td>
<td>33.8 ± 1.27</td>
</tr>
<tr>
<td>II (Carcinogen)</td>
<td>27.2 ± 0.89</td>
<td>34.1 ± 1.84</td>
</tr>
<tr>
<td>III (Curcumin 10 mg/kg)</td>
<td>28.1 ± 0.53</td>
<td>38.0 ± 0.76</td>
</tr>
<tr>
<td>IV (AMLE 30 mg/kg)</td>
<td>22.9 ± 0.66</td>
<td>30.3 ± 1.80</td>
</tr>
<tr>
<td>V (AMLE 100 mg/kg)</td>
<td>26.6 ± 0.85</td>
<td>24.7 ± 1.41</td>
</tr>
<tr>
<td>VI (AMLE 300 mg/kg)</td>
<td>27.3 ± 0.56</td>
<td>29.5 ± 1.65</td>
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*Values expressed as mean ± S. E. M, *(Group II) at p<0.05, *(Group III) at p<0.05: significance level between treated group and carcinogen control: (Group II) at p<0.05.
Significantly reduced the tumor incidence, tumor burden and tumor volume to 14.29%, 1 and 1.05 respectively when compared to carcinogen control. In contrast, there was no tumor formation in all doses of AMLE-treated groups (Group IV, V, VI) during both peri-initiation and promotion phase. Thus, AMLE completely inhibited tumor initiation and promotion in this phase. (Figure 3). Neither skin papillomas appeared in the animals topically treated only with AMLE at highest dosage of 500 mg/kg, nor death was observed during the entire observation period of 12 weeks (data not shown).

In histopathological analysis, Figure 4a showed normal histology of mouse skin from all the skin tissue sections from Group I (Vehicle Control). Carcinogen control-treated group (Group II) in all protocols exhibited extensive epidermal hyperplasia (indicated from the thickened epidermal layer) together with the numerous numbers of keratin pearls and rete ridges (Figure 4b). Furthermore, in some of the tissue sections, parts of the basement membrane were disrupted indicating the tumors have progress to premalignant stage. Skin tissue samples from the curcumin-treated group (Group III) in all protocols displayed the lower degree of epidermal hyperplasia (however, more apparent when compared significantly reduced the tumor incidence, tumor burden and tumor volume to 14.29%, 1 and 1.05 respectively when compared to carcinogen control. In contrast, there was no tumor formation in all doses of AMLE-treated groups (Group IV, V, VI) during both peri-initiation and promotion phase. Thus, AMLE completely inhibited tumor initiation and promotion in this phase. (Figure 3). Neither skin papillomas appeared in the animals topically treated only with AMLE at highest dosage of 500 mg/kg, nor death was observed during the entire observation period of 12 weeks (data not shown).

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to all AMLE treated groups) and no presence of keratin pearls and rete ridges. The basement membrane was intact and not invaded into dermis, thus the tumors formed were considered as benign.

In peri-initiation study, slight hyperplasia of epidermis was observed in Group V and VI (100 and 300mg/kg AMLE). Whilst, histological observation in Group IV (30mg/kg AMLE) developed papilloma showed hyperplasia but no premalignant lesions, rete ridges and keratin pearls were observed in contrast with carcinogen and curcumin-treated (Figure 4c). The same scenario was shown in promotion phase. Whilst, AMLE treated groups in peri-initiation and promotion phase showed only slight thickening of epithelial layer without any presence of keratin pearls and rete ridges (Figure 4d, 4e).

Discussion

Human beings have been exposed to a variety of carcinogenic agents which may act as initiator and promoter to the tumor formation. In fact, the initiation of carcinogenesis may occur many years before it is being promoted (Gills et al., 2005). Thus, the chemopreventive agents are preferable to slow, reverse or completely halted multiple steps in the carcinogenesis process (Sanja and Mukhtar, 2002). Therefore, a new science of chemoprevention has appeared as an attractive alternative to control malignancy (Kapadia et al., 2000).

Two-stage skin carcinogenesis in mice model is used extensively to investigate the epithelial carcinogenesis which consists of initiation phase and promotion phase. Initiation phase is an irreversible reaction activated by initiator (DMBA), while promotion stage which is reversible and long term is triggered by repetitive application of promoter (croton oil). In accordance, development of anti-tumor-promoter is considered the most effective method in cancer chemoprevention due to the reversible nature of the promotion phase (Takao and Midori, 2000).

A large numbers of agents including natural and synthetic compounds have been identified to possess potential cancer chemopreventive value, inhibiting mutagenesis, hyperproliferation or induce apoptosis or differentiation, which are critical characteristics of chemoprevention (Keloff, 2000). Similarly, the present findings clearly indicate that topical administration of A. muricata L leaves during initialisation as well as tumor promotional stage of papillomagenesis significantly reduced the occurrence of skin papillomas induced by DMBA/croton oil in mouse skin after 12 weeks without causing any toxic effect. AMLE extracts in any stages appreciably decreased tumor burden by 58-100%, tumor volume by 20-100% and tumor incidence by 25-100% in the AMLE–treated experimental groups (Group IV, V and VI) as compared to the carcinogen treated control (Group II).

Previous studies have shown that several plants exhibit chemopreventive properties by disrupting the different stages of multi step skin carcinogenesis, especially tumor promotion (Javed et al., 1998; Zhao et al., 1999). Therefore it can be presumed that A. muricata may act in the similar way as it increased the average latency period of tumor occurrence by 10-20%, thereby prolonging the promotional stage by delaying tumor formation and reducing the number of tumors in mouse skin as compared to the carcinogen treated control. Furthermore, carcinogen treated control demonstrated progression of tumors into premalignant stage, whilst, in all AMLE treated groups, they showed a much delayed tumor progression with absence of premalignant lesion in the cutaneous sections. In addition, even AMLE at lower dose applied ie 30 mg/kg was as equipotent as curcumin.

Curcumin, one of the most studied chemopreventive agent is a natural compound isolated from Curcuma longa L that allows suppression, retardation or inversion of carcinogenesis. Most chemopreventive agents known until today are plant extracts subdivided into two categories: (i) blocking agents which inhibit the initiation step by preventing carcinogen activation and (ii) suppressing agents, which inhibit malignant cell proliferation during promotion and progression stages of carcinogenesis. Curcumin belongs to both categories as it presents multiple mechanisms of action (Duvoix et al., 2005). To date, curcumin has undergone clinical trial and was nanoformulated as anti-cancer potential for therapy (Yallappu et al., 2012). Interestingly, the higher dose of AMLE (100 and 300 mg/kg) was able to inhibit the tumor formation completely, and in this present study, it was even more potent than results obtained for curcumin.

The chemopreventive effects of A. muricata leaves might be attributed to the presence of a class of compound obtained from Annona species, acetogenins. Previous studies revealed the effect of several acetogenins act as a DNA topoisomerase I poison, arrested cancer cells at the G1 phase and induced apoptotic cell death in a Bax- and caspase-3- related pathway, and inhibited NADH-ubiquinone oxidoreductase (complex I) in mitochondria (Lopez et al., 2001; Yuan et al., 2003; Kojima et al., 2010). It has been reported that the main antitumorous compound, annonacin was effective against various in vitro cancer cell lines as well as in vivo lung-induced cancer (Oberlies et al., 1995; Wang et al., 2002; Tormo et al., 2003). Nowadays, even without any scientific validation, many cancer patients and health practitioners are adding the natural leaf and stem of A. muricata (with over 40 documented naturally-occurring acetogenins including annonacin) as a complementary therapy to their cancer protocols. After all, A. muricata has a long history of safe use as a herbal remedy for other conditions for many years, and research indicates that the antitumorous acetogenins are selectively toxic to just cancer cells and not healthy cells—and in miniscule amounts.

Oxidative stress has been implicated in the pathology of many diseases such as inflammatory conditions, cancer, diabetes and aging (Marx, 1987). Free radicals induced by peroxidation have gained much importance because of their involvement in several pathological conditions such as atherosclerosis, ischemia, liver disorder, neural disorder, metal toxicity and pesticide toxicity (Pandey et al., 1994). Reactive oxygen species (ROS) such as superoxide anions, hydroxyl radical and nitric oxide inactivate enzymes and damage important cellular
component causing injury through lipid peroxidation and covalent binding (Geesin et al., 1990). Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanism and thus prevent disease (Youdim and Joseph, 2001). It is ubiquitously known that most antioxidants are flavonoids, like quercetin, rutin or myricetin (Kandaswami and Middleton, 1994). In contrast, A. muricata leaves has been shown to possess antioxidant properties, and this may be attributed to the presence of acetogenins, which probably play a role as an effective free radical scavenger, and hence an active antitumorous agent (Baskar et al., 2007).

Although the insights made in the present study provide a very small aspect of the modulation of carcinogenesis process, yet it can be concluded that A. muricata acts as a modulator of two stage skin papillomagenesis in ICR mice since it prevents the tumor formation, delayed the tumor promotion and progression, elicited by DMBA/croton oil. Since A. muricata is a plant rich in acetogenins, it can be suggested that the synergistic effects of phytochemicals present in this plant including acetogenins may be the underlying principle behind the chemopreventive potential of A. muricata.

In overall, the ethanolic extract A. muricata L. leaves exhibited chemopreventive potential towards skin tumor growth in DMBA/croton oil induced papillomagenesis even at low dosage (30mg/kg). Nevertheless, additional study is required to elucidate the exact anti-cancer mechanism underlying this prevention effect. Further investigation for its possible use as chemopreventive agent against other types of tumors is also demanded. Moreover, isolation and characterization of the exact bioactive compounds are also vital to study its chemical, biochemical and pharmacological effect in depth and by identifying the molecular pathway, genetic alterations and cellular targets that are essential in chemoprevention.

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