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Full Length Research Paper

Anticarcinogenic activity of *Labisia pumila* against 7, 12- dimethylbenz (a) anthracene (DMBA)/croton oil-induced mouse skin carcinogenesis

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Labisia pumila **or locally known as Kacip Fatimah, is one of the most popular medicinal herb in Malaysia. Anticarcinogenic activity of this medicinal herb however, has not been reported until today. In this paper, the *in vivo* anticarcinogenic activity of *L. pumila* ethanol extract on two -stage mouse skin carcinogenesis model is reported. In the present study, we investigated whether *L. pumila* ethanol extract have an effect on tumor growth *in vivo*. Therefore, varying doses (25, 50 and 100 mg/kg bwt) of *L. pumila* ethanol extract were tested on 7, 12-dimethylbenz(a)anthracene (DMBA)/croton oil-induced mouse skin carcinogenesis. At the end of the experiment of 20 weeks, animals in carcinogen control group developed a mean number of 5.70 ± 1.3 skin tumors per tumor-bearing mouse and on the 16th week prompted a tumor incidence of 100%. Animals that have been treated with 25, 50 and 100 mg/kg bwt of *L. pumila* extract topically for 30 min developed a mean number of 3.60 ± 1.1, 3.20 ± 0.8 and 2.40 ± 0.7 skin tumors per tumor-bearing mouse with tumor incidence of 90, 60 and 50%, respectively. The tumor volume per tumor-bearing mice of carcinogen control animals was 121.03 ± 3.46 mm^3, which was significantly ($p<0.05$) reduced to 92.27 ± 2.68, 69.24 ± 3.93 and 54.24 ± 4.38 mm^3 for the animals treated with 25, 50 and 100 mg/kg bwt of *L. pumila* extract, respectively. In terms of tumor incidence and tumor burden, the highest dose (100 mg/kg bwt) of *L. pumila* ethanol extract was almost equipotent with curcumin (10 mg/kg bwt). The extract of *L. pumila* not only decreased the tumor incidence, tumor burden and tumor volume in DMBA/croton oil-induced mice but also delayed the skin tumor growth as compared to carcinogen control group. Further histopathological examination revealed that tumors from animals that have been treated with *L. pumila* showed intact basement membrane as compared to the tumors from the untreated animals. This finding suggested that *L. pumila* extract was able to suppress the progression of benign tumors to malignant stage in DMBA/croton oil-induced mice. Further studies should be carried out in order to identify the active compound responsible for the anticarcinogenic activities and the mechanism of action of *L. pumila* at the molecular level.**

Key words: Chemoprevention, chemical carcinogenesis, *Labisia pumila*.

INTRODUCTION

Plant has been one of the sources of medicine to treat various illness and diseases since ancient time. As cancer persists as a major health problem in many countries (Jemal et al., 2008), the search for biologically active compounds with anticarcinogenic activity from plant sources have been carried out extensively (Lee et al., 2006, Issa et al., 2006, Cragg and Newman, 2005; Jordan and Wilson, 2004; Ahmad and Mukhtar, 2001; Gupta and Mukhtar, 2001).

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Numerous animal models have been used to study inhibition of chemical carcinogenesis and one of the better characterized models is the two-stage mouse skin carcinogenesis (Oyama et al., 2009; Owens et al., 1999; Warren and Slaga, 1993; DiGiovanni, 1992). The established two-stage skin carcinogenesis protocol using animal models, which has been initially introduced by Berenblum (1941 a, b) and Mottram (1944 a, b) is a multi step process including initiation, promotion and progression (DiGiovanni, 1992; Agarwal and Mukhtar, 1991) and it is an ideal biological system that has provided essential model not only for studying the stepwise development of tumors and manipulation of genetic on tumor initiation, promotion and progression but it is also the best established *in vivo* models to study both novel skin cancer prevention strategies and inhibitors of chemical carcinogenesis as well as to determine the efficacy of potential chemopreventive agents (McCormick and Moon, 1986; Slaga et al., 1980). Efficacy is evaluated based upon the percent inhibition of tumor incidence and/or multiplicity, or increased tumor latency in comparison to carcinogen-treated controls (Steele et al., 1994 and Boone et al., 1992).

Labisia pumila syn. *Labisia pothoina* (Myrsinaceae) or locally known as *Kacip Fatimah or Kachit Fatimah, Kunchi Fatimah, Akar Fatimah, Rumput Siti Fatimah, Selusuh Fatimah, Pokok Pinggang, Bunga Belangkas Hutan* (Burkill, 1993) is one of the most popular Malaysian medicinal herbs that has been used by the Malay women for more than 400 years and therefore meet the criteria to be the “queen of Malaysian herbs” (Choi et al., 2009). There are three varieties of *L. pumila* that were identified in Malaysia, namely var. *alata*, var. *pumila* and var. *lanceolata* (Stone, 1988). The water decoction of *L. pumila* is usually taken as a health tonic drink for pre- and post-partum treatment (Zakaria and Mohd, 1994; Burkill, 1993). Other uses include treatment of dysentery, flatulence, dysmenorrhea, gonorrhea excessive gas in the body and “sickness in the bones” (Zakaria and Mohd, 1994; Stone, 1988; Burkill, 1966). Owing to the many good claims of *L. pumila*, the herbs has been widely commercialized as supplement capsules and health tonic for women, hence there is an increasing demand for the supply of *L. pumila* in pharmaceutical and food industries.

The long history of usage of *L. pumila* in Malaysia clearly indicates that this plant is safe to be consumed. Recent findings from a few scientific studies on safety issues of *L. pumila* further confirmed that the plant and its byproduct are safe for human use (Ezumi et al., 2007). The aqueous extract of *L. pumila* was found to contain oestrogen-like compounds (Husniza, 2002) and lowered the cortisol levels in pregnant rats with no effect on the immune status (Pandey et al., 2008). In another study, various extracts of *L. pumila* have been found to exhibit positive correlation between antioxidant capacities of *L. pumila* and individual antioxidative compounds in the order of -carotene > flavonoid > vitamin C > total anthocyanins > phenolics (Norhaiza et al., 2009) . This finding is further confirmed by the study of Choi et al. (2009) that *L. pumila* extract indeed has strong antioxidant activity comparable to that of ascorbic acid. In the same study, *L. pumila* extract also was found to have strong protective effect against human dermal fibroblasts from cell damage induced by UV irradiation and showed better performance than ascorbic acid in protecting skin against UV- induced photoaging. Apart from these studies, very little is known about the ability of *L. pumila* to modify the genotoxic effects of carcinogens.

Our earlier *in vitro* studies showed that the ethanol extract of *L. pumila* exerted its antiproliferative action through apoptosis in cancer cell lines (data not published). In view of this, in the present study, the two-stage mouse skin carcinogenesis model was manipulated to test the efficacy of *L. pumila* ethanol extract in inhibiting the tumor promotion by croton oil (tumor promoter) in the ICR mouse skin previously initiated with DMBA. In this paper, the anticarcinogenic activity of *L. pumila* is reported and to the best of our knowledge, this is the first *in vivo* report on the effect of topical applications of *L. pumila* ethanol extract on two-stage mouse skin carcinogenesis model.

MATERIALS AND METHODS

Chemicals

Croton oil (CO), Curcumin and 7, 12- dimethylbenz(a)anthracene (DMBA) were obtained from Sigma, St Louis, USA. Acetone and ethanol were purchased from BDH Laboratory Supplies (Merck Ltd, United Kingdom).

Animals

Ninety random-bred female ICR mice, 6 to 7 weeks of age, were obtained from the Animal House of Faculty of Science and Technology, National University of Malaysia. The animals were acclimatized at the animal house facility for a maximum of 10 days, housed 10 animals/cage in polypropylene cages. The mice were fed standard pellets (Barastoc, Australia) and drinking water *ad libitum*. Two days prior to the start of the study, the dorsal skin (2 × 2 cm) of all experimental animals was shaved with an electric clipper for small animals (Oster A2, USA) . Only mice with no regrowth of hair were used in the experiment.

Plant material

Dried powder of *L. pumila* var *pumila* whole plant was kindly provided by Prof. Dr. Azimahtol Hawariah Lope Pihie (National University of Malaysia).

Preparation of extract

The extraction was done based on a procedure described by Wagner et al. (1983) and Harborne (1984). Five hundred grams of dried powder of *L. pumila* whole plant were subjected to extraction with 90% ethanol by using a Soxhlet apparatus at 60 to 80°C until the solvent became clear. The crude ethanol extract obtained was

then evaporated to dryness by using a rotary evaporator and was dissolved in acetone based on the required amount for the dosing.

Experimental protocol

Based on the preliminary *in vitro* experiments (data not shown), the anticarcinogenic effects of varying doses (25, 50 and 100 mg/kg bwt) of *L. pumila* ethanol extract were tested against DMBA/croton oil-treated mice based on the work by Das et al. (2005) and Duuren and Melchionne (1983) with some modifications.

Ninety mice were randomly divided into 9 groups and each experimental group consisted of 10 mice.

Group N: All animals in this group were subjected to topical applications of acetone, the vehicle (0.1 ml/mouse) on the shaved skin area twice week until the end of 20 weeks of experiment.

Group DMBA: All animals in this group were subjected to a single topical application of DMBA (the initiator) at 390 nmol/0.1 ml/mouse on the shaved skin area. Ten days after the initiation, the treated skin was topically applied with 0.1 ml of distilled water twice a week until the end of experiment.

Group CO: All animals in this group were topically applied with 0.1 ml of croton oil (0.5%, v/v in acetone) the promoter twice a week throughout the experiment.

Group Lp: All animals in this group received topical applications of *L. pumila* ethanol extract at 100 mg/kg bwt (the highest dose tested) twice a week throughout the experiment.

The above-mentioned four groups (Group N, DMBA, CO and Lp) of negative control were included in the experiment to eliminate unrelated effects of the chemicals or test samples used in the study.

Group DMBA-CO: Animals in this group were initiated with a single topical application of DMBA (390 nmol/0.1 ml/mouse). Ten days after the initiation, the shaved skin of each animal was promoted with 0.1 ml of croton oil (0.5%, v/v in acetone) twice a week until the end of 20 weeks of experiment. This group served as the carcinogen control.

As for **Group Lp25, Group Lp50 and Group Lp100** the animals in these groups received the same treatment as group DMBA- CO, but *L. pumila* extract (25, 50 and 100 mg/kg bwt) was topically applied 30 min prior to croton oil treatment until the end of 20 weeks.

Group Curcumin: All animals in this group received the same treatment as group DMBA-CO, but curcumin (10 mg/kg bwt) was topically applied 30 min prior to croton oil treatment until the end of 20 weeks. This group served as the positive control.

Morphological observations

During the course of the study, the animals were shaved weekly for an easy application of tested agents/extracts and skin lesion observation. A weekly observation was carefully performed to count and record the incidence of skin tumor, the number of tumors per tumor-bearing mice and to measure the volume of the skin tumors formed.

Skin growth with a diameter greater than 1 mm that persisted for at least two (2) consecutive observations was identified as skin tumor and included in the cumulative counts. Growth that regressed after one observation was not considered for counting. The diameter of each identified skin tumor was measured weekly by using a dermatology ruler and the tumor volume was calculated based on the formula below (Fujiwaki et al., 1997),

Volume (mm^3) = (/6) × (length of the tumor) × (width of the tumor) × (height of the tumor).

Histological evaluation of treated skin tissue

At the end of 20 weeks, all mice were sacrificed by cervical dislocation. The skin from the treated area from each mouse was sampled and kept in 12% buffered formalin for at least 12 h. Skin tissues were trimmed and processed for histological evaluation based on Drury and Wallington (1980). All skin samples were dehydrated in a graded series of ethanol and water (from 70 to 100% ethanol), embedded in paraffin wax and cut into sections of 4 µm thick. Three adjoining sections of a tissue sample were placed on glass slides, stained with Hematoxylin-Eosin and observed under the light microscope. At least 10 fields from each slide and 10 slides from each group were examined (Dellmann and Eurell, 1998; Faccini et al., 1990).

Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed on the data and the Univariate Analysis of Variance (ANOVA) was used followed by the Duncan post hoc test to determine if there was any significant difference between the treated and controlled groups. P values of less than 0.05 ($p<0.05$) was considered as indicative of significance.

RESULTS

Effect of *L. pumila* extract on experimental skin carcinogenesis

In the study, the anticarcinogenic effects of varying doses (25, 50 and 100 mg/kg bwt) of *L. pumila* ethanol extract were evaluated in DMBA-initiated, croton oil-promoted ICR mice. The percentage of tumor incidence (the number of mice with skin tumors), tumor burden (the number of skin tumor per tumor-bearing mouse), tumor size (mean tumor volume per tumor-bearing mouse) and latency period of tumor formation were determined. The tumor data were compared not only with the carcinogen control group but also with the group that was treated with curcumin (10 mg/kg bwt), a natural antitumor compound from *Curcuma longa* plant.

As shown in Figures 1 to 3 and Table 1, topical applications of *L. pumila* extract prior to that of croton oil in DMBA- initiated mouse skin resulted in a dose-dependent inhibition of skin carcinogenesis. The inhibition was apparent when the data were expressed as the percentage of tumor incidence (Figure 1), tumor burden (Figure 2) and tumor size (Figure 3).

At the end of the experiment of 20 weeks, none of the mice in the control groups (Group N, DMBA, CO and Lp) developed skin tumors. As can be seen from the data shown in Table 1, *L. pumila* exhibited significant anticarcinogenic activity. It means the number of tumor

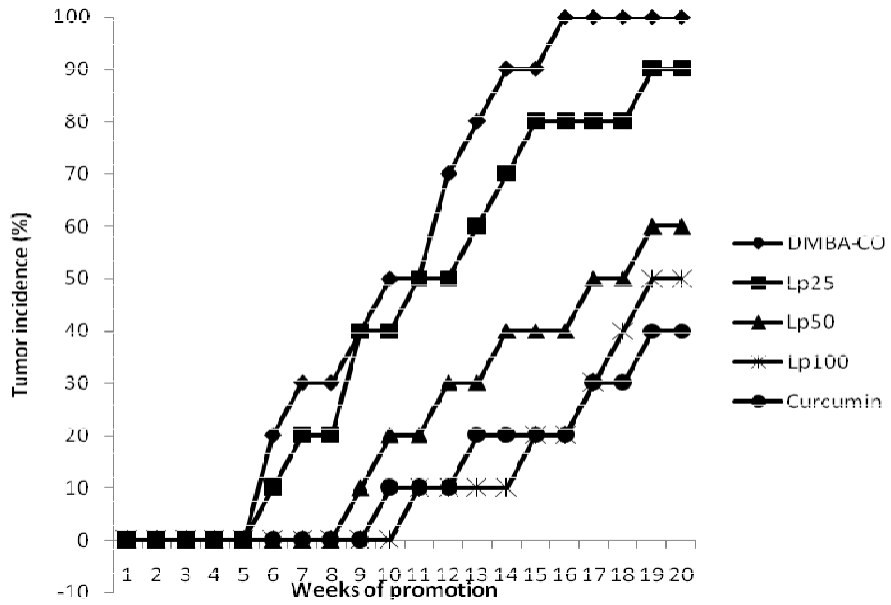


Figure 1. Effect of varying doses of *L. pumila* ethanol extract on tumor incidence in DMBA- croton oil-induced mice. Tumor incidence (%) of carcinogen control mice (DMBA-CO, closed diamonds), mice treated with 25 mg/kg bwt of *L. pumila* extract (Lp25, closed squares), 50 mg/kg bwt of *L. pumila* extract (Lp50, closed triangles), 100 mg/kg bwt of *L. pumila* extract (Lp100, cross) and 10 mg/kg bwt of curcumin (closed circles). Values are mean ± SEM. Each data point represents the tumor incidence (percentage of tumor-bearing mice) in the treated groups.

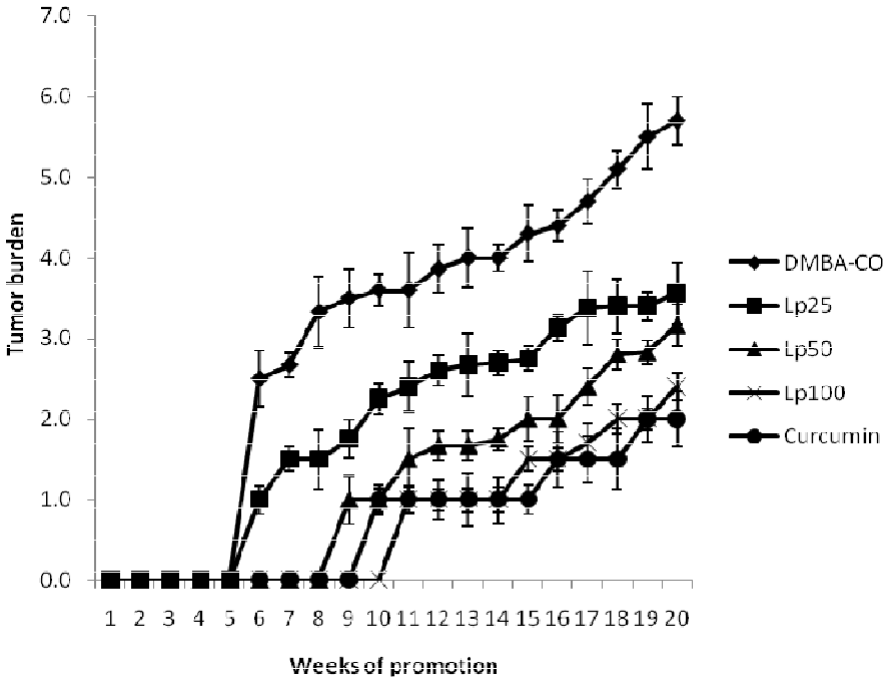


Figure 2. Effect of varying doses of *L. pumila* ethanol extract on tumor burden in DMBA-croton oil-induced mice. Tumor burden of carcinogen control mice (DMBA-CO, closed diamonds), mice treated with 25 mg/kg bwt of *L. pumila* extract (Lp25, closed squares), 50 mg/kg bwt of *L. pumila* extract (Lp50, closed triangles), 100 mg/kg bwt of *L. pumila* extract (Lp100, cross) and 10 mg/kg bwt of curcumin (closed circles). Values are mean ± SEM. and each data point represents the tumor burden (number of tumors per tumor-bearing mouse) in the treated groups.

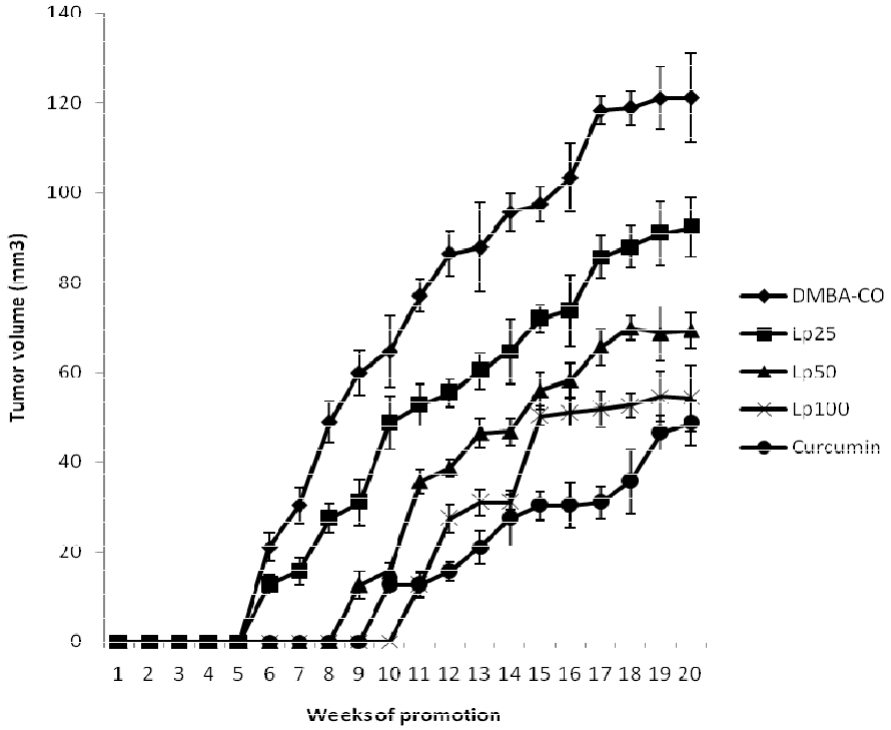


Figure 3. Effect of varying doses of *L. pumila* ethanol extract on tumor volume in DMBA-croton oil-induced mice. Tumor volume (mm^3) of carcinogen control mice (DMBA-CO, closed diamonds), mice treated with 25 mg/kg bwt of *L. pumila* extract (Lp25, closed squares), 50 mg/kg bwt of *L. pumila* extract (Lp50, closed triangles), 100 mg/kg bwt of *L. pumila* extract (Lp100, cross) and 10 mg/kg bwt of curcumin (closed circles). Values are mean ± SEM. and each data point represents the mean tumor volume per tumor-bearing mouse in the treated groups.

Table 1. Effects of the topical application of ethanolic extract of *Labisia pumila* on tumor incidence, tumor burden, tumor volume and tumor latency of DMBA-croton oil-induced skin tumor in mice at the end of 20 weeks of promotion.

Group	Tumor incidence (%)	Tumor burden (%)	Tumor size (tumor volume per mouse, mm^3)
Carcinogen control,	100	5.70 ± 1.3^a	121.03 ± 6.46^a
Curcumin (10 mg/kg bwt)	40	2.00 ± 0.5^b	48.65 ± 3.96^b
L. pumila (25 mg/kg bwt)	90	3.60 ± 1.1^c	92.27 ± 2.68^c
L. pumila (50 mg/kg bwt)	60	3.20 ± 0.8^c	69.24 ± 3.93^c
L. pumila (100 mg/kg bwt)	50	2.40 ± 0.7^b	54.24 ± 4.38^b

Values are means ± SEM, n=10. Means with the same letter indicates no significant difference. If letters differ then $p<0.05$.

developed is less, than in the case of the carcinogen control, the volume of the developed tumor also decreased. In terms of tumor incidence and tumor burden, the highest dose (100 mg/kg bwt) of *L. pumila* ethanol extract was almost equipotent with curcumin (10 mg/kg bwt) (Figures 1 and 2, Table 1).

Topical applications of higher doses of *L. pumila* extract (50 and 100 mg/kg bwt) in DMBA-initiated, croton oil-promoted mice not only significantly ($p<0.05$) inhibited the tumor incidence and tumor burden, but also the size of skin tumors formed (Table 1) when compared with the carcinogen control group.

Again, the highest dose (100 mg/kg bwt) of *L. pumila* ethanol extract was found almost equipotent with curcumin (10 mg/kg bwt) (Figure 3, Table 1) in reducing tumor volume in DMBA-croton oil-induced mice. Interestingly, the lowest dose of *L. pumila* extract (25 mg/kg bwt) was able to significantly ($p<0.05$) reduce the tumor burden and the size of the tumor in DMBA-initiated, croton oil-promoted mice as compared to carcinogen control group mice.

The extract of *L. pumila* not only decreased the tumor incidence, tumor burden and tumor volume in DMBA-croton oil-induced mice but also delayed skin tumor

development (Figure 1, Table 1). In the carcinogen control group, the first skin tumor appeared after 6 weeks of tumor promotion, while in groups that were treated with 50 and 100 mg/kg bwt of *L. pumila* ethanol extract, the first skin tumor appeared after 9 and 11 weeks of tumor promotion, respectively. The lowest dose (25 mg/kg bwt) of *L. pumila* ethanol extract tested, however, failed to show an inhibitory effect on the tumor latency of the treated mice (Figure 1). Mice treated with DMBA alone, *L. pumila* ethanol extract alone and croton oil alone produced no skin tumors (data not shown).

Findings from the morphological studies of the tumors revealed that at the termination of experiment, animals that were treated with higher doses (50 and 100 mg/kg bwt) of *L. pumila* ethanol extract showed less tumor numbers as well as smaller size as compared to the carcinogen control group. In addition, the skin tumors formed on the back of carcinogen control mice were asymmetrical in feature, dark red in color and some were ulcerated on the surface and sometimes with black necrotic tissues, whereas the tumors that formed on the back of mice that have been treated with higher dose of *L. pumila* (50 and 100 mg/kg bwt) were generally round and lobulated with pinkish red in color. No ulcerated tumors were noted in this group.

The effect of topical applications of *L. pumila* ethanol extract on mouse skin histology of DMBA/croton oil-induced mice over 20 weeks of tumor promotion is shown in Figure 4. From the histological observations, cutaneous tissue of acetone (vehicle)-treated control mice showed distinct uniformly arranged layers (Figure 4A). The cutaneous tissue sections from the carcinogen control group however, demonstrated parts of basement membrane were breached and some of the epidermal cells were found in the dermal layer (black arrow) (Figure 4B). Most of these invasive tumor cells were polymorphic with hyperchromatic nuclei and altered nucleo-cytoplasmic ratio (Figure 4B1). As for the groups that have been treated with higher doses of *L. pumila* extract (50 and 100 mg/kg bwt), cutaneous sections from this group exhibited intact basement membrane and no epidermal cells were observed in all levels of the dermal layer examined (Figuer 4C). Under higher magnification (400×) (Figure 4C1), the epidermal cells in this group showed normochromatic nuclei with normal nucleo-cytoplasmic ratio.

DISCUSSION

The search for potential inhibitors of carcinogenesis has become important in cancer research (Lee et al., 2006, Issa et al., 2006, Cragg and Newman, 2005; Jordan and Wilson, 2004; Ahmad and Mukhtar, 2001 and Gupta and Mukhtar, 2001). Based on the results obtained from our previous *in vitro experiments* (data not shown), the anticarcinogenic effects of *L. pumila* were studied *in vivo* using a two-stage skin carcinogenesis model for the first time. Results obtained from the study pointed out the anticarcinogenic effect of *L. pumila* ethanol extracts in DMBA/croton oil-induced mice. The key findings indicated that the ethanolic extract of *L. pumila* at higher doses (50 and 100 mg/kg bwt) significantly inhibited the progression of mouse skin carcinogenesis at the promotional stage.

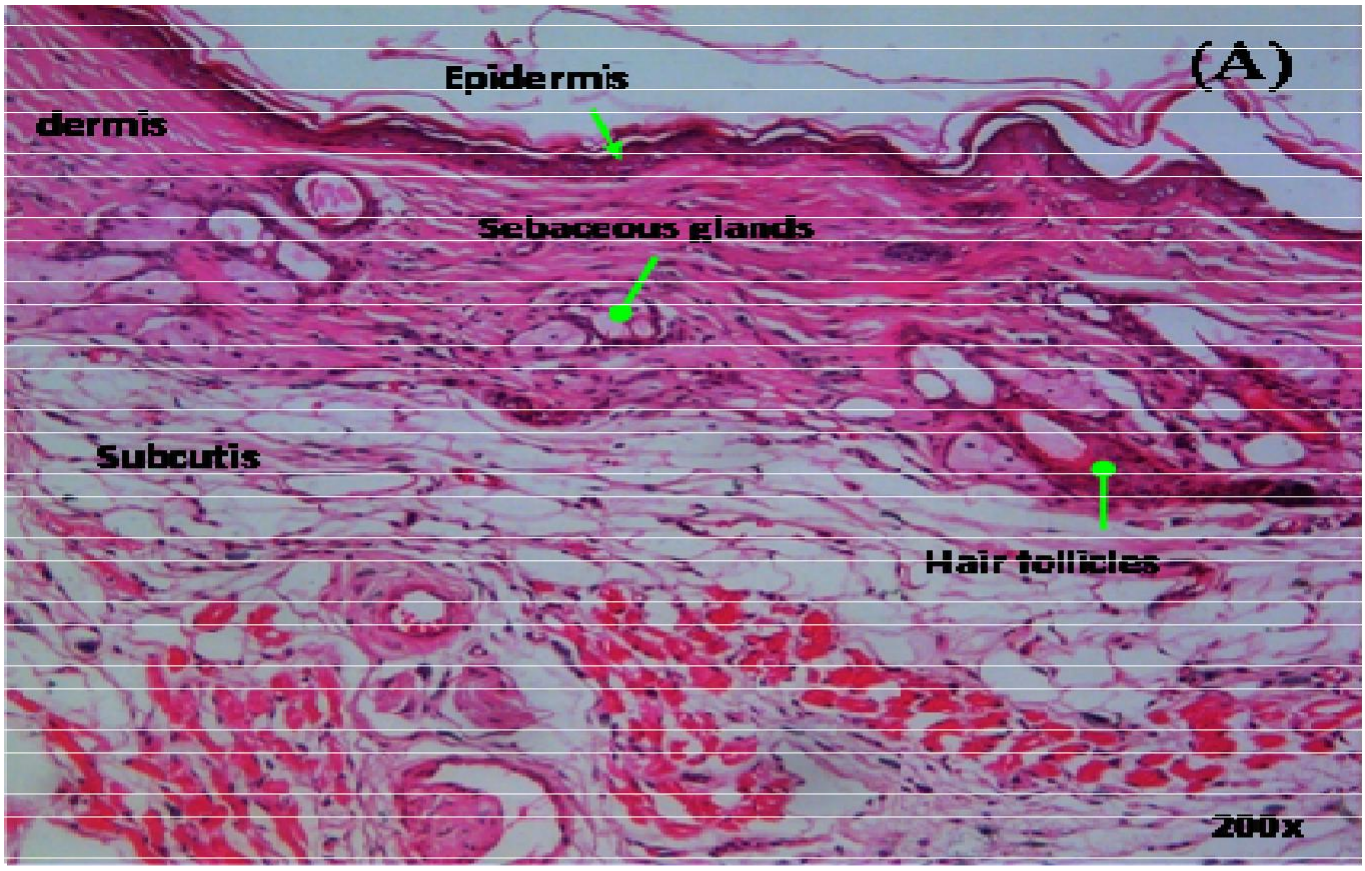
The preapplication of *L. pumila* extracts prior to croton oil topical application exhibited protective effects when the tumor data were considered as the percentage of tumor incidence, total number of tumors per tumor-bearing mouse and the tumor volume per mouse. The ability of *L. pumila* ethanol extract to delay the onset of tumor formation in DMBA/croton oil-induced mice further indicated the plant's anticarcinogenic potential. The prolonged latency period of skin tumor formation showed by the *L. pumila*- treated groups as compared to the carcinogen control group further suggested that *L. pumila* extract was able to restrain the proliferation of tumor cells in DMBA/croton oil-induced mice.

As treatment with *L. pumila* ethanol extract was carried out 30 min prior to croton oil application, it is assumed that the constituents in the ethanol extract may have prohibited the direct action of croton oil with the active form of DMBA and/or modify the carcinogen metabolism or blocking its reactive electrophiles from reaching the critical target sites in the cellular environment (Wattenberg, 1992).

Results from the histopathological examinations carried out further confirmed the anticarcinogenic activity of *L. pumila* extract. Findings from the microscopic observations revealed that the cutaneous tissue sections from carcinogen control group exhibited parts of the basement membrane were disrupted and some of the epidermal cells were present in the dermal layer and most of these cells were found polymorphic with hyperchromatic nuclei and altered nucleo-cytoplasmic ratio. These findings obviously indicated that the invasion of tumorous cells has been occurred and that the skin tumors in the carcinogen control group were invasive in behavior and had progressed to premalignant stage. As for the animals that have been treated with higher doses of *L. pumila* extract (50 and 100 mg/kg bwt), microscopic observations done disclosed that the structure of the basement membrane was still intact and there were no epidermal cells observed in all levels of the dermal layer examined. These observations clearly indicated that the skin tumors formed in this group were benign in nature and that *L. pumila* extract were able to restrain the tumor progression in DMBA/croton oil-induced mouse skin carcinogenesis.

Conclusion

The ethanolic extract of *L. pumila* possessed acticarcin-ogenic potential that should be considered for further


(A)
Epidermis
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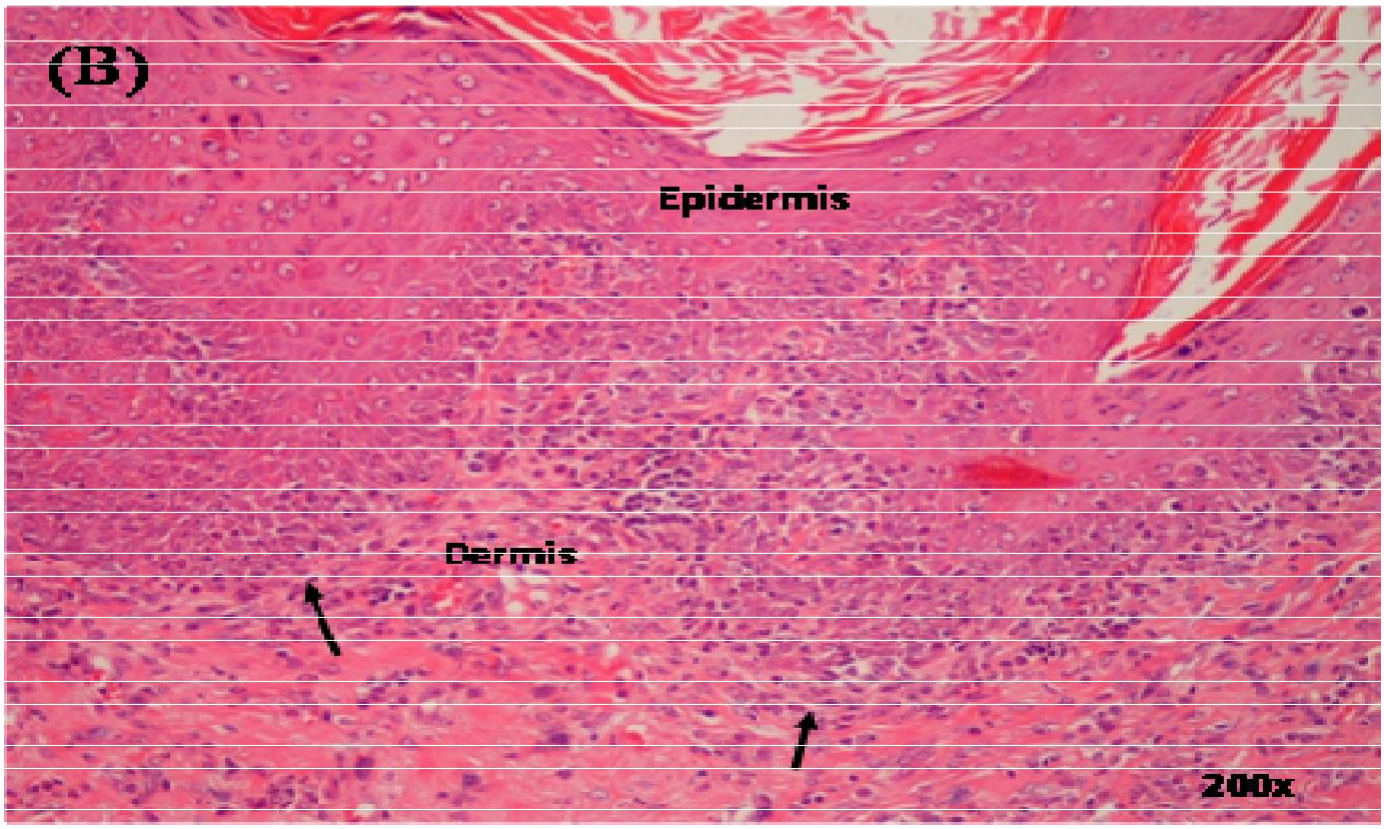
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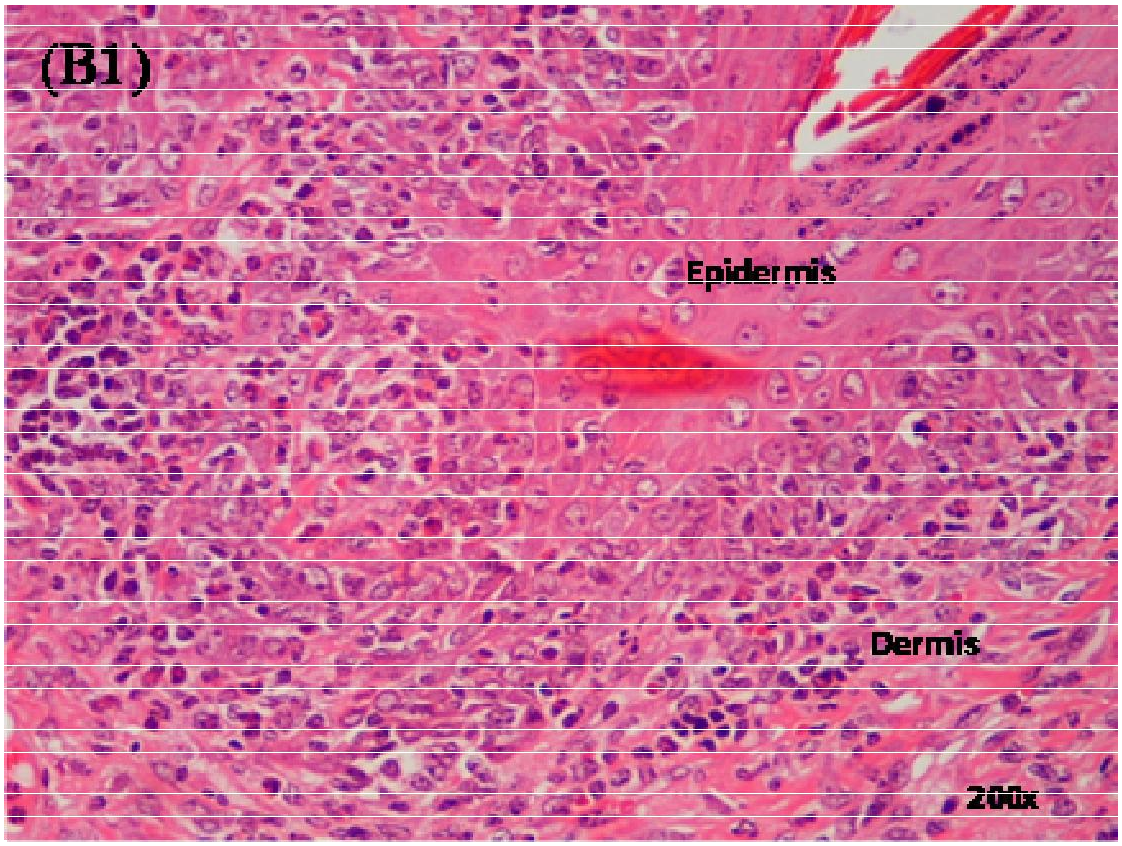
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(C)
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Dermis
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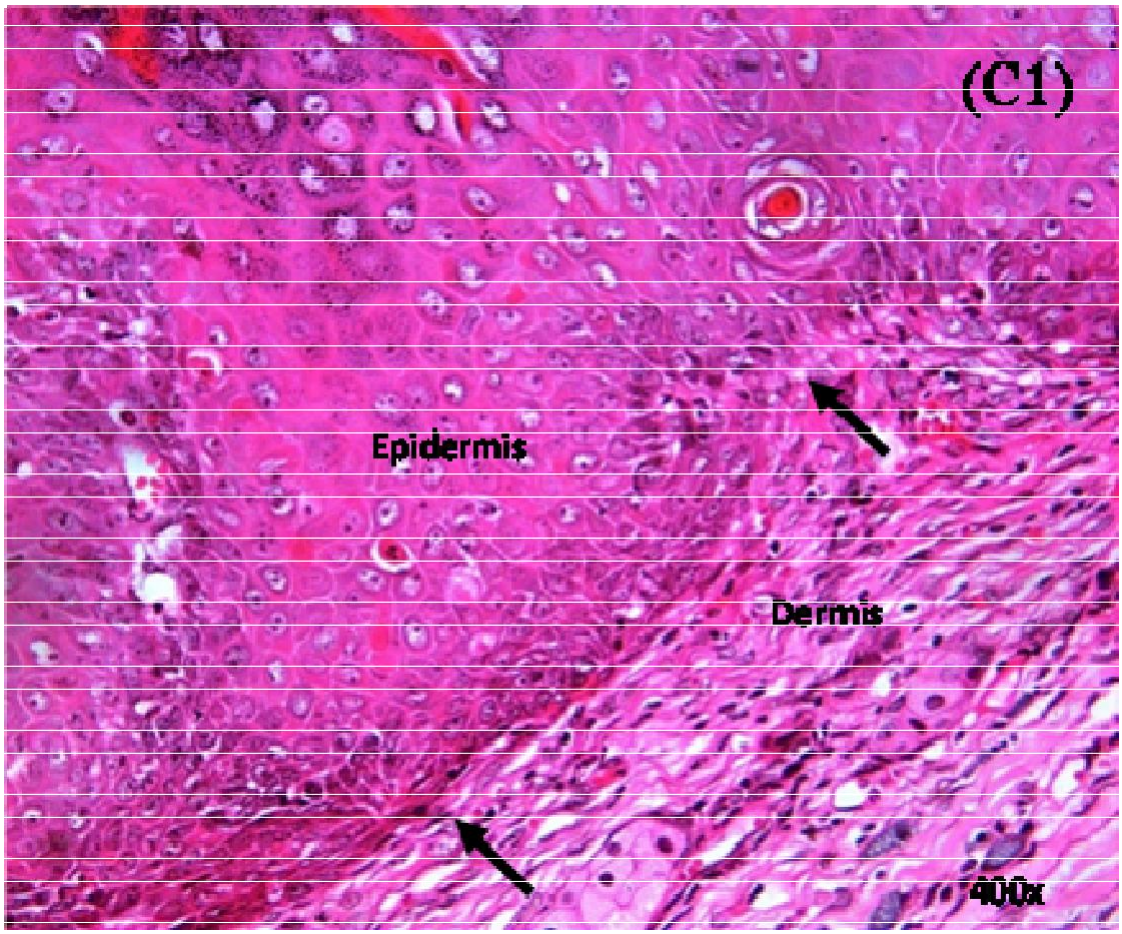

Figure 4. Representative microphotographs of H&E-stained cutaneous tissue sections obtained from histologic examinations of skin tumors from carcinogen control and *L. pumila*-treated mice in the two-stage skin carcinogenesis experiment. (A) Section of cutaneous tissue of acetone (vehicle)-treated control mice (200×). (B) Section of cutaneous tissue of carcinogen (DMBA/croton oil)-treated control mice (200×). B1, a magnified portion of B showed disrupted basement membrane and the epidermal keratinocytes that invaded into the dermis (200×). (C) Cutaneous section of DMBA-initiated mice treated with *L. pumila* extract (100 mg/kg bwt) 30 min before each croton oil (promoter) topical application (200×). (C1), a magnified portion of C showed the fully intact basement membrane (black arrow) (400x).

studies. However, we as yet, are not able to suggest the compound(s) responsible and the mode(s) of action involved for this activity. Further studies on the characterization of active principles of *L. pumila* and elucidation of its mechanism of action in two-stage skin carcinogenesis models are needed.

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