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Chemopreventive potential of methanol extract of *Dicranopteris linearis* leaf on DMBA/croton oil-induced mouse skin carcinogenesis

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The present study was carried out to elucidate the chemopreventive potential of methanol extract of *Dicranopteris linearis* (MEDL) in a two-stage mouse skin carcinogenesis model due to the interrelated inflammation, oxidative stress and tumor promotion pathways. MEDL was prepared in a dose range of 30 to 300 mg/kg body weight. A total of 48 imprinting control region (ICR) female mice (6 to 8 weeks old) were randomly assorted into six groups. To induce skin tumor formation, a single topical application of 7,12-dimethylbenz[a]anthracene (DMBA) at 100 µg/100 µl was applied to the shaved dorsal region of mice, followed by repetitive administration of 1% croton oil, twice weekly for 15 weeks. Topical application of MEDL, 30 min prior to the croton oil application significantly reduced the tumor incidence to 12.5% in 300 mg/kg MEDL-treated group as compared to 87.5% in carcinogen control. The latency period of tumor formation was increased from sixth week in the carcinogen control to ninth and fifteenth weeks in 100 and 300 mg/kg MEDL-treated groups, respectively. The tumor burden of MEDL-treated groups (30, 100, and 300 mg/kg) were significantly lessen (5.67 ± 1.28, 5.00 ± 1.13, and 1.00 ± 0.13), as compared to carcinogen control (7.86 ± 2.37). The tumor volume was also significantly reduced from 9.00 ± 2.27 mm^3 in carcinogen control to 3.70 ± 0.96, 2.39 ± 0.54 and 0.26 ± 0.03 mm^3 in 30, 100 and 300 mg/kg MEDL-treated groups, respectively. In conclusion, the MEDL exhibited anti-carcinogenic effect in a dose-dependent manner, indicating its chemopreventive potential, which worth further study.

Key words: *Dicranopteris linearis*, leaves, methanol extract, anti-carcinogenic effect.

INTRODUCTION

Cancer is a major public health concern worldwide and the incidence of cancer continues to increase. It is now one of the leading causes of death globally. According to World Health Organization (WHO), about 7.6 million of cancer deaths were reported in 2008 and the cancer death are believed to continue rising, with an estimated 13.1 million deaths in 2030 (Siegel et al., 2011). Besides, one in four deaths due to cancer is estimated in the United State alone (Siegel et al., 2011). Moreover, the worldwide cancer burden was found to increase

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dramatically, especially in the developing countries where the contributing factors include population aging and growth, and the trend of practicing cancer-associated lifestyle such as smoking, physical inactivity, and westernized diets (Jemal et al., 2011).

Based on the statistics released by the Ministry of Health Malaysia in 2007, cancer was the third common cause of death, accounting for 11.28% of all deaths, after heart diseases and diseases of pulmonary circulation (16.49%) and septicemia (13.38%). A total of 18,219 new cancer cases were diagnosed in the same year and registered in the National Cancer Registry. Among the cancer cases, 8,123 (44.6%) cases were diagnosed in males and 10,096 (55.4%) cases for females. The age standardized incidence rates (ASR) were 85.1/100,000 in males and 94.4/100,000 in females (Ariffin and Saleha, 2011).

Under normal physiological conditions, the process of cell division or cell proliferation occurs in a control manner. This is to ensure that the body is at the state of equilibrium between proliferation and apoptosis. However, if the equilibrium is disturbed, the cells will grow and proliferate unrestrained, which finally will lead to tumor formation (Ames et al., 1993; Athar, 2002). The process of tumor development is also known as tumorigenesis. It is a multistep process involving a series of genetic and epigenetic alterations, including activation of oncogenes and inactivation of tumor-suppressor genes in a cell. These mutations accumulate in the cells and results in uncontrolled growth that eventually lead to invasion of cancerous cells into the surrounding tissue and/or finally metastasize to distant sites (Wu and Pandolfi, 2001). Although there are many different forms of cancer, the basic multistage process by which various tumors develop is similar for all cancers. The process of cellular deregulation can be caused by exposure to carcinogens, such as external exposure of body to chemicals, radiation and viruses that in turn, damage DNA and hence leads to cancer development (Greenwald and Mcdonald, 2002).

Current strategies are still not capable to effectively manage overall cancer incidence, even though efforts have been put on early cancer detection and treatment approaches. Hence, in recent years, one of the efforts in developing effective novel strategies to control cancer development is the approach of chemoprevention (Kelloff et al., 2000), a process which was first described by Sporn (1976). It is a means of cancer control where the cancer formation can be intervened in the early precancerous stages and entirely suppressed, reversed and slowed down by administration of synthetic or natural compounds (Nair et al., 1995; Lamson and Brignall, 2001; Abdullaev and Espinosa-Aguirre, 2004). An ideal chemoprevention agent should possess three important criteria first, it should be able to inhibit the development of cancer; second, it is expected to have minimal toxicity to the user; and third, it should act differentially on the premalignant or malignant cells, leaving normal cells unaffected (Lippman et al., 1998).

The most popular model used to study the anti-carcinogenic effect of extracts/compounds is the two-stage chemical-induced mouse skin carcinogenesis model. This model is a multi-factorial, multi-step process that consists of three distinct stages: initiation, promotion and progression, utilizing 7,12-dimethylbenz[a]anthracene (DMBA) and croton oil as tumor initiator and tumor promoter, respectively (Bowden et al., 1995). By separating the process of carcinogenesis mechanistically and temporally into three stages, the action of chemopreventive agent in either initiation, promotion (or premalignant progression), or malignant progression can be monitored (Yuspa, 1994).

The use of natural products started thousands of years ago and the herbal medicines are the oldest remedies known to mankind (Brown, 1980). The use of herbs as anticancer agents has a long history since traditional medicine systems (Sharma et al., 2009). Recent years, cancer prevention by natural products has gained escalating attention (Naithani et al., 2008) and undergone extensive laboratory and clinical testing due to its promising impact on human health (Cseke et al., 2006). In an effort to identify new chemopreventive agent, the current study was carried out to investigate the anticarcinogenic potential of methanol leaf extract of *Dicranopteris linearis* using a two-stage mouse skin carcinogenesis protocol.

METHODOLOGY

Plant collection

The leaves of *D. linearis* (Burm. f) Underw. were collected in September, 2011 from the roadside vicinity to Institute of Biosciences (IBS), UPM, Serdang, Selangor, Malaysia. Only the green, fresh and not shrivel leaves were collected. The young leaves and fiddleheads were all excluded. A voucher specimen was then deposited in the herbarium of IBS, with the voucher specimen number of SK 1987/11.

Preparation of MEDL

The leaves of *D. linearis* were rinsed with tap water to clean up the dirt or dusts and then air-dried at room temperature (27 ± 2°C) until dryness. The dried leaflets (pinnae) were ground into fine powder using laboratory mill (Mill Power Tech RT series, China). After that, the leaf powder was weighed and soaked in methanol in the ratio of 1:20 (w/v) for 72 h and repeated for three times. The supernatant was collected and first filtered using cotton wool, followed by Whatman no. 1 filter paper. The process was repeated three times and the last residue was air-dried and kept for reference. The filtered supernatant was concentrated until dryness in a rotary evaporator at 40°C under reduced pressure. The paste form of product was obtained and labelled as leaf methanol extract of *D. linearis* (MEDL). The stock MEDL was then stored at 4°C for future use (Zakaria et al., 2006, 2008).

Drugs and chemicals

The chemicals used in the experiment were methanol, 7,12 dimethylbenz[a]anthracene (DMBA) purchased from Sigma-Aldrich, croton oil (Sigma-Aldrich), curcumin (Sigma-Aldrich) and acetone. DMBA and croton oil served as tumor initiator and tumor promoter, respectively while curcumin was used as positive control. Acetone that was used as vehicle to dissolve all the drugs or chemicals was purchased from Mallinckrodt Chemicals (US). The tumor initiator, DMBA was prepared at the concentration of 100 µg/100 µl per mouse (Sharma et al., 2009; Chaudhary, 2011) by dissolving 100 µg of DMBA in 100 µl of acetone for each mouse. On the other hand, croton oil that serves as tumor promoter was diluted in acetone to give a concentration of 1% (w/v) (Das et al., 2005; Sharma et al., 2009; Chaudhary, 2011), based on the fact that 1% solution contains 10 mg/ml dissolved substance. Thus, 1% of croton oil was prepared by dissolving 1 mg of croton oil in 100 µl of acetone for each mouse. Curcumin was used as positive control due to its potent anti-tumor property (Park et al., 1998; Roslida et al., 2011). It was prepared at the dosage of 10 mg/kg (Roslida et al., 2011). The weight of curcumin needed to dissolve in 100 µl of acetone was calculated based on the mean body weight of Group 4 mice.

Phytochemical screening

The phytochemical screening tests were carried out based on 5.0 g of dried powder material and 100 mg of extract (organic).

Alkaloids test

Sample was macerated in chloroform followed by addition of ammoniacal chloroform. The mixture was then treated with sulphuric acid 10% and further tested with Mayer's reagent. Formation of white precipitates indicates the presence of alkaloids.

Saponins test

The methanol extract of sample was mixed with distilled water in a test tube. Formation of stable froth for at least 15 min indicates the presence of saponins.

Flavanoids test

The methanolic extract of sample was dissolved in ether and shaken in 10% ammonia solution. Formation of yellow colour in ammonia layer indicates the presence of flavonoids.

Tannins and polyphenolic compounds test

The methanolic extract of sample was mixed with 1% ferric solution. Formation of blue black colour indicates the presence of hydrolysable tannins, while brownish-green indicates that of condensed tannins.

Triterpenes/steroids test

The methanolic extract of sample was analysed using Liebermann-Buchard reagent. Formation of reddish colour indicates the presence of triterpenes and greenish colour for steroids.

Animals

The animal models used for *in vivo* study consisted of a total of 48 healthy 6 to 8 weeks old female ICR mice (Park et al., 1998), with the body weight of 15 to 25 g. The mice were divided into six groups, with eight mice per group and housed in polypropylene cage, fed with a standard laboratory pellet diet and water *ad libitum*. The animals were housed in Animal House of Faculty of Medicine and Health Sciences, at room temperature of 25 ± 4°C and alternating light and dark cycle. The animals were acclimatized for one week prior to the commencement of experiment. Three days before the experiment, all mice were dorsally shaved with electric hair clipper, with an area of 2 cm × 2 cm and 1 cm off tail (Sharma et al., 2009; Chaudhary, 2011).

Two-stage mouse skin carcinogenesis

Experimental design

All mice were randomly assigned into six groups, namely Group 1 to 6 (n = 8). The six experimental groups were composed of three treatment groups (Group 1 to 3) and three control groups (Group 4 to 6). During the promotional stage, each mouse from the three treatment groups, which are Groups 1, 2 and 3 were topically applied with 100 µl of 30, 100 and 300 mg/kg of MEDL, respectively. On the other hand, each mouse in Group 4 (positive control) was topically administered with 100 µl of curcumin at the dose of 10 mg/kg body weight of mice, while Group 5 (negative or carcinogen control) was assigned with no pre-treatment. Meanwhile, each mouse in the Group 6 (vehicle control) received acetone only throughout the experiment.

Two-stage mouse skin carcinogenesis protocol

In the *in vivo* two-stage mouse skin carcinogenesis model, the initiation phase was accomplished by a single application of sub-carcinogenic dose of mutagen that acts as tumor induction agent. This was followed one week later by repeated application of tumor promoter, twice a week, for up to 15 weeks. All the mice in all experimental groups were topically applied with 100 µg/100 µl of DMBA. After one week of waiting/retention period, the promotion phase started and lasted for 15 weeks. The three treatments groups (Group 1, 2, and 3) were topically administered with 30, 100, and 300 mg/kg of MEDL, respectively, followed by 1% croton oil at 30 min later, twice a week. For the positive control group (Group 4), the mice received 10 mg/kg of curcumin, 30 min prior to application of 1% croton oil promotion, twice a week. In contrast, mice in negative control group (Group 5) received only 1% croton oil without any pre-treatment, twice a week. Animals in vehicle control group (Group 6) were applied only with acetone (twice a week) throughout the whole experiment. All the chemicals or drugs that applied onto dorsal part of mice were at the volume of 100 µl each.

Papilloma detection

Along the 15 weeks of tumor promotion duration, the following macroscopic parameters were recorded at weekly intervals: (i) body weight of mice, (ii) latency period of tumor formation, (iii) tumor

incidence, (iv) tumor burden, and (v) tumor volume. The latency period of tumor formation was defined as the onset of first tumor in a group, expressed in terms of weeks. The percentage of tumor incidence denoted the number of mice carrying at least one tumor (Sharma et al., 2009; Chaudhary, 2011) while the tumor burden indicated the average number of tumors per tumor-bearing mouse (Sharma et al., 2009; Chaudhary, 2011). The measurements of tumor volume applied the formula of ellipsoid volume that is based on three axes (length, width and height axes) as it was the most accurate method for estimating the tumor growth (Girit et al., 2008). The experiment was terminated by the end of 15th week of tumor promotion. All the mice were scarified by cervical dislocation method (Sharma et al., 2004) followed by the skin histopathological analysis.

Morphological observation

Throughout the 15 weeks of tumor promotion, the dorsally shaved part of all mice was observed regularly for detection of any lesions and tumors development (Cibin et al., 2010). The hair of mice was shaved prior to every treatment so that the hair could not impede the absorption of chemicals onto the skin and hinder the tumor from being clearly observed during tumor size measurement, using caliper technique (Girit et al., 2008). Skin tumor was defined as reddish elevation from dorsal skin, polypoidal in shape, pedunculated, and cauliflower-like in appearance (Sharma et al., 2004). The palpable mass with a diameter greater than 1 mm and persisted at least for two consecutive weeks was considered for counting. Those skin papillomas that regressed after one week observation was excluded from data analysis (Das et al., 2005; Abel et al., 2009; Sharma et al., 2009). Since there was a couple of treatment day per week, the tumors was also observed and measured by using caliper technique (Girit et al., 2008), twice a week, to ensure the consistency of the tumors existence. Then, the two measurement readings were averaged so that only one reading was presented for each tumor. Apart from that, the body weight of each mouse was also recorded weekly for the purpose of dosage calculation in drugs preparation step and manipulation of causal factors if fluctuations of body weight occurred. Any death case in mouse should be recorded along with time of death.

Histopathological observation

The harvested skin was grossed by cross section of skin samples for both normal and tumor region. The skin samples were then kept in labelled cassettes and fixed in 10% buffered formalin for at least 24 h. Then, the fixed skin samples were sent for tissue processing in an automated tissue processor (Leica TP1020, Germany). The processed skin samples were then embedded in paraffin wax by an embedder machine (Leica EG1160, Germany) before subjected to trimming and sectioning, using a microtome (Leica RM2135, Germany) to obtain skin tissue sections with 4 µm thickness. Next, the tissue sections were fished on 40°C water bath, onto the frosted end glass slide. The slides were then stained with Hematoxylin and Eosin (H&E) stains using routine protocol in an automated slide stainer or autostainer (Sakura Tissue-Tek Prisma-E2S, Japan). Stained slides were finally mounted with DPX and viewed under biological microscope (40× and 100× magnification). Under the microscope, the histology of skin tissues was examined.

Statistical analysis

All statistical calculations were carried out with statistical package for social sciences (SPSS) software program (version 20.0). All of the values were expressed as the mean ± standard error of mean (SEM), analyzed by one-way analysis of variance (ANOVA) and significant difference among multiple means was determined using least significant difference (LSD) multiple comparison test at the level of 0.05.

RESULTS

Morphological observation

Figure 1 illustrates the representative photographs of animals of each experimental group captured at the end of study (15th week of tumor promotion). The tumors formed on the dorsal skin of mice appeared as reddish, cauliflower-like, and polypoidal in shape, ranging from 1 to 8 mm in diameter and 1 to 5 mm in height. Comparison among all the experimental groups showed that Group 5 (carcinogen control) demonstrated the highest number and largest size of tumor formation (Figure 1E), in contrast to Group 6 (vehicle control) which showed no tumor formation at all (Figure 1F). Among the MEDL-treated groups, both the tumor number and tumor size were reduced in Group 1, which has been treated with MEDL at 30 mg/kg body weight (Figure 1A), compared to Group 2, which has been treated with MEDL at 100 mg/kg body weight (Figure 1B), and almost null in Group 3, which has been treated with MEDL at 300 mg/kg body weight (Figure 1C). Throughout the 15 weeks of tumor promotion period, the number and body weight of mice, latency period of tumor formation, percentage of tumor incidence, tumor burden and tumor volume were recorded at weekly interval, which are summarized in Table 1.

Body weight

The body weight of mice was statistically analyzed for group factor, at the first week of tumor promotion (initial body weight) and fifteenth week of tumor promotion (final body weight). Results show that there was no significant different ($p > 0.05$) among experimental groups for both initial and final body weight. Figure 2 shows a net increment in body weight from week 1 to 15 of tumor promotion for all the experimental groups

Gross morphological observation

The onset of first tumor ranged from sixth to fifteenth week for different experimental groups (Figure 3). The first tumor appeared simultaneously at week 6 of tumor promotion period, for Group 1, which has been treated with MEDL at 30 mg/kg body weight and Group 5 (carcinogen control). The latency period of tumor formation were then increased from week 8 for Group 4 (positive control) to week 9 for Group 2, which has been

Table 1. Effect of methanol extract *of Dicranopteris linearis*(MEDL) leaf on two-stage mouse skin carcinogenesis at the promotion stage. The terms ‗initial' and ‗final' indicate the first and fifteenth week of tumor promotion, respectively.

Group	No. of animal		Body weight (g)		Tumor latency period (weeks)	Tumor incidence (%)	Tumor burden	Tumor volume (mm^3)
	Initial	Final	Initial	Final				
30 mg/kg MEDL	8	8	25.00 ± 0.87^{A}	29.50 ± 1.27^{A}	6	75.0^{B}	$5.67\pm1.28^{a,b,C}$	$3.70\pm0.96^{b,B}$
100 mg/kg MEDL	8	8	23.88 ± 0.88^{A}	30.00 ± 0.60^{A}	9	62.5^{B}	$5.00\pm1.13^{b,B}$	$2.39\pm0.54^{b,A,B}$
300 mg/kg MEDL	8	8	26.00 ± 1.02^{A}	32.38 ± 1.00^{A}	15	$12.5^{a,b,A}$	$1.00\pm0.13^{a,b,A}$	$0.26\pm0.03^{b,A}$
Positive control	8	8	25.25 ± 0.59^{A}	32.13 ± 0.92^{A}	8	75.0^{B}	$4.83\pm1.02^{b,A,B}$	$3.06\pm0.78^{b,A,B}$
Carcinogen control	8	8	25.13 ± 1.09^{A}	30.25 ± 0.98^{A}	6	87.5^{B}	$7.86\pm2.37^{a,D}$	$9.00\pm2.27^{a,C}$
Vehicle control	8	8	24.13 ± 1.00^{A}	30.38 ± 1.27^{A}	-	$0.0^{a,b,A}$	$0.00\pm0.00^{a,b,A}$	$0.00\pm0.00^{b,A}$

Values are expressed as mean ± S.E.M, except latency period of tumor formation and tumor incidence. ap < 0.05: significantly different from the positive control (Group 4). bp < 0.05: significantly different from the carcinogen control (Group 5). A,B,C,Dp < 0.05: comparison among all the experimental groups. The values followed by the same superscript (capital letter) are not significantly different (p > 0.05). Percentage of tumor incidence was calculated as ration of number of tumor-bearing mice in a group/number of mice in a group × 100. Tumor burden was calculated by dividing total number of tumor in a group by number of tumor-bearing mice in a group. Tumor volume was calculated using the formula V = π/6 × length × width × height.

treated with MEDL at 100 mg/kg body weight, and further increased to week 15 for Group 3, which has been treated with MEDL at 300 mg/kg body weight.

The percentage of tumor incidence was recorded at weekly interval and displayed in Figure 4, where it indicates the percentage of tumor-bearing mice in a group. The highest incidence of tumor formation was recorded in Group 5 (carcinogen control) (87.5%), followed by 75.0% observed in both the Group 4 (positive control) and Group 1 (MEDL treatment at 30 mg/kg body weight). The percentage of tumor incidence was further reduced to 62.5% in Group 2 (MEDL treatment at 100 mg/kg body weight) and the lowest percentage of tumor incidence was found in Group 3 (MEDL treatment at 300 mg/kg body weight) (12.5%) with only one mouse showing tumor. Group 6 (vehicle control) showed no tumor formation at all throughout the experiment.

The statistical analysis of percentage of tumor incidence (Figure 5) indicates that only Group 3, which has been treated with MEDL at 300 mg/kg body weight, showed significantly decrease ($p < 0.05$) in tumor incidence, as compared to other experimental groups, including both positive and carcinogen controls. The percentage of tumor incidence was not significantly different ($p > 0.05$) among positive control (75.0%), carcinogen control (87.5%), Group 1, treated with MEDL at 30 mg/kg body weight (75.0%), and Group 2, treated with MEDL at 100 mg/kg body weight (62.5%).

Tumor burden was also recorded at weekly interval (Figure 6) and is defined as the average number of tumors formed on a tumor-bearing mouse. Group 5 (carcinogen control) documented the highest number of tumor per tumor-bearing mice (7.86 ± 2.37), followed by Group 1, treated with MEDL at 30 mg/kg body weight (5.67 ± 1.28), and then Group 2, treated with MEDL at 100 mg/kg body weight (5.00 ± 1.13). Group 4 (positive control) recorded tumor burden of 4.83 ± 1.02, while the least tumor burden was found in Group 3 with MEDL at 300 mg/kg body weight (1.00 ± 0.13). Group 6 (vehicle control) showed no tumor formation at all throughout the experiment.

The statistical analysis of tumor volume reflected that all the tumor volumes were significantly smaller ($p < 0.05$) in experimental groups, as compared to carcinogen control. When comparing with positive control, only Group 3 treated with MEDL at 300 mg/kg body weight demonstrated significantly decrease ($p < 0.05$) in tumor volume. Besides, when comparing among the MEDL-treated groups, the tumor volume in Group 3 is significantly lesser ($p < 0.05$) than Group 1, which has been treated with MEDL at 30 mg/kg body weight, as well as Group 2, which has been treated with MEDL at 100 mg/kg body weight. There was no significant difference ($p>0.05$) between Group 1 and 2.

In summary, Group 5 (carcinogen control) showed the lowest latency period of tumor formation (week 6), greatest tumor incidence (87.5%), highest tumor burden (7.86 ± 2.37) and largest tumor volume (9.00 ± 2.27 mm^3). In contrast, Group 3,

Table 2. Phytochemical constituents in methanol crude extracts and powder of *Dicranopteris linearis*.

Sample	*Alkaloids	**Saponins	***Flavonoids	***Tannins and polyphenolic compounds	***Triterpenes	***Steroids
D.linearis extract	-	3+	2+	2+Condensed tannins detected	3+	3+
D.linearis powder	-	3+	1+	2+Condensed tannins detected	-	2+

*1+ negligible amount of precipitate; 2+ weak precipitate; 3+ strong precipitate. **1+ 1 to 2 cm froth; 2+ 2 to 3 cm froth; 3+ > 3 cm froth. ***1+ weak colour; 2+ mild colour; 3+ strong colour.

the highest dose of MEDL-treated group (300 mg/kg body weight) demonstrated the highest latency period of tumor formation (week 15), lowest tumor burden (12.5%), lowest tumor burden (1.00 ± 0.13) and smallest tumor volume (0.26 ± 0.03 mm^3).

Histopathological analysis

The effect of leaf methanol extract of MEDL was further assessed by histopathological studies after 15 weeks of tumor promotion. Microscopic exami-nation of the dorsal skin of mice has been carried out. The histopathological features observed in the dorsal skin tissues of mice in control and treatment groups are depicted in Figures 1 to 5.

The histological studies on vehicle control mice showed normal skin structure (Figure 7). The skin epidermis seen in acetone-treated mouse skin was approximately a single layer of basal cells overlaid by flattened squamous cells. Keratin was found in the stratum corneum. Hair follicles and sebaceous glands can be found in dermis layer. The skin tissues from carcinogen control demonstrated severe papillomatosis with severe hyperkeratosis (thickening of the stratum corneum) and severe acanthosis (thickening of the stratum spinosum) of the epidermis (Figure 8). In general, the hyperplastic lesions with intact epidermis indicated that all the papillomas formed were benign in nature. On the other hand, the papilloma formed in positive control was smaller, as compared to carcinogen control. Histological features observed in positive control were characterized by mild papillomatosis with mild hyperkeratosis and mild acanthosis of epidermis (Figure 9).

When comparing the cutanenous histological chracteristics among MEDL-treated groups, Group 1 (MEDL treatment at 30 mg/kg body weight) displayed a moderate papillomatosis with moderate hyperkeratosis and moderate acanthosis of epidermis (Figure 10), whereas Group 2 (MEDL treatment at 100 mg/kg body weight) showed mild papillomatosis with mild hyperkeratosis and mild acanthosis of epidermis (Figure 11). On the other hand, Group 3 (MEDL treatment at 300 mg/kg body weight) exhibited only mild hyperplasia of epidermis, with no papillomatosis observed (Figure 12).

Phytochemical tests

Phytochemicals screening of *D. linearis* was done in two forms which are powdered *D. linearis* and *D. linearis* extract to detect the presence of alkaloids, saponins, flavonoids, tannins and polyphenolic compounds, triterpenes and steroids as shown in Table 2. Both sample showed no presence of alkaloids but high amount of saponins. Moderate amount of flavonoids were present in *D. linearis* extract and low amount in the powder. Tannins and polyphenolic compounds were moderately found in both *D. linearis* extract and *D. linearis* powder. In comparison to *D. linearis* extract, *D. linearis* powder has no presence of triterpenes at all. Steroids were found in both sample extracts containing the higher amount while powder only contained a moderate amount.

DISCUSSION

The effort of screening natural products which can inhibit or reverse the process of carcinogenesis and thereby turn out into promising chemopreventive agent which gained increased attention (Craig, 1997; Kellen, 1999; Nishino et al., 2005).

According to Park et al. (1998), the inflammatory, oxidative stress and tumor promotion pathways are interrelated. Thus, a compound that displays anti-inflammatory and/or antioxidative properties is expected to act as an anti-tumor promoter as well (Park et al., 1998). This formed the basis of the current study to investigate the chemopreventive potential of *D. linearis* in the promotion stage of DMBA/croton oil-induced mouse skin carcinogenesis. In DMBA/croton oil-induced mouse skin carcinogenesis protocol, topical applications of DMBA and croton oil was able to induce skin papilloma formation through the process of chronic inflammation, reactive oxygen species (ROS) overproduction and oxidative stress-related DNA damage in skin (Das and Bhattacharya, 2004).

The proposition of an inflammation-cancer connection has arisen since the 19th century where the role of inflammation in carcinogenesis has gained more evidence (Balkwill and Mantovani, 2001). Numerous laboratory and population-based studies have proposed that some

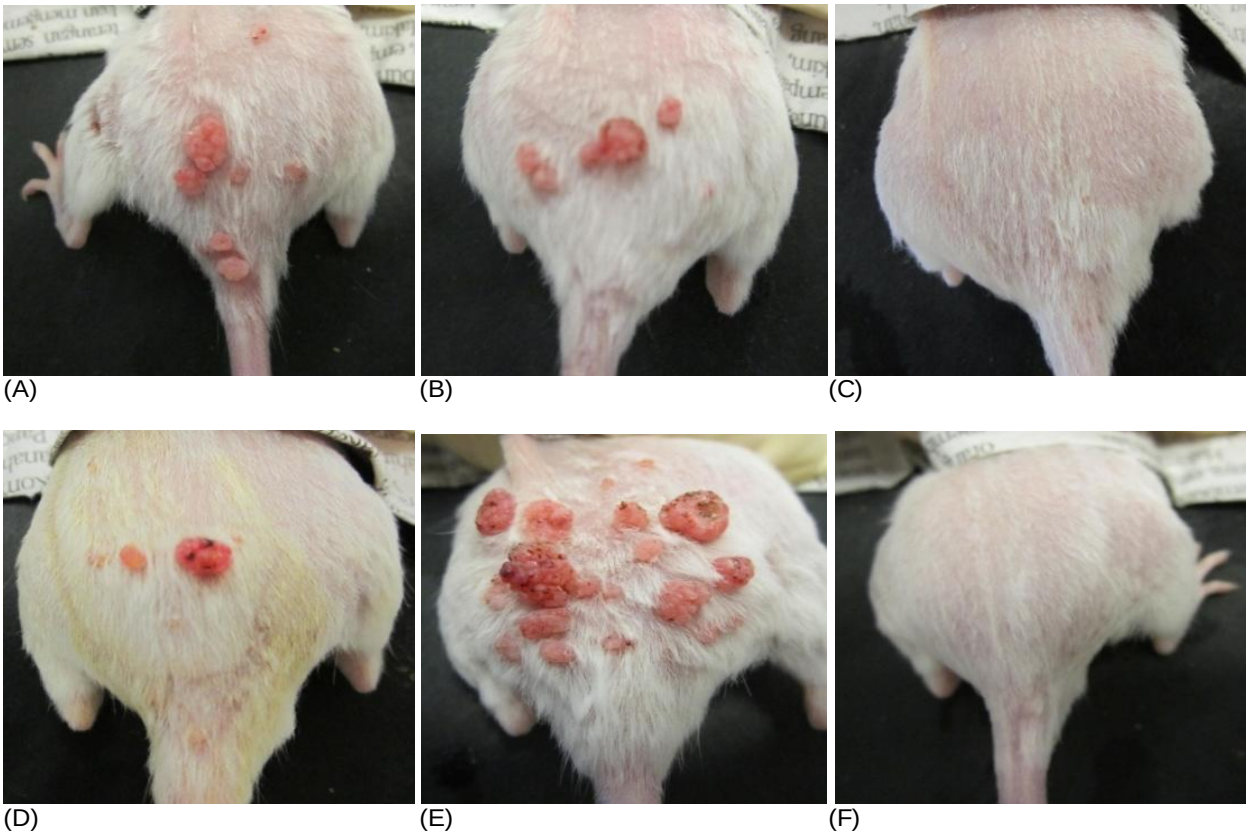


Figure 1. Representative photographs for an animal from each experimental group captured at the end of study (15 weeks). (A) MEDL treatment at 30 mg/kg; (B) MEDL treatment at 100 mg/kg; (C) MEDL treatment at 300 mg/kg; (D) positive control, curcumin treatment at 10 mg/kg; (E) carcinogen control, application of 1% croton oil only at the promotion stage, without any pre-treatment; (F) vehicle control, application of acetone only, throughout the experiment.

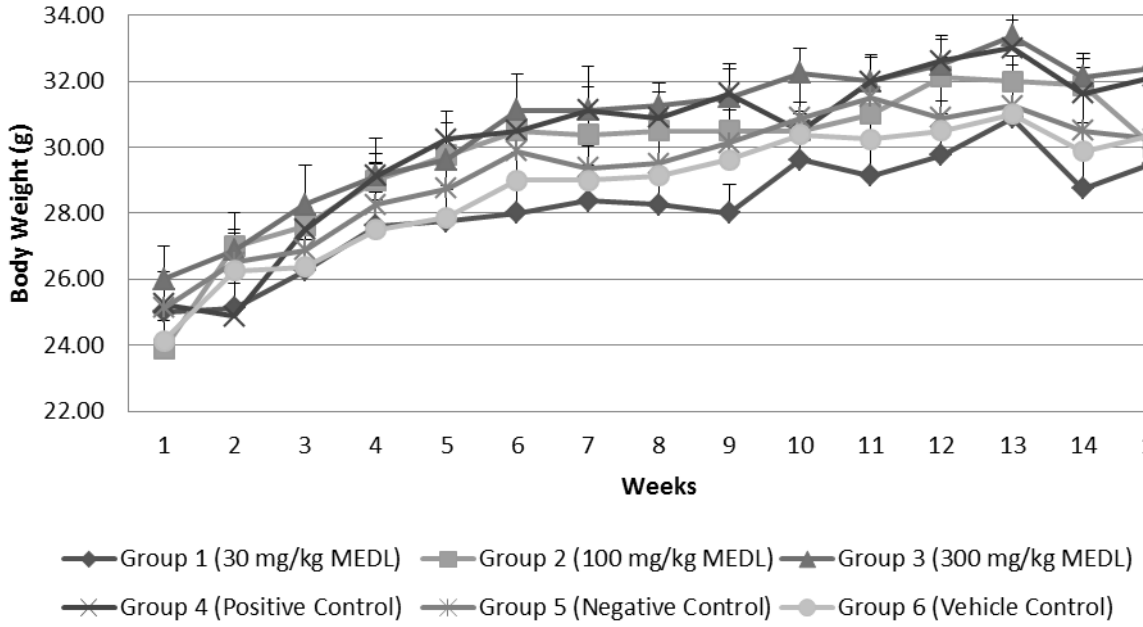


Figure 2. Effect of methanol extract *of Dicranopteris linearis*(MEDL) leaf on body weight of mice.

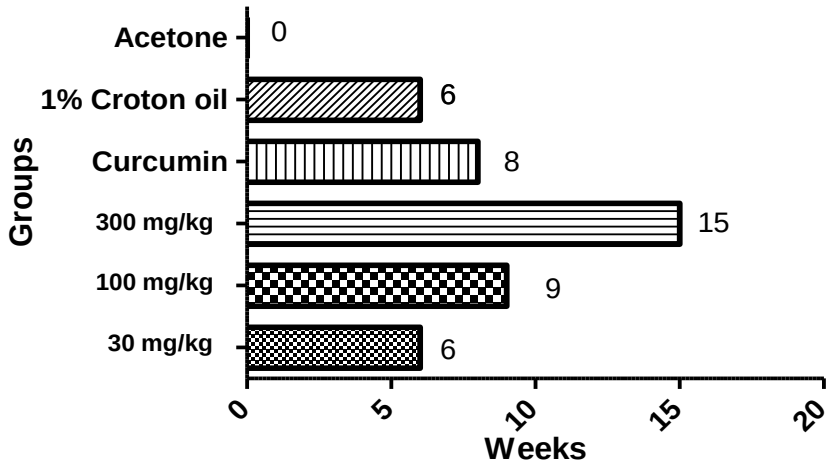


Figure 3. Effect of methanol extract of *Dicranopteris linearis* (MEDL) leaf on latency period of tumor formation in mice. Group 1 (MEDL at 30 mg/kg body weight); Group 2 (MEDL at 100 mg/kg body weight); Group 3, (MEDL at 300 mg/kg body weight); Group 4 (positive control); Group 5 (carcinogen control); and Group 6 (vehicle control).

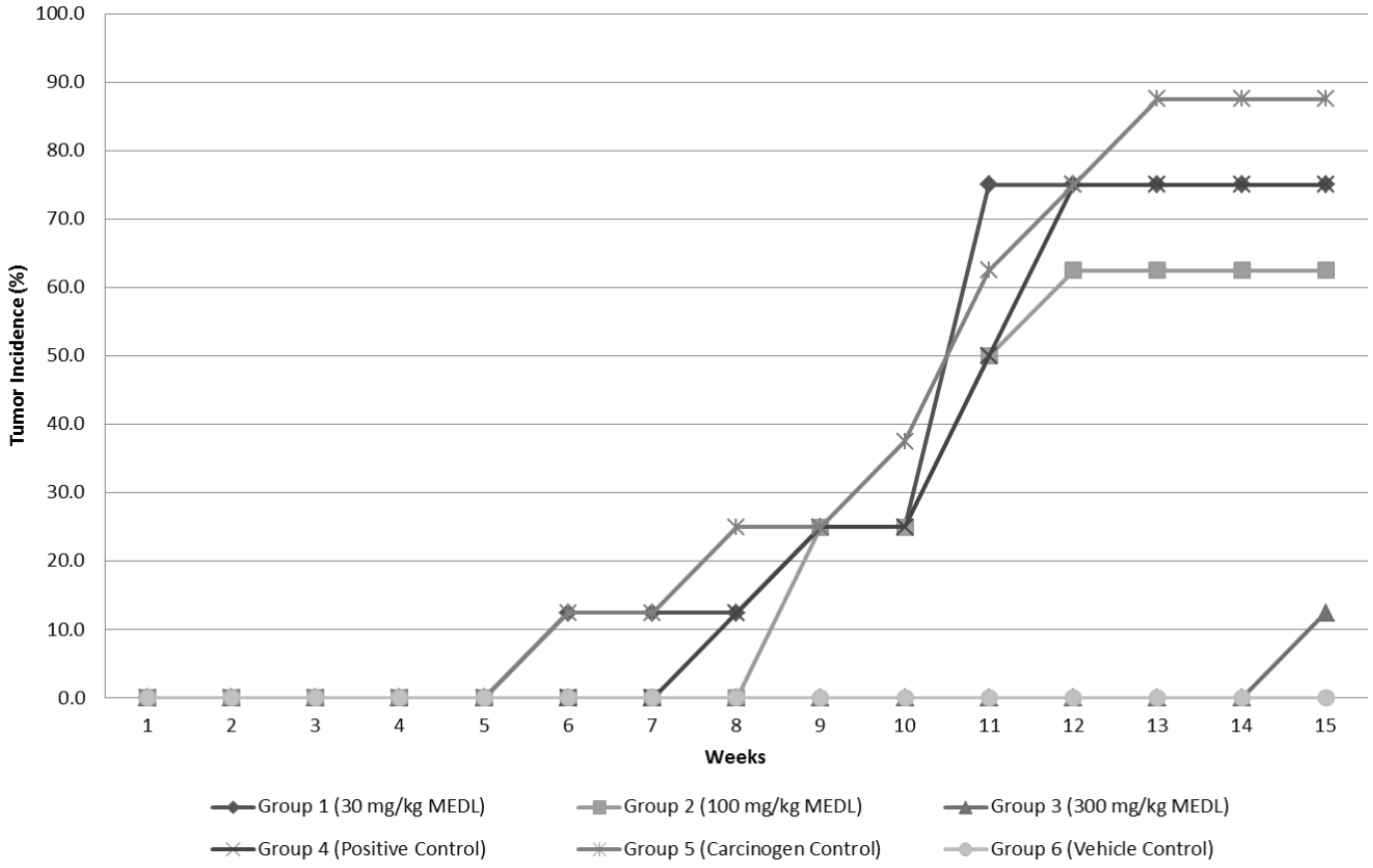


Figure 4. Effect of methanol extract *of Dicranopteris linearis*(MEDL)leaf on percentage of tumor incidence in mice. At the end of the study, highest tumor incidence was reported in carcinogen control group (87.5%), followed by positive control and Group 1 (75.0%), and then Group 2 (62.5%) whereby the lowest percentage of tumor incidence has been seen and in Group 3 (12.5%). Vehicle control showed no tumor formation at all throughout the experiment.

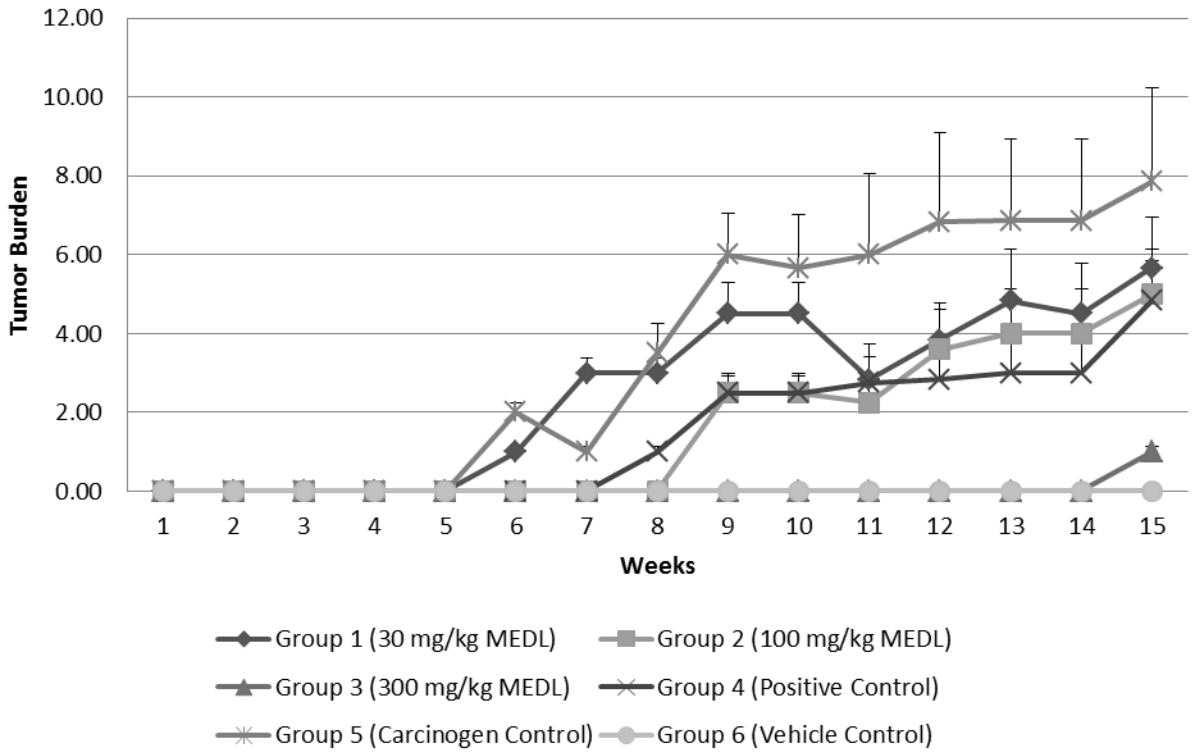


Figure 5. Effect of methanol extract *of Dicranopteris linearis*(MEDL)leaf on tumor burden.

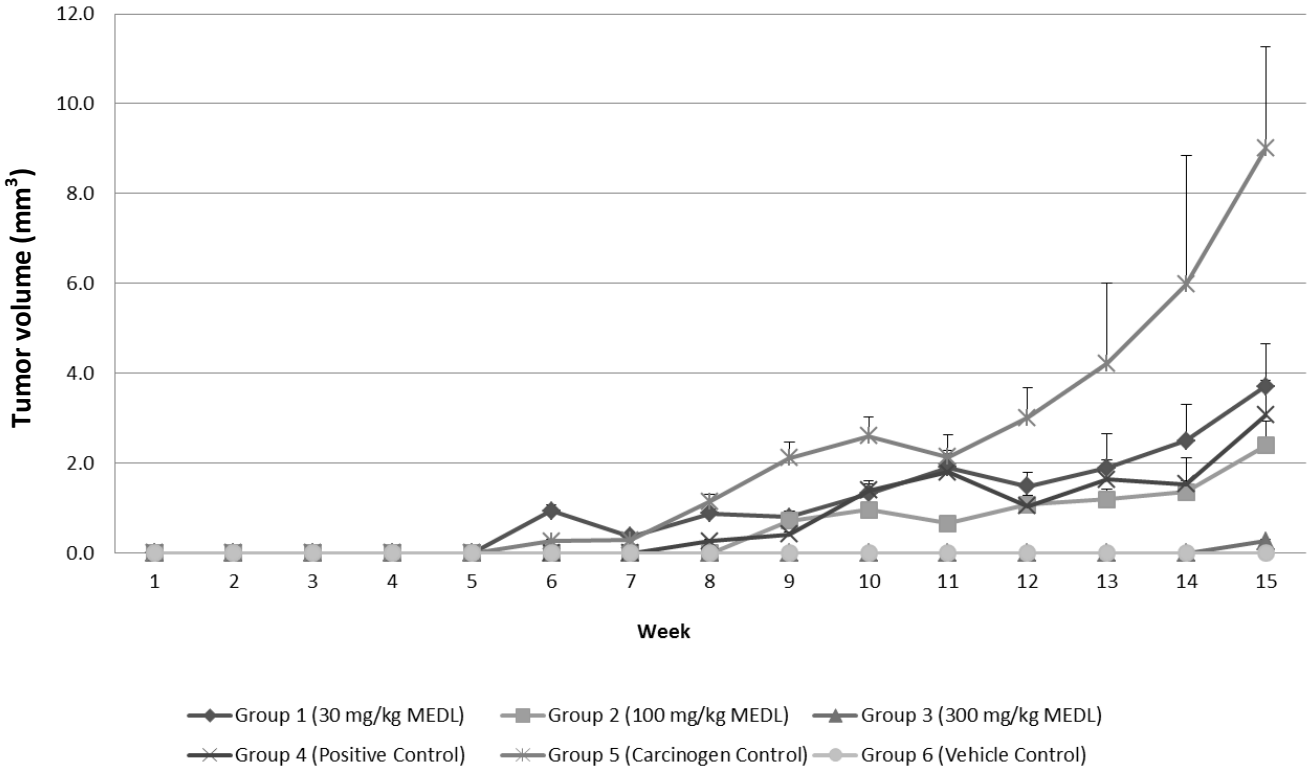


Figure 6. Effect of methanol extract *of Dicranopteris linearis*(MEDL) leaf on tumor volume in mice.

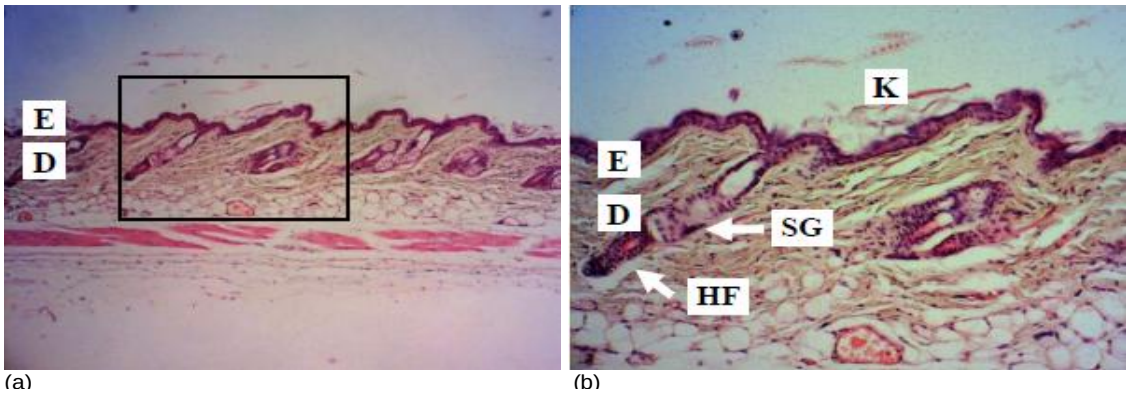


Figure 7. Representative microphotograph taken from H&E stained mouse skin of Group 6, vehicle control (acetone-treated group). (i) Normal skin structure can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows approximately a single layer cell thick of epidermis (E) overlaid by keratin (K). Hair follicles (HF) and sebaceous glands (SG) can be observed in dermis (D) (magnification 100×).

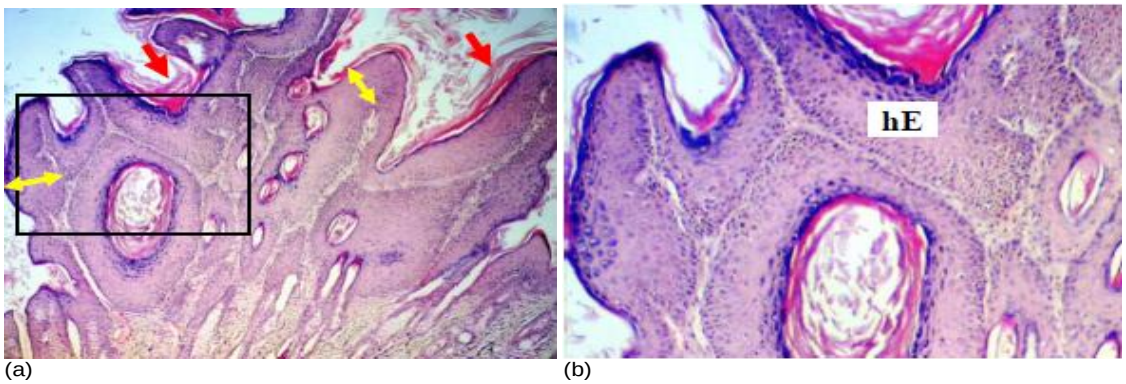


Figure 8. Representative microphotograph taken from H&E stained mouse skin of Group 5, carcinogen control (DMBA/croton oil-treated group). (i) Severe papillomatosis with severe hyperkeratosis (red arrow) and severe acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact (magnification 100×).

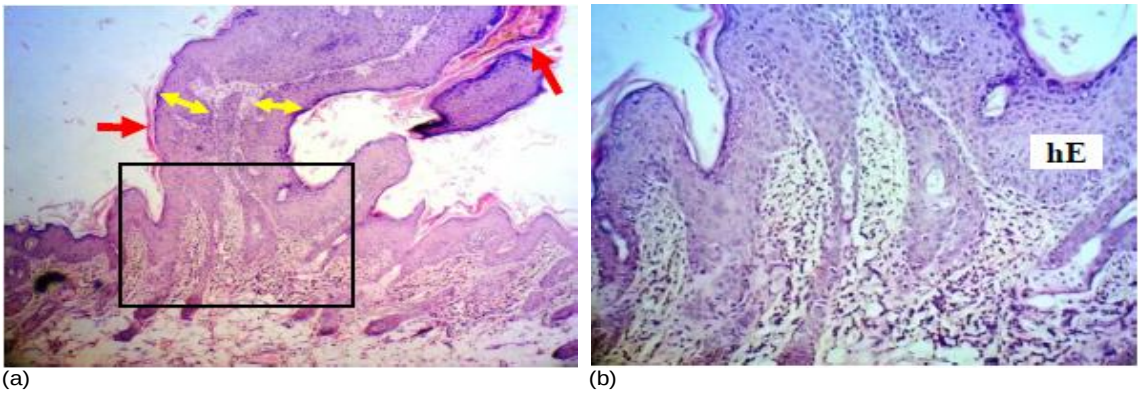
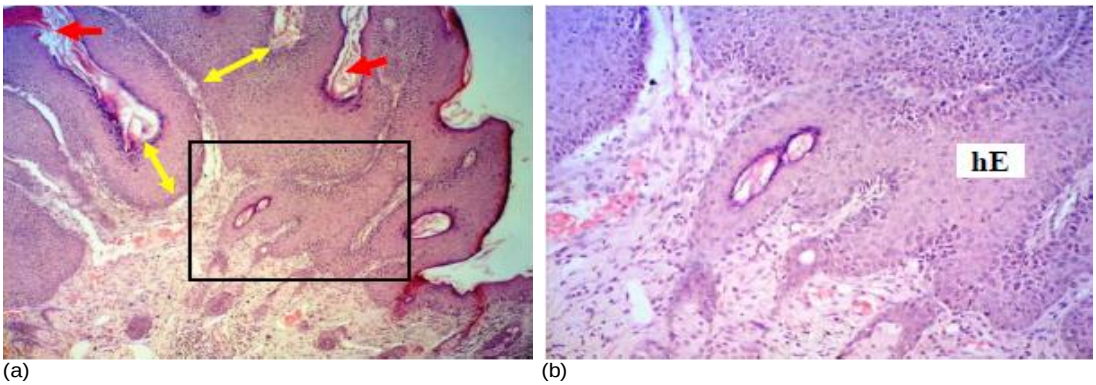
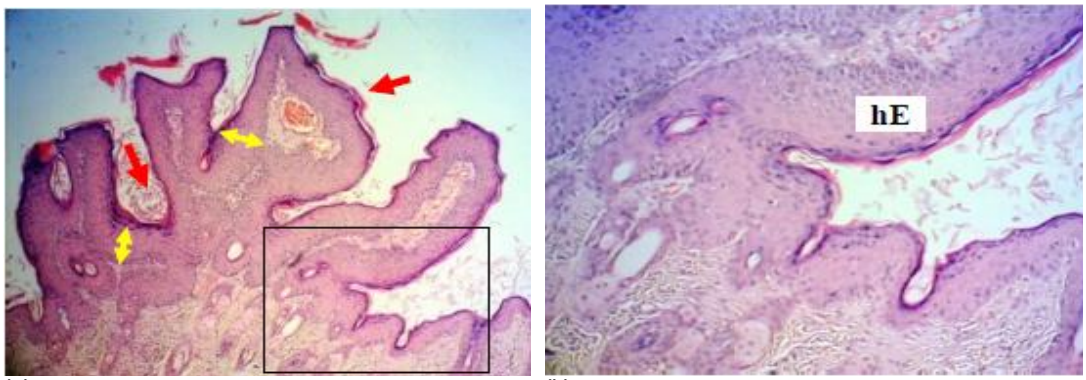


Figure 9. Representative microphotograpgh from positive control, Group 4 (curcumin treatment at 10 mg/kg body weight). (i) Mild papillomatosis with mild hyperkeratosis (red arrow) and mild acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact (magnification 100×).



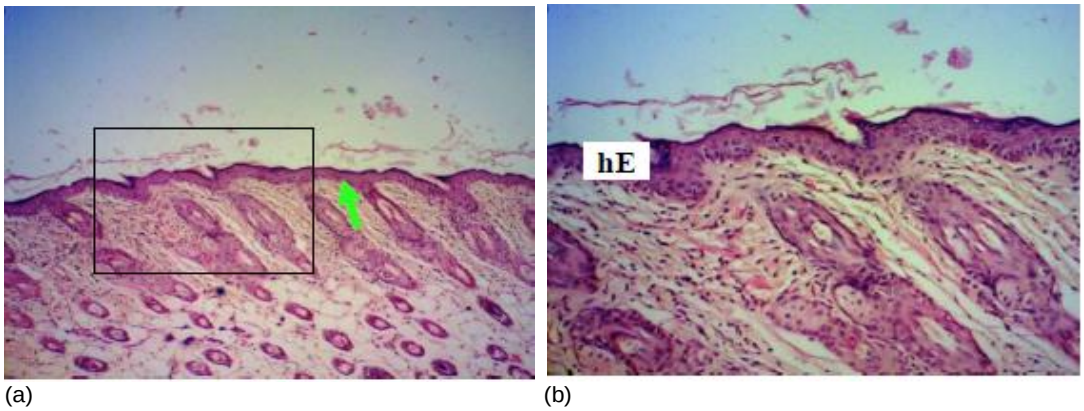
(a) (b)

Figure 10. Representative microphotograph taken from H&E stained mouse skin of Group 1 (MEDL treatment at 30 mg/kg body weight). (i) Moderate papillomatosis with moderate hyperkeratosis (red arrow) and moderate acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact. (magnification 100×).



(a) (b)

Figure 11. Representative microphotograph taken from H&E stained mouse skin of Group 2 (MEDL treatment at 100 mg/kg body weight). (i) Mild papillomatosis with mild hyperkeratosis (red arrow) and mild acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact (magnification 100×).



(a) (b)

Figure 12. Representative microphotograph taken from H&E stained mouse skin of Group 3 (MEDL treatment at 300 mg/kg body weight). (i) Mild hyperplasia of epidermis (green arrow) can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows thickening of epidermis. The hyperplastic epidermis (hE) was well-defined and intact with the absence of characteris of invasion (magnification 100×).

malignancies evolved from tissues that are severely damaged by chronic inflammation (O'Byrne and Dalgleish, 2001; Itzkowitz and Yio, 2004; Kundu and Surh, 2008). Various pro-inflammatory mediators (cytokines, cyclooxygenase 2, prostaglandins, nitric oxide and et cetera) can contribute to carcinogenesis through the mechanisms of:

(1) induction of chromosomal instability, (2) alterations in epigenetic events and error in the subsequent gene expression, (3) enhancement of cell proliferation, (4) evasion from apoptosis, (5) stimulation of angiogenesis, (6) invasion through basement membrane, and (7) promoting the metastatic movement (Perwez Hussain and Harris, 2007; Kundu and Surh, 2008; Colotta et al., 2009; Porta et al., 2009). Previous studies done by Zakaria et al. (2006, 2008) showed that *D. linearis* possess positive anti-inflammatory effect, thus suggesting the possibility that *D. linearis*-based extracts might contribute to the action of chemoprevention, by interrupting any of the inflammation mechanisms that lead to tumor formation.

The role of ROS-mediated oxidative stress in pathogenesis of cancer has been well documented (Ray and Husain, 2002). Oxidative stress created by free radicals often causes DNA damage such as DNA bases mutation, single- and double-strand breaks, DNA cross-linking and chromosomal breakage and rearrangement (Ames et al., 1993). Previous experiments have shown that a variety of plants exert chemoprotective effect by interrupting the tumor promotion stage (Javed et al., 1998; Zhao et al., 1999), the stage where the role of ROS is mostly emphasized (Huang et al., 1997). It is therefore implied that agents that can reduce the generation of free radicals *in vivo* including *D. linearis* may be presumed as a chemopreventive agent (Huang et al., 1992).

Previous phytochemical screening done on *D. linearis* showed the presence of flavonoids (Zakaria et al., 2006, 2008). The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify carcinogens, and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters (Canivenc-Lavier et al., 1996; Shih et al., 2000; Steenkamp et al., 2013). Therefore, it is possible that these flavonoids are responsible for the *D. linearis* anticarcinogenesis.

In the present study, the chemopreventive potential of *D. linearis* was evaluated *in vivo* by monitoring the latency period of tumor formation, percentage of tumor incidence, tumor burden, tumor volume as well as microscopic examination by observing histological changes on the dorsal skin of mice. The body weight of mice was also monitored weekly to obtain information of growth condition of mice. At end of the study, the number of mice survived was maintained at eight, which is same as the initial number. The body weight of mice also showed a net increment. In addition, all the mice were free from any toxicity symptoms. Therefore, the mice were assumed to be tolerable to all the doses of methanol extract of MEDL used, even at the highest dose (300 mg/kg).

Statistical analysis indicated that there is significant ($p < 0.05$) positive effect in all the parameters measured when comparing the three doses (30, 100 and 300 mg/kg body weight) of MEDL-treated groups with the carcinogen control (DMBA/croton oil-treated group), particularly at the highest dose (300 mg/kg body weight). The results also showed that MEDL exhibits its activity in a dose-dependent manner in all the parameter measured. The latency period of tumor formation was increased from week 6 to week 9 and to week 15 in MEDL-treated groups at 30, 100 and 300 mg/kg body weight, respectively. In addition, the histopathological examinations showed that MEDL was able to suppress the skin tumor formation with less extent of histological changes observed in MEDL-treated group at 300 mg/kg body weight, as compared to 30 mg/kg body weight. Further histopathological examination suggested that all the skin tumors formed was begin tumors. This finding is parallel to the studies done by Abel et al. (2009) which suggested that papilloma was expected to be converted to squamous cell carcinoma after 20 weeks of tumor promotion. It has been reported that 52 weeks are needed to achieve the maximum number of malignancy conversion (Abel et al., 2009).

In this study also, positive control mice that have been treated with curcumin at 10 mg/kg body weight showed significant ($p < 0.05$) reduction in the tumor burden and tumor volume as compared to carcinogen control. However, the percentage of tumor incidence was not significantly ($p < 0.05$) different from the carcinogen control. This similar result was observed in a study conducted by Limtrakul et al. (1997). They reported the difference in the number of animals developing tumors was not statistically significant between 1% curcumin diet-fed group versus control diet-fed group, though the number and size of tumors developed in the curcumin-treated group were significantly lower (Limtrakul et al., 1997). They agreed with the previous study which claimed that 2% turmeric diet significantly suppressed DMBA-induced skin tumors in mice (Azuine and Bhide, 1992). Thus, one of the determining factors of high incidence of tumor formation in positive control might be due to the low concentration of curcumin used in the study.

Flavonoids which also act as antioxidants have the potential of inhibiting carcinogenesis. Apart from inhibiting angiogenesis, there are also some flavonoids stated as to be potent inhibitors of cell proliferation such as fisetin, apigenin, and luteolin (Nijveldt et al., 2001). Flavonoids play a role in cancer prevention. Identified mechanisms of action includes carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation,

inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms. It is demonstrated that flavanoids are able to inhibit carcinogenesis *in vitro* and substantial evidence indicates that they can also do so *in vivo*. This carcinogenesis inhibition may occur by affecting the molecular events in the initiation, promotion, and progression stages. Flavonoids could inhibit tumor initiation as well as tumor progression through animal studies and investigations using different cellular models (Ren et al., 2003).

Tannins which are in the category of polyphenolics have been found to reduce the carcinogenic activity of a number of carcinogens. These anticarcinogenic and antimutagenic potential has been related to their antioxidative property that is known important in protecting cellular oxidative damage including lipid peroxidation (Bhattacharya, 2012). Phenolic compounds can be found in plants consumed by humans (Selim et al., 2013). There is still lack of evidence in proving that dietary phenols are anticarcinogens but it is believed to do so due to their antioxidative properties. Inhibition of tumorigenesis by plant polyphenols has been described in many publications. Polyphenols that are effective when given during the post-initiation period, that is by inhibiting tumor promotion and progression, are believed to be more useful in preventing cancer in humans than are polyphenols, which are effective only when given before and during the carcinogen treatment. Although extensive studies on the inhibition of carcinogenesis by dietary polyphenols had been done, the molecular mechanisms of action and their applicability to human cancer prevention are unclear. Recently, *in vitro* studies have been published on the modulation of oncogenes, tumor suppressor genes, cell cycle, apoptosis, angiogenesis, and related signal transduction pathways by polyphenols (Yang et al., 2001). Tea polyphenols has the ability to inhibit the level of activated carcinogens, oxidative stress-induced cellular damage, carcinogen-DNA adduct formation, and possibly the initiation of carcinogenesis, the inhibition of post-initiation events. Numerous mechanisms for the action of tea polyphenols include inhibition of MAP kinases and the PI3K/AKT pathway, inhibition of NFκB- and AP-1-mediated transcription, inhibition of growth factor-mediated signalling, inhibition of aberrant arachidonic acid metabolism and other activities (Yang et al., 2009).

Saponins are in a diverse group of plant glycosides showing soap like frothing with water. High amount of saponins present in MEDL has contributed to anticarcinogenic activity exhibited by this study (Bhattacharya, 2012). They possess surface-active characteristics that are due to the amphiphilic nature of their chemical structure and proposed mechanisms of anticarcinogenic properties of saponins include direct cytotoxicity (Rao and Sung, 1995).

Phytochemical investigations of various triterpenes were found to possess several pharmacological properties, including anti-inflammatory, anti-tumor, antioxidative and anti-diabetes effects (Barkatullah et al., 2013). The triterpenes-rich fraction and several isolated triterpenes showed the inhibitory effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation and Epstein-Barr virus early antigen (EBV-EA) activation induced by TPA in mice. It was also reported that the major triterpenes, ursane types, such as corosolic acid (CA) and ursolic acid (UA), and oleanane types, such as maslinic acid (MA) and oleanolic acid (OA) were present. These major triterpenes showed anti-proliferative activities against gastric cancer cells (NCI-N87), colorectal cancer (HCT15), cervical cancer (HeLa), glioblastoma (U291, U373 and T98G) and colon cancer (HT29) cell lines (Uto et al., 2013).

Conclusion

The methanol extract of MEDL leaves exhibited some level of inhibition of tumor promotion in a dose-dependent manner in DMBA-initiated and croton oil-promoted mice skin carcinogenesis protocol, with the highest dose (300 mg/kg body weight) showing the greatest activity. This suggested that *D. linearis* is a potential chemopreventive agent.

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