ORIGINAL ARTICLE

Pre-Initiation Effect of Oleuropein towards Apoptotic and Oxidative Stress Levels on the Early Development of Two-Stage Skin Carcinogenesis

Dayang Noor Suzliana John[1], Tengku Hasmira Tengku Mamat[1], Omchit Surien[1], Izatus Shima Taib[1,] Siti Fathiah Masre[1]*

[1]Biomedical Science Programme, Centre of Health and Applied Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Aziz, 50300 Kuala Lumpur, Malaysia

Abstract:

Background: Oleuropein is a form of phenolic compound which can be majorly found in the olive leaves. Previous studies have shown several biological functions of oleuropein against different cancer cells. *Aim and Objectives:* This research was designed to study the pre-initiation effect of oleuropein on the early skin tumour development using a mouse model. *Material and Methods:* Female Institute of Cancer Research (ICR) mice (n=6 per group) were divided into 2 groups (group 1 as a carcinogen control and group 2 as a vehicle control) and 1 treatment group (group 3: 10 mg/kg of oleuropein). *Results:* After 10 weeks, Group 3 showed delay in epidermal hyperplasia formation and a significant reduction ($p<0.05$) in the epidermal thickness as compared to Group 1. Data were also displayed a significant increase ($p<0.05$) in the apoptotic rate in Group 3 as compared to Group 1 and 2. For biochemical assays, the level of Malondialdehyde (MDA) was significantly ($p<0.05$) decreased whilst the levels of Glutathione (GSH) and Superoxide Dismutase (SOD) were significantly ($p<0.05$) increased in Group 3 as compared to Group 1. *Conclusion:* Our results indicate that pre-initiation treatment of oleuropein may prevent skin tumour development through its antioxidant and apoptotic activities.

Keywords: Chemoprevention, Carcinogenesis, Oleuropein, Skin Cancer, Olive

Introduction:

Skin cancer is one of the most common forms of human cancer, in which increasing new skin cancer cases are diagnosed every year worldwide [1]. Skin cancer is mainly caused by various environmental carcinogens, inflammatory agents, UV radiations, and tumour promoters [2]. Conversion of benign stage to malignancy is considered as one of the most significant stages of carcinogenesis. Whether an initiated cell will form a benign tumour and undergo malignant conversion is unpredictable. Thus, preventing cancer at the early stage is most likely anticipated rather than at the late stage which in many cases leads to mortality. In accordance with this, the great discoveries have been made in the natural product-based treatment on several types of cancer. Oleuropein, the most common phenolic compound that can be found abundantly in olive leaves, seeds, pulp and skin of unripe olives have shown its miraculous effects on various diseases [3].

Studies in the past exhibited that oleuropein possesses a variety of pharmacological roles which includes being antioxidant [4], anti-inflammatory [5], antimicrobial [6] and anticancer [7]. A recent study showed the anti-tumour effects of orally administered oleuropein to induce tumour regression in mice bearing spontaneous tumours [8]. However, the effect of oleuropein on the pre-initiated stage by using a 7, 12-Dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate

(DMBA/TPA)-induced skin carcinogenesis in mouse model is still undetermined.

The two-stage skin carcinogenesis model by DMBA/TPA is an excellent in vivo model that can well annotate stages of carcinogenesis including initiation and promotion [9, 10]. It is transpired that the development of initiated pre-neoplastic cells can be stimulated by the presence of oxidative stress [11] leading to a decrease in apoptosis or an increase in proliferation [12]. Oxidative stress happens when there is an overproduction of reactive oxygen species rather than the antioxidant. The present study was conducted to investigate whether treatment of oleuropein before the initiation stage could inhibit the two-stage skin carcinogenesis model. Moreover, this study has also looked on the effect of oleuropein to apoptotic rate and oxidative stress levels.

Material and Methods:
Chemicals and Reagents

DMBA, TPA and oleuropein were purchased from Sigma-Aldrich, America. All the other reagents used were of highest purity and were commercially available.

Animals

Total of 18 female Institute of Cancer Research (ICR) mice around seven to eight-week old (25-30g) were obtained from the Animal Resource Unit, Faculty of Health Sciences, National University of Malaysia (UKM). All animals were maintained in polypropylene cages at room temperature with free access to standard mouse pellet diet and fresh tap water *ad libitum*. All animal procedures in this study were approved by the Animal Ethics Committee of UKM with the approval number FSK/2016/FATHIAH/28-SEPT./795-SEPT.-2016-APR.-2018.

Two-stage mouse skin carcinogenesis model

Two days before commencement of the experiment, each mouse was dorsally shaved with an electric hair clipper. Mice were randomly divided into three groups (n: 6 mice per group): Group 1 as a carcinogen control received DMBA (200 nmol in 100µl acetone) and TPA (20 nmol in 100µl acetone) (13); Group 2 as a vehicle control received acetone alone (100µl); and Group 3 as a treatment group received topical application of oleuropein, 10 mg/kg body weight (14) before DMBA and TPA. Mice in Group 3 were pre-treated with oleuropein daily for 2 weeks before continued with DMBA-initiated agent and followed by TPA-promoted agent twice a week for 10 weeks. Body weight of animals were recorded every week.

Histopathological analysis

The skin tissues from the treated sites were biopsied and part of the tissues was stored in 10% neutral buffered formalin for histology processing. The tissues were embedded in paraffin wax and were cut to 5 µm sections using a microtome before stained with Haematoxylin and Eosin (H&E). The final sections were examined under light microscope for histopathological changes. The epidermal thickness was measured using the Image J software under the magnification of 40x.

Analysis of apoptotic activity

Apoptotic activity was analysed by Immuno-histochemistry (IHC) analysis on caspase-3protein expression. The 5 µm section was deparaffinized in xylene and dehydrated in absolute alcohol. The deparaffinized section was boiled for 2-4 minutes in commercial citrate buffer for antigen retrieval. Then, each section was treated with 3% H_2O_2 to suppress the endogenous

peroxidase activity. Primary antibody of an activated caspase-3 (Cell Signalling Technology) was incubated to the section for overnight at 4°C. On the next day, the sections were washed with Phosphate Buffered Saline (PBS) before and were incubated with secondary horseradish peroxidase-conjugated antibody at room temperature for 1 hour. The brown colour was developed on the section with DAB-peroxidase reaction. The level of apoptotic activity was expressed as the percentage (%) of total nuclei that was positively labelled for active caspase-3 [15].

Analysis of biochemical parameters

The skin tissue was homogenized using a glass-Teflon homogenizer in PBS (0.1M, pH 7.4). The homogenized tissue was centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was aliquoted for estimation of Malondialdehyde (MDA), Reduced Glutathione (GSH) and Superoxide Dismutase (SOD). The protein content was assessed using the Lowry method. The MDA level was measured to indicate oxidative stress activity by lipid peroxidation. The principle of the method of MDA measurement was based on MDA reaction with Thiobarbituric Acid (TBA) that yields TBA reactive substances (TBARS) in pink chromogen colour. The pink chromogen colour was then measured using spectrophotometer at 532 nm wavelength to determine the MDA concentration [16]. GSH level was measured based on the principle of GSH reaction with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) [17]. Colour production was measured using spectrophotometer at 412 nm wavelength to determine the GSH concentration. In principle, the SOD level was determined based on superoxide reaction with SOD that causes Nitro Blue Tetrazolium (NBT) reduction in purple colour [18]. The coloured product was then measured using spectrophotometer at 560 nm wavelength to determine the SOD concentration as unit of enzyme/mg protein.

Statistics

SPSS software version 22 was used for statistical analysis. The level of significance between different groups was analyzed using one-way Analysis of Variance (ANOVA). Data obtained were presented as Mean and Standard Error of Mean (Mean ± SEM), and values of $p < 0.05$ were considered statistically significant.

Results:

Histopathological analysis

Fig. 1 shows the histopathological changes in the early development of two-stage mouse skin carcinogenesis model on week 10. Data indicate that oleuropein pre-treated group showed a reduction in hyperplasia development on the mouse model (Fig. 1a) whilst carcinogen control group displayed thick hyperplasia with disorganised epithelial layers on the same week (Fig. 1b). On the other hand, vehicle control group remained normal with a single layer of epidermal cells (Fig. 1c). Results from the histopathological evaluation were supported by the measurement of epidermal thickness (µm). Pre-treatment with oleuropein has resulted in a significant reduction ($p < 0.05$) in the thickness of epidermal layers (35.59±0.15 µm) as compared to the carcinogen control group (146.35±12.5 µm) (Fig. 2).
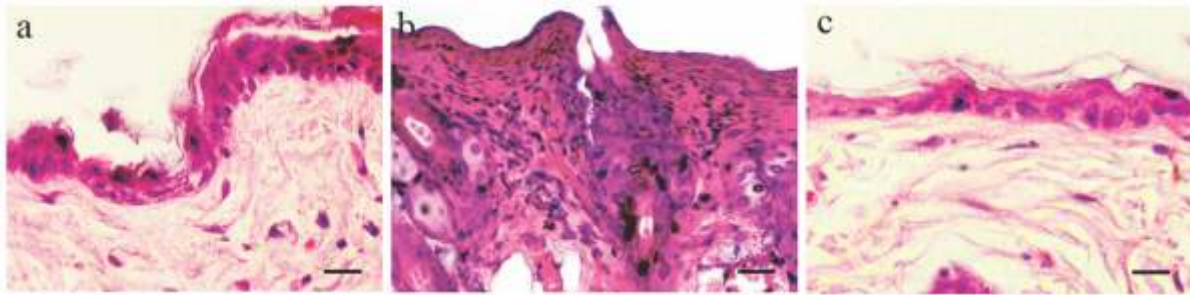
Fig. 1: Histological Analysis of Two-Stage Skin Carcinogenesis Mouse Model

(a): Oleuropein pre-treated group showed delay in hyperplasia formation. This reflects the ability of pre-treated oleuropein to interrupt initiated cells from proliferation during the DMBA/TPA-induced carcinogenic period).

(b): DMBA/TPA group display thick hyperplasia appearance together with disarrangement of epidermal layers which indicates an increase in proliferation and high number of cells.

(c): Vehicle control group remained normal with a single layer of epidermal cells. (Magnification 40x)
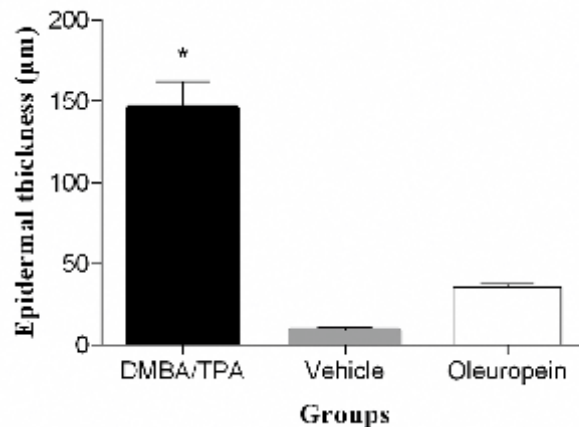
Fig. 2: The Measurement of Epidermal Thickness (μm) in the DMBA/TPA, Vehicle Control and Oleuropein Pre-Treated Groups

*Significant increase in the epidermal thickness in the carcinogen control group as compared to vehicle control and oleuropein groups. (Data were presented in Mean ± SEM).

Apoptotic activity

Apoptotic activity was evaluated by IHC staining that targeted the` apoptotic cells through the expression of the active caspase-3 protein. The percentage of apoptotic activity by activated caspase-3 labelling was assessed in each group (Fig. 3 and Table 1). The oleuropein pre-treated group showed a significantly ($p<0.05$) higher apoptotic rate ($16.39 \pm 2.21\%$) as compared to the DMBA/TPA group ($2.56 \pm 0.70\%$) and vehicle control group ($1.33 \pm 0.91\%$).
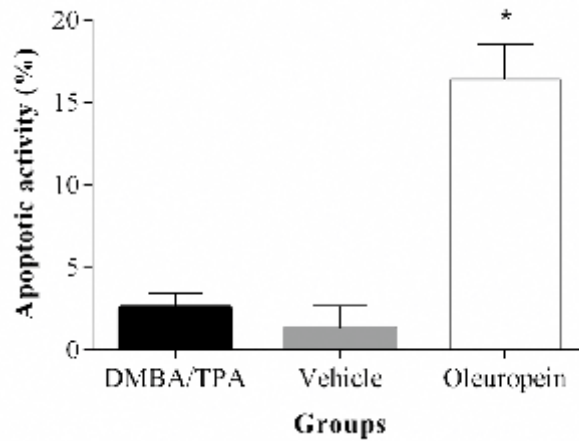
Fig. 3: **Percentage of Apoptotic Activity by Activated Caspase-3 Labelling in DMBA/TPA, Vehicle Control and Oleuropein Pre-Treated Groups**

[*]Significant increase in apoptotic rates in the oleuropein pre-treated group as compared to the DMBA/TPA and vehicle control groups (p<0.05). (Data were presented in mean ± SEM).

Table 1: Apoptotic Activity (%) in All Animal Groups

Groups	DMBA/TPA	Vehicle	Oleuropein
Apoptotic activity (%)	2.56±0.70	1.33±0.91	*16.39±2.21

Oleuropein group showed a significant increase of apoptosis as compared to the DMBA/TPA group

**Evaluation of biochemical parameters**

The effects of oleuropein on lipid peroxides MDA level in two-stage mouse skin carcinogenesis was demonstrated in Table 2 and Fig. 4. DA level was significantly increased in DMBA/TPA group in comparison to the oleuropein pre-treated (p<0.05) and control groups (p<0.05). Whilst there was no significant difference in the MDA levels between oleuropein pre-treated and control groups.

MDA level in oleuropein group showed a significant decrease while both GSH and SOD levels in oleuropein group showed a significant increase as compared to the DMBA/TPA group.

Table 2: Evaluation of Biochemical Parameters in All Animal Groups

Groups	DMBA/TPA	Vehicle	Oleuropein
MDA level (nmol/mg)	20.40±1.63	8.36±0.73	*12.84±1.72
GSH level (nmol/mg)	0.027±0.01	0.039±0.01	*0.037±0.02
SOD level (U/mg)	7.10±0.01	11.74±0.01	*11.42±0.05
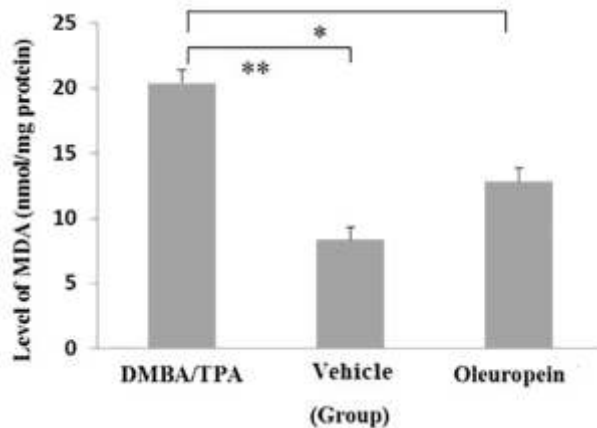
Fig. 4: Effects of Oleuropein on MDA Level in Two-Stage Skin Carcinogenesis in Mouse Model.

*,**Significant increase of the MDA level in the DMBA/TPA group as compared to the oleuropein pre-treated and vehicle control groups at $p<0.05$. Significant decrease in MDA levels in pre-treated group indicates a reduction of oxidative stress, thus showing a protective potential of oleuropein against skin carcinogenesis.
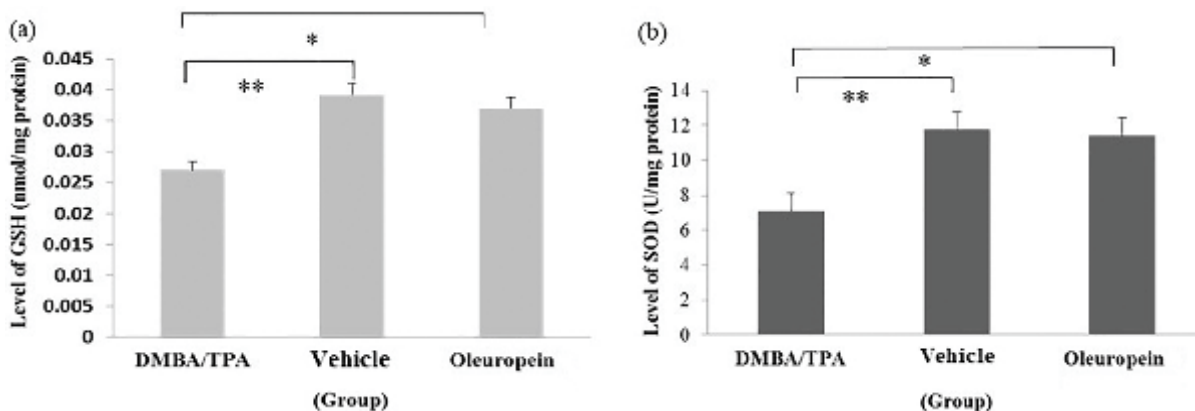
Fig. 5: Effects of Oleuropein on (a) Non-Enzymatic Antioxidant Glutathione (GSH) and (b) Enzymatic Antioxidant Superoxide Dismutase (SOD).

*,**Oleuropein pre-treated group and vehicle control group showed a significant increase ($p<0.05$) in the level of GSH and SOD in comparison to the DMBA/TPA group. This reflects the potential of oleuropein to halt the process of oxidative stress-induced carcinogenesis.

Fig, 5 and Table 2 showed the effects of oleuropein on GSH and SOD levels in skin carcinogenesis. Both GSH and SOD levels were significantly increased in the oleuropein pre-treated group compared with the DMBA/TPA group ($p<0.05$). Data also displayed significant differences between DMBA/TPA and control groups ($p<0.05$) on the GSH and SOD levels in the skin carcinogenesis model.

Discussion:

The usage of natural compounds as a chemo-preventive agent has gained much attention in the research of various types of cancers. In this study, we reported the pre-treatment effects of oleuropein in the early development of skin carcinogenesis. As the two-stage skin carcinogenesis in mouse model represented the best sequential development of tumours from initiation stage to promotion stage [19]. Thus, hyperplasia is expected within 10 weeks of DMBA/TPA application before development of papilloma within 10 to 20 weeks and progression to carcinoma after 20 weeks [20].

Pre-treatment of oleuropein before DMBA/TPA application clearly showed delay in hyperplasia formation at 10th week in this study. The histological observation of skin sections obtained from experimental mice at week 10th demonstrated a visible reduction in the thickness of epidermal layers in the oleuropein pre-treated group compared to the DMBA/TPA treated and control groups. Our findings supported previous researches that showed a topical application of oleuropein after acute and chronic UVB exposure could significantly inhibit increases in skin thickness and tumour growth [14, 21].

In this current study, we also examined the apoptotic activities in the early development of two-stage skin carcinogenesis model and our findings showed significant increase in apoptotic rates in the oleuropein pre-treated group. Several studies have demonstrated the ability of oleuropein to induce the cell death by stimulating the pro-apoptotic factor in colorectal cancer [22] and breast cancer cells [23]. Oleuropein also have consistently been reported to discriminate between cancer and normal cells, inhibiting proliferation and inducing apoptosis in cancer cells [24]. Thus, current results in this study showed that the delay in hyperplasia formation at week 10th of DMBA/TPA induction may be due to apoptotic effects by oleuropein.

The protective effects of oleuropein on oxidative stress were demonstrated through the evaluation of the formation of lipid peroxidation by-product, MDA. In this study, the level of MDA as a marker for oxidative stress was measured and our data showed significant reduction of MDA levels in the oleuropein pre-treated group as compared to the control and DMBA/TPA treated groups. The level of MDA was significantly elevated in the DMBA/TPA treated group and this indicated that the oxidative stress condition during skin carcinogenesis event induced lipid peroxidation, leading to massive production of MDA. Previous research has shown the inhibitory effect of oleuropein on oxidative stress with a significant decrease in MDA level in alloxan-induced diabetic rabbits [25].

Furthermore, there was a significant increase in the level of GSH in the oleuropein pre-treated group as compared to the DMBA/TPA group. This finding was apparent as seen in the pre-treatment with oleuropein group that restored the level of GSH though has been induced by DMBA/TPA application. GSH played an important role in defense mechanisms to protect cells against Reactive Oxygen Species (ROS) and it also acted as a protective antioxidant in scavenging free radicals in response to oxidative damage [26].

Moreover, measurement of the SOD enzyme activity levels in this study showed significant increase in the group pre-treated with oleuropein

compared with DMBA/TPA treated group. It is essential to measure the enzymatic antioxidant such as SOD due to its role as the first line of defence against the oxygen free radicals that damage the cells by catalyzing superoxide radicals to hydrogen peroxide and [27]. Our current findings in SOD activity is in line with previous research [28] that has shown the ability of oleuropein to increase the activity of SOD in ethanol-induced gastric damages in rats. Thus, increase in the GSH level and SOD activity in this study reflects the ability of oleuropein to halt the process of oxidative stress-induced carcinogenesis.

Conclusion:

From this study, it can be concluded that early development of skin carcinogenesis was delayed by pre-treatment of oleuropein. Therefore, it is suggested that oleuropein may act as a chemo-

preventive agent through its antioxidant and apoptotic activities on the pre-cancerous cells. However, the limitation can be due to the duration of skin carcinogenesis model which was done until week 10 in this study. Thus, there is still no papilloma or carcinoma formation for further histo-pathological analysis. Hence, additional studies by extending the treatment period and looking on the effect of oleuropein in the promotion stage will be carried out to solidify the potential of oleuropein as a chemopreventive agent.

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Author for Correspondence: Siti Fathiah Masre, Biomedical Science Programme, Centre of Health and Applied Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Aziz, 50300 Kuala Lumpur, Malaysia Email: sitifathiah@ukm.edu.my