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Oxyresveratrol from *Artocarpus lakoocha* Roxb. Inhibit Melanogenesis in B16 Melanoma Cells through the Role of Cellular Oxidants

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Abstract

Overproduction of melanin pigment is well recognized in hyperpigmentation disorders and melanoma progression. In this study, we investigated the anti-oxidant activity of oxyresveratrol from *Artocarpus lakoocha* Roxb. The effects of oxyresveratrol on melanogenesis, tyrosinase activity, and cellular oxidants in B16 cells were also obtained. Toxic doses of oxyresveratrol were confirmed by using an *in vitro* micronucleus method in V79 cells. Oxyresveratrol had a potent anti-oxidant activity. It also showed a statistical significance in the inhibition of melanogenesis, tyrosinase activity, and cellular oxidants in B16 cells at 10 and 12.5 μg/ml. These effective doses had no toxicity in V79 cells. Oxyresveratrol had a potent inhibition of melanogenesis in B16 cells through the role of cellular oxidants relating to tyrosinase activity. Effective doses of this agent may be used to control hyperpigmentation disorders and to be applied in chemopreventive drugs for melanoma.

Keywords: Oxyresveratrol, melanin, tyrosinase, oxidative stress, melanoma cell

Introduction

Melanin is a biological pigment of skin and hair in mammals, and has an important role in skin protection from ultraviolet (UV) radiation [1]. The tyrosinase enzyme is a rate limiting step enzyme in melanin biosynthesis [2]. Abnormal hyperpigmentation, such as age spots, melasma, and actinic damage are well recognized as being associated with oxidative stress [3]. High levels of free radicals can induce melanogenesis through the ERK/JNK pathway in melanocytes [4]. In addition, oxidative stress is associated with abnormal proliferation, apoptotic resistance, and cancer metastasis in melanoma cells [5,6]. Treatment of melanoma cells with resveratrol showed a potential to balance the redox status in melanoma therapy [7]. Therefore, the inhibitory effect on cellular oxidants may be useful in inhibiting melanogenesis and melanoma cell progression.

Some phytopolyphenols, especially in family of stilbenes, have shown anti-oxidant activity [8,9]. An increase in the OH group of 4-substituted resorcinol has been mostly related to tyrosinase inhibition [10]. Oxyresveratrol, trans-tetrahydroxy stilbene containing 4-substituted resorcinol, was found in the heartwood of *Artocarpus lakoocha*, known in Thai as 'Ma-Haad' [11,12]. This agent also had the benefit of free radical scavenging activity [13]. The focus of this work is to determine the effect of oxyresveratrol from *Artocarpus lakoocha* Roxb. on melanogenesis through the biological roles of tyrosinase activity and anti-oxidant property in B16 melanoma cells. Cellular genotoxicity of the effective dose for melanogenesis inhibition was also investigated.

Materials and methods

Preparation of oxyresveratrol

Dried extract of oxyresveratrol from *Artocarpus lakoocha* Roxb. was kindly provided by Dr. Nasapon Povichit, Detox (Thailand) Co., Ltd. The heartwood of *A. lakoocha* was dried at 50 °C and then ground into powder and macerated in 95 % ethanol for 6 h in dark conditions. The crude extract was centrifuged at 1,500 rpm for 15 min. The dried extract was obtained by drying the supernatant in a rotary evaporator at 50 °C and 50 mm/Hg. Phytooxyresveratrol was isolated by high performance liquid chromatography (HPLC) which was separated by an Alltech Altima C18 column (250×4.6 mm) and a C18 guard column (Phenomenex 40×3.0 mm). The separation was carried out using water as mobile phase A and 1 % acetic acid in 35 % MeOH as mobile phase B, with 1.0 ml/min of the flow rate. The elution profile was: 10 - 55 % B (50 min), 55 - 100 % B (10 min), and 100 - 10 % B (5 min). Its structure was confirmed with the ^{1}H and ^{13}C–NMR spectroscopic method. The sample was dissolved by 40 % ethanol and filtrated with 0.45 µm polytetrafluoroethylene (PTFE) filter nylon. The sample was diluted with complete DMEM medium as 1 % ethanol contained in the final concentration before being used in the experiment.

DPPH radical scavenging activity

A working solution of DPPH was freshly prepared by dissolving and diluting it with 95 % ethanol to obtain an absorbance of 0.85±0.05 units at 540 nm. 0.2 ml of the extracted sample was mixed with 1.8 ml of the DPPH working solution. The mixture was immediately measured at 540 nm against a blank by a spectrophotometer (UV-2650, Labomed, USA). The radical scavenging activity of each sample was measured as a decrease in the absorbance of the DPPH solution. Vitamin C was used as standard. The total anti-oxidant activity in the DPPH test was expressed as mg VitC equivalent/g sample. The assay was carried out in triplicate.

ABTS radical decolorization assay

An ABTS working solution was prepared by mixing 7 mM of an ABTS solution and 2.4 mM of a potassium persulfate solution (2:3 v/v) and allowing them to react for 12 h in dark conditions. Diluting with 95 % of ethanol obtained an absorbance of 0.85±0.05 at 734 nm by using the spectrophotometer (UV-2650, Labomed, USA). 0.2 ml of the sample was reacted with 1.8 ml of the ABTS working solution. After 30 min, the absorbance was measured at 734 nm by spectrophotometer. Trolox was used as a standard. Total scavenging activity in ABTS test was expressed as mg Trolox equivalent/g sample. The assay was carried out in triplicate.

Ferric reducing anti-oxidant power (FRAP) assay

The working FRAP reagent was prepared by mixing 25 ml of 0.1 M acetate buffer (pH 3.6), 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 2.5 ml ferric chloride hexahydrate ($FeCl_3 6H_2O$). In dark conditions, 20 µl of extracted sample was allowed to react with 180 µl of FRAP reagent in 96 well plates for 15 min. A reading of ferrous tripyridyltriazine complex product at 595 nm was measured by using an automated microplate reader (1420 Victor 2, Wallac, USA). In the result, iron (II) sulfate heptahydrate ($FeCl_3 6H_2O$) was used as a standard. The analysis was performed in triplicate.

Total phenolic content

Twenty µl of the extracted sample was mixed with 1580 µl of distilled water and then mixed with 100 µl of Folin-Denis reagent. The mixture was then incubated with 300 µl of 7.5 % w/v of sodium bicarbonate for 30 min at room temperature in dark conditions and its absorbance was measured at 765 nm by using the spectrophotometer (UV-2650, Labomed, USA). Gallic acid was used as a standard. Results were expressed as mg Gallic acid equivalent/g sample. The analysis was performed in triplicate.

B16 melanoma cells

B16 cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 3.7 mg/ml of $NaHCO_3$ at 37 °C and 5 % CO_2.

Tetrazolium dye methylthiotetrazole (MTT) assay

B16 Cells viability was determined by MTT assay [14]. Briefly, 1.5×10^4 B16 cells were seeded in the 96-well microculture plates in the presence of different concentrations of oxyresveratrol extract at a final volume of 100 µl for 24 h at 37 °C and maintained for 24 h. MTT at 0.5 mg/ml was added to each well. After 2 h of additional incubation, 100 µl of 0.04 N HCl in isopropanol was added to dissolve the crystals. B16 cells viability were determined by an automatic microplate reader (1420 Victor 2, Wallac, USA) at a wavelength 570 nm. DMSO was used as a blank. The result was expressed as the percentage of viable cells (% cell viability).

Melanin content

Melanin content in B16 cells was determined by using a modified method [15]. In brief, 1.5×10^5 cells/ml B16 cells were seeded into 24-well plates and incubated in DMEM for 24 h, treated with various safety doses of sample at 0, 2.5, 5.0, 7.5, 10, and 12.5 µg/ml, and incubated for 4 days. To determine the melanin content of the cells, B16 cells were washed with PBS and then lysed with 100 µl of NaOH 1 N and incubated at 60 °C for 1 h. After centrifugation at 12,000 g for 5 min at 4 °C, the supernatant was measured by using a plate reader (1420 Victor 2, Wallac, USA) at 405 nm. Sodium L-Lactate (SLL) at 50 mM was used for positive melanin inhibition. Melanin content was expressed as a percentage of the control, 100 % for untreated cells.

Melanin pigments in B16 cells

The melanin pigment accumulation and cellular morphology of B16 cells were observed by a light microscope without staining and fixation [16]. In brief, 3.0×10^5 cells/ml of B16 cells were seeded into 6 well plates and incubated in DMEM for 24 h, treated with various safety doses of sample at 0, 2.5, 5.0, 7.5, 10, and 12.5 µg/ml, and incubated for 4 days. Cellular morphology and melanin containing cells were observed by a light microscope at 400× of magnification. Sodium L-Lactate (SLL) at 50 mM was used for positive melanin inhibition. Percent melanin containing cell frequency was expressed as a percentage of total cells counted in 1,000 cells.

Cellular tyrosinase activity

Tyrosinase activity was assayed as dihydroxyphenylalanine (DOPA) oxidase activity using the previous method [17]. B16 cells (1.5×10^5 cells/ml) were seeded into the 24 well plates and incubated for 24 h. Cells were treated with various safety doses of the sample and incubated for 72 h. The cells were washed twice with ice-cold phosphate buffer saline (PBS) and lysed with 100 µl PBS (pH 6.8) containing 0.1 % Triton X-100 and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) (pH 7.5). The mixture was frozen at –20 °C for 120 min, followed by incubation at 37 °C for 10 min. Then, the lysate was clarified by centrifugation at 12,000 g, 4 °C for 30 min. With the supernatant, 80 µl was transferred to 96 well plates and 20 µl of 20 mM L-DOPA was added. Tyrosinase activity was determined at 0, 30, 60, 90, and 120 min by using a microplate reader (1420 Victor 2, Wallac, USA), absorbance 492 nm. The linear plot of tyrosinase activity was calculated and expressed as a percentage of the control (100 % for untreated cells). Sodium L-Lactate (SLL) at 50 mM was used for positive control.

Cellular oxidants

Intracellular oxidants were detected with fluorescent 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) in the modified method [18]. Fifteen thousand cells/ml of B16 cells were seeded in 96 well plates and incubated at 37 °C, 5 % CO_2 for 24 h. Cells were treated with various safety doses of the sample for 1 h. Cultured cells were removed from the conditioned medium. Ten µM of DCFH-DA

medium was added and incubated at 37 °C, 5 % CO_2 for 1 h. DCF fluorescence intensity was immediately assessed for cellular oxidants at excitation/emission wavelengths of 485/535 nm by using a fluorescence microplate reader (1420 Victor 2, Wallac, USA). Five mM of N-acetyl cysteine (NAC) was used as a positive anti-oxidant with 100 % control for untreated cells.

***In vitro* micronucleus**

In vitro micronucleus assay was performed according to the Organization for Economic Co-operation and Development (OECD) guidelines [19]. Two hundred thousand cell/ml of Hamster lung fibroblast, V79 cells were treated with oxyresveratrol at 10 and 12.5 µg/ml in 10 % fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) for 3 h at 37 °C with 5 % CO_2, then washed and incubated with fresh DMEM for 18 h with the same condition. Three µg/ml of Cytochalasin B in DMEM was added and incubated for 3 h in 37 °C with 5 % CO_2. Mitomycin C (MMC) at 1.0 µg/ml was used as a positive control. Treated cells were collected and stained for binucleated cells. Micronuclei (Mn) per 1,000 cells were examined under light microscope.

Statistical analysis

All results were expressed as a mean ± standard deviation of three determinations. One way analysis of variance (ANOVA) was used to determine the differences of means among the samples. A Pearson correlation test was used to assess the correlation. A significance difference was considered at the level of $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results and discussion

Previously, an over production of melanogenesis has been known to be involved in an imbalance of redox state by an excess of free radicals, both of the reactive oxygen species (ROS) and reactive nitrogen specie (RNS) [20]. Their radicals have a crucial role in melanogenesis relating tyrosinase activity [21,17]. In this study, we demonstrated that oxyresveratrol scavenged free radicals in all *in vitro* tests with ABTS and DPPH methods. It showed reducing activity by using FRAP methods. The radical scavenging activity and reducing activity of oxyresveratrol gradually increased with a dose dependent manner. Anti-oxidant equivalences of ABTS and DPPH were 2.430±0.082 mg Trolox equivalent/g sample and 37.967±0.579 mg Vit C equivalent/g sample, respectively. Ferric reducing activity by FRAP method was 91.997±3.802 M Fe^{2+}/g sample, and total phenolic content of oxyresveratrol had Gallic acid equivalence at 0.294±0.028 mg Gallic acid equivalent/g sample (**Table 1**). Additionally, we found that total phenolic content of oxyresveratrol showed a strong relation to anti-oxidant activities and reducing activity in ABTS ($r = 0.938$, $p < 0.05$), DPPH ($r = 0.961$, $p < 0.05$), and FRAP ($r = 0.994$, $p < 0.001$) methods.

Table 1 Free radical scavenging and total phenolic content of oxyresveratrol.

Sample	Free Radical scavenging activities		Ferric reducing activity	Total phenolic content
	ABTS (mg Trolox equivalent/g sample)	DPPH (mg Vit C equivalent/g sample)	FRAP (M Fe^{2+}/g sample)	Folin-ciocalteu (mg Gallic acid equivalent/g sample)
Oxyresveratrol	2.430 ± 0.082	37.967 ± 0.579	91.997 ± 3.802	0.294 ± 0.028

No significance modification was found in B16 cells treated with oxyresveratrol at various concentrations (2.5, 5, 7.5, 10, and 12.5 µg/ml) (**Figure 1a**). Non-toxic doses of oxyresveratrol were designed to determine the effects on melanogenesis, tyrosinase activity, and cellular oxidants. Untreated

B16 melanoma cells were indicated and used as a control (100 % control) in all tests. In this study, we used DMEM containing 1 % of ethanol, which had no effect on the melanogenesis, tyrosinase activity and cellular oxidants in B16 cells (data not shown). Treated B16 cells with oxyresveratrol showed the diminution trend of the melanogenesis, and significantly reduced by 27 % ($p < 0.01$) and 46 % ($p < 0.01$) at the doses 10 and 12.5 μg/ml, respectively (**Figure 1b**). Oxyresveratrol at concentrations 10 and 12.5 μg/ml also suppressed the activity of tyrosinase enzyme with statistical significance at $p < 0.01$ (**Figure 1c**). The effect of oxyresveratrol on melanogenesis was highly associated with tyrosinase activity at $r = 0.949$, $p < 0.01$. Sodium L-lactate (SLL) at 50 mM was used as an inhibitor for melanogenesis and tyrosinase activity. This result conforms to a previous study which showed oxyresveratrol's anti-oxidant capacity to establish phytopolyphenol, especially in family of stilbenes. It had a capacity to scavenge free radicals, and also played a crucial role in melanin inhibition [22]. In this study, oxyresveratrol had a potential role to inhibit melanogenesis and tyrosinase activity with a dose dependent manner at 10 and 12.5 μg/ml. The results are in agreement with other reports; oxyresveratrol contained 4-substituted resorcinol structure strongly scavenged free radicals and was also mostly related with tyrosinase inhibition [23,24]. In addition, B16 cells treated with oxyresveratrol showed low levels of cellular oxidants with a dose dependent manner (**Figure 1d**). NAC at 5 mM was used as an anti-oxidant reference. The anti-cellular oxidant activity of oxyresveratrol had a trend to associate with melanin inhibition ($r = 0.932$, $p < 0.05$) and anti-tyrosinase activity ($r = 0.980$, $p < 0.01$). The results are supported by other studies; natural and synthetic agents have been associated with melanogenesis through the role of cellular oxidants relating to tyrosinase activity [25,27]. Therefore, the modulation of cellular oxidants by oxyresveratrol may play a crucial role in melanogenesis inhibition through tyrosinase activity. Previously, cellular oxidative stress modulation also reduced malignant transformation and progression in melanoma cells through the regulation of redox sensitive signals [28,29]. Therefore, the oxyresveratrol may help to suppress melanoma progression.

In this study, we also obtained melanin pigment in B16 cells. B16 cells treated with oxyresveratrol were not found to morphologically change at 10 μg/ml (**Figure 2b**) and 12.5 μg/ml (**Figure 2c**) when compared with untreated cells (**Figure 2a**). The treated cells showed a tendency to reduce melanin production with dose manner by 31 and 23 % with oxyresveratrol treated cells at 10 and 12.5 μg/ml, respectively. SLL at 50 mM was used as melanin inhibitor (**Figure 2d**). In addition, the effective doses of oxyresveratrol at 10 and 12.5 μg/ml were designed to check the genotoxic properties. *In vitro* micronucleus assay of V79 cells was used to verify the genotoxic damage in interphase cells (**Figure 3b**). The micronuclei of chromosome aberration is provided by the micronucleus in binucleated cells (**Figure 3D**). Treatment of oxyresveratrol on V79 cells at concentrations 10 and 12.5 μg/ml were 5.33±0.943 and 6.33±0.943 Mn/1,000 binucleated cells, respectively. No statistical significance of micronucleus was found among groups of the treated and untreated cells. Mitomycin C (MMC) was used as a genotoxic positive control and it provided strongly increased micronucleus frequency with statistical significance at $p < 0.001$ when compared with untreated cells (**Figure 3e**).

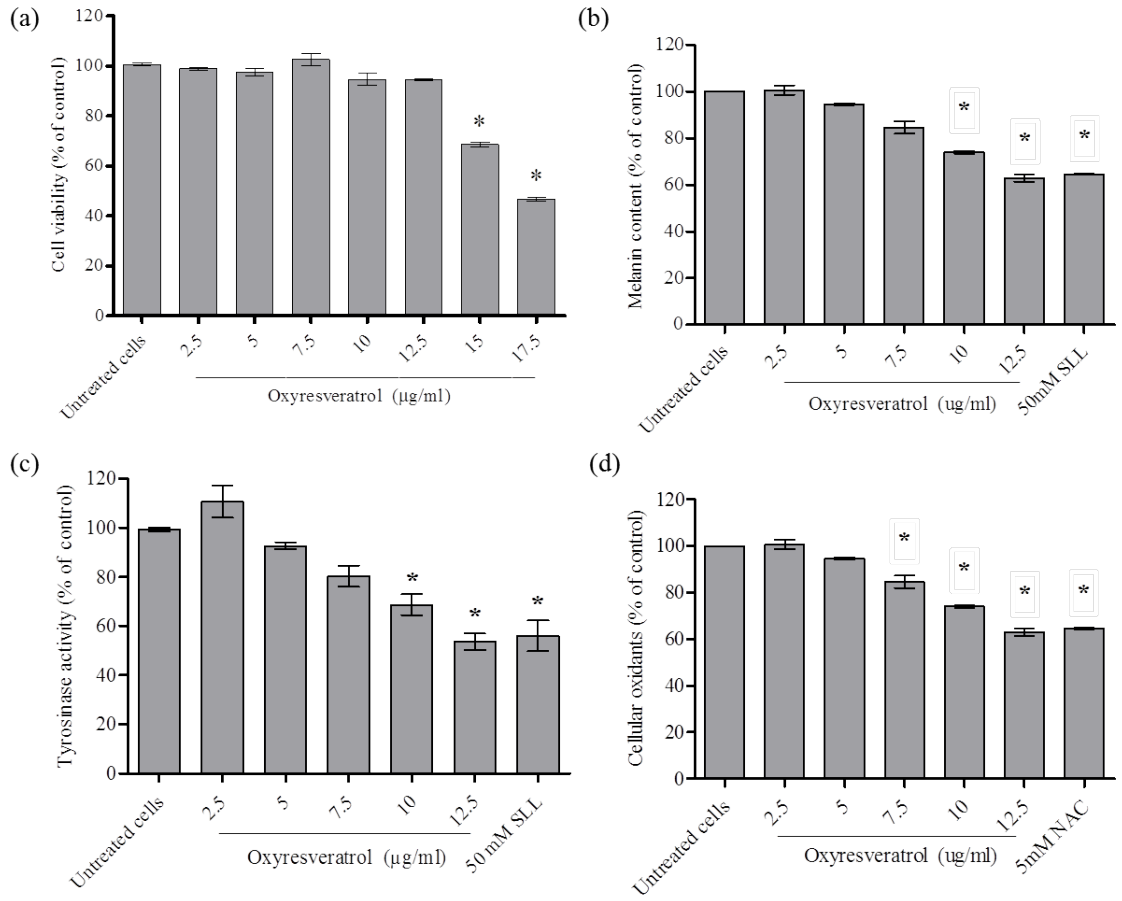

Figure 1 Oxyresveratrol treated B16 cells at various concentrations. The effects of oxyresveratrol on cell viability were obtained by MTT assay (a), melanogenesis (b), tyrosinase activity (c), and cellular oxidants (d). Sodium L-lactate (SLL) at 50 mM was used for melanogenesis and tyrosinase inhibition. N-acetyl cysteine (NAC) at 5 mM was used as an anti-oxidant reference. The results are expressed as a mean ± SD. * = the statistical significance at $p < 0.01$. The analysis was performed in triplicate.

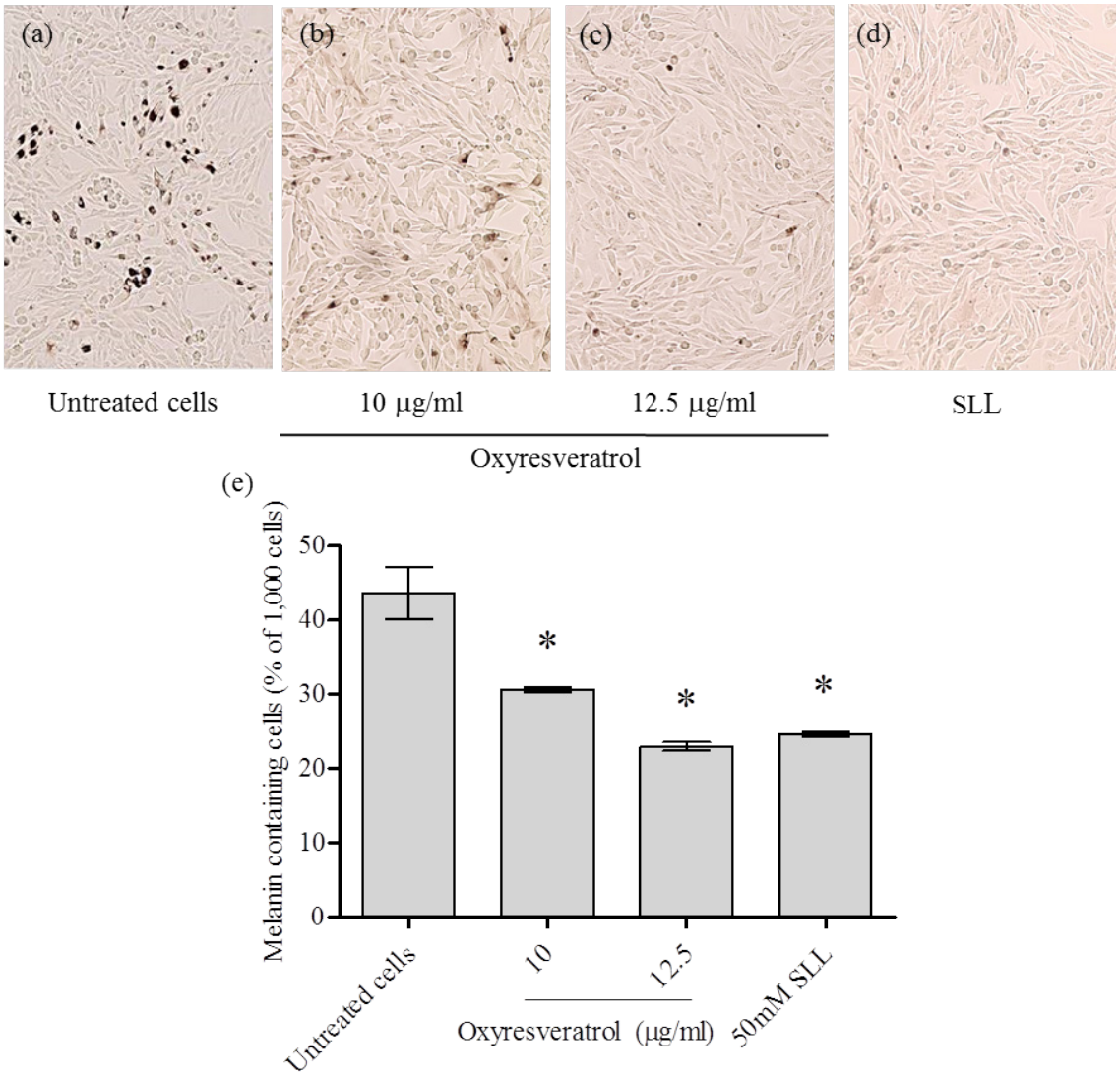

Figure 2 The effect of oxyresveratrol treatment on B16 melanoma cells, showing cellular morphology and melanin accumulation of untreated cells (a), 10 µg/ml (b), and 12.5 µg/ml (c) of oxyresveratrol treated cells. SLL at 50 mM was used as melanin inhibition (d). The results were visualized by light microscope (magnification, 40×) and expressed in a graph (e). The results are expressed as a mean ± SD. * = the statistical significance at $p < 0.05$.

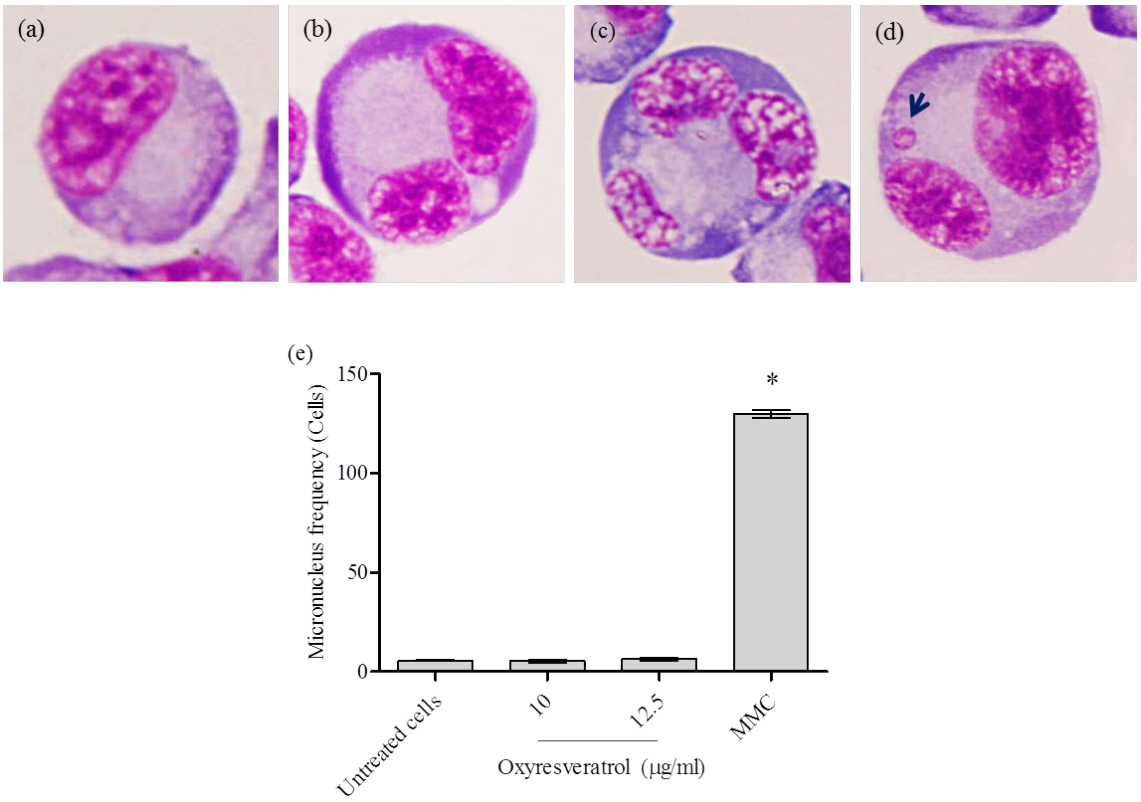

Figure 3 Effective doses of oxyresveratrol treated V79 cells. The morphology of V79 cell line was observed through light microscope with giemsa staining. Mononucleated cell (a), binucleated cell (b), trinucleated cell (c), and binucleated cells (d) with micronucleus (an arrow). Micronucleus frequency was observed from 1,000 binucleated cells (e); Mitomycin C (MMC) was used as a genotoxic positive control. The results are expressed as a mean ± SD. * = the statistical significance at $p < 0.001$.

Conclusions

The modulation of cellular oxidants by oxyresveratrol plays a crucial role in melanogenesis inhibition through tyrosinase activity. Also, the effective doses of oxyresveratrol showed no genotoxicity activity with *in vitro* micronucleus. Thus, oxyresveratrol from *Artocarpus lakoocha* Roxb. may be used as a candidate for developing pharmaceutical products for anti-hyperpigmentary disorders or chemopreventive drugs for melanoma progression.

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