ORIGINAL ARTICLE

The radical scavenging activity of vanillin and its impact on the healing properties of wounds

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ABSTRACT

Vanillin, an extract from the *Vanilla planifolia* plant, is reported to possess potent antioxidant properties. The ability of vanillin to protect skin cells from reactive oxygen species (ROS)-induced damage and its potential use in the treatment of wounds were studied. Cytocompatibility and cytoprotective properties against ROS-induced damage were examined using keratinocyte and fibroblast cell models. Vanillin's effect on cell migration was studied using the scratch wound healing assay. Vanillin exhibited cytocompatibility and cytoprotective properties against cell damage induced by ROS. Human keratinocytes and fibroblast cells showed >80% survival when exposed to vanillin (10–500 μM). Both cells showed no evidence of necrosis or apoptosis, which was confirmed by acridine orange/propidium iodide staining. Both examined cells were exposed to 750 μM hydrogen peroxide to cause oxidative stress, and vanillin demonstrated the ability to inhibit ROS-induced cell death. In addition, a considerable increase in cell migration suggested that vanillin had the ability to heal wounds *in vitro*. Vanillin is safe and potentially useful in wound healing treatments.

Key words: Antioxidant, reactive oxygen species, vanillin, wound healing

INTRODUCTION

A wound is an injury to the skin that impairs its structure and functionality. The healing process initiates immediately after a wound occurs. A disruption in the wound healing process results in a nonhealing wound or chronic wound.[1,2] At the site of the wound, an excessive amount of radicals and reactive oxygen species (ROS) are produced during the inflammatory phase. These radicals prolong inflammation and promote oxidative stress, which impairs wound healing.[3-5] ROS at low levels is beneficial for tissue defense against infection and for generating cell survival signals that support a normal wound healing response. However, excessive ROS leads to oxidative stress, which in turn damages cells.[1,2] To prevent the development of a chronic wound and accelerate the healing process, antioxidants should be applied to wound tissue to lower ROS to a safe level.[1,4]

Vanillin ($C_8H_8O_3$) is extracted from *Vanilla planifolia* pods. The most significant pharmacological evidence for vanillin is its antioxidant activity.[6] In ABTS, ORAC, and reducing power assays, vanillin had been found to have more potent antioxidant properties than Trolox, notably in the ABTS assay.[7] Vanillin has been demonstrated in Costantini studies to inhibit free radicals and inflammatory mediators while promoting the regeneration of periodontal tissue.[8] Vanillin has also been demonstrated to inhibit ROS-induced *in vivo*

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reactions.[8,9] The use of vanillin, a common natural flavoring compound, to protect wounds from oxidative stress and even provide additional advantages to the healing process is therefore interesting. According to our knowledge, no research has been conducted on the efficacy of vanillin in accelerating the healing of skin wounds. Therefore, the goal of the current study is to evaluate vanillin's cytocompatibility, cytoprotective effects, and *in vitro* wound healing capabilities in human keratinocytes (HaCaT) and primary dermal fibroblast cells.

SUBJECTS AND METHODS

Materials

Vanillin, hydrogen peroxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), acridine orange (AO), propidium iodide, and dimethyl sulfoxide (DMSO) were obtained from Sigma and Aldrich (USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin antibiotic, and fetal bovine serum (FBS) were obtained by Gibco® (USA).

Cell culture condition

HaCaT or primary skin fibroblasts (ATCC® CRL 2097, USA) were cultured in DMEM medium with 10% FBS and 100 U/mL penicillin/streptomycin (Gibco®, USA). The cells were cultured in a 5% CO_2 incubator at 37°C. Every 2 days, the medium was changed. The cells were harvested by gently shaking, and a new single-cell suspension was cultured in freshly finished media.

Cell viability assay

A 96-well plate was seeded with HaCaT keratinocyte or skin fibroblast cells (5×10^3 cells/well) and incubated for 12 h at 37°C with 5% CO_2. Vanillin or hydrogen peroxide (H_2O_2) was diluted to 10–1000 μM or 50–2000 μM with freshly prepared medium, respectively. Each well received 100 μL of a specific sample concentration, whereas the completed medium was employed as a negative control. Cell viability after a 24-h exposure to the samples was assessed using the MTT assay. Briefly, the cell supernatants were removed, and 20 μL of MTT solution (5 mg/mL) and 80 μL of new medium were then added. Additional incubation was done for 4 h, then the medium was removed, and a developing formazan salt was dissolved in 100 μL of DMSO. At 570 nm, the absorbance of formazan was measured with a Biohit 830 microplate reader (Biohit®, Helsinki, Finland). Calculated cell viability was compared to a negative control.

Vanillin-induced apoptosis cell death

The apoptosis of HaCaT keratinocytes or skin fibroblast cells following vanillin exposure was assessed using propidium iodide (PI)/AO double-staining assay. In a brief procedure, vanillin was introduced to the cells for 24 h at a dose that did not result in necrotic cell death. Within 30 min, the collected cells were dyed with AO/PI fluorescent dyes and evaluated under an Olympus BX51 ultraviolet-fluorescent microscope.

Protective effect of vanillin against reactive oxygen species-induced cell death

In this study, 750 μM H_2O_2 was used to harm HaCaT keratinocytes or skin fibroblast cells. Vanillin's ability to protect both tested cells from ROS damage was tested at a safe dose.

In a 96-well plate, 50 μL of 1×10^5 cells/mL of HaCaT keratinocyte or skin fibroblast cells were seeded. The plate was incubated for 24 h at 37°C, 5% CO_2. The cells were given a 24 h exposure to vanillin before being changed to medium containing 750 μM H_2O_2 and incubated for a further 24 h. The well exposure to H_2O_2 without vanillin served as an untreated control. The MTT assay was used to measure the vitality of the cells under investigation.

In vitro wound healing effect of vanillin

The effect of vanillin on the promotion of HaCaT keratinocyte or primary skin fibroblast migration was examined using the scratch wound healing assay. In 96-well plates, 3×10^4 cells/well were seeded and incubated overnight. After incubation, the DMEM was completely withdrawn, and the adhering cell layer was scraped with a sterile yellow pipette tip. Rinse the well with phosphate buffered saline to remove the certain detached cellular debris. The cells were then incubated for 24 h in completed media with or without vanillin. The image of the scratch area was recorded every 8 h for the first 24 h under bright field microscopy at ×10. The Olympus DP controller software was used to measure the wound area. The difference in the area of the vanillin-treated cells at 8, 16, and 24 h compared to 0 h in each experiment was used to calculate the relative cell migrations.

Statistical analysis

The data from at least three separate studies are reported as mean values with standard deviation. A one-way ANOVA with a *post hoc* test was used to make statistical comparisons (SPSS, SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was used to establish statistical significance.

RESULTS

Cytocompatibility of vanillin against skin cell culture

Vanillin was tested for cytotoxicity in HaCaT keratinocytes and skin fibroblasts at concentrations ranging from 10 to 1000 μM, as shown in Figure 1a and b, respectively. Vanillin at concentrations ranging from 10 to 500 μM was safe for both cells, with nearly 100% viability. However, compared to the untreated negative control, 1000 μM vanillin resulted in significantly lower cell viability ($P < 0.05$). Therefore, vanillin in the range of 0–50 μM was chosen for subsequent experiments based on cell viability data.

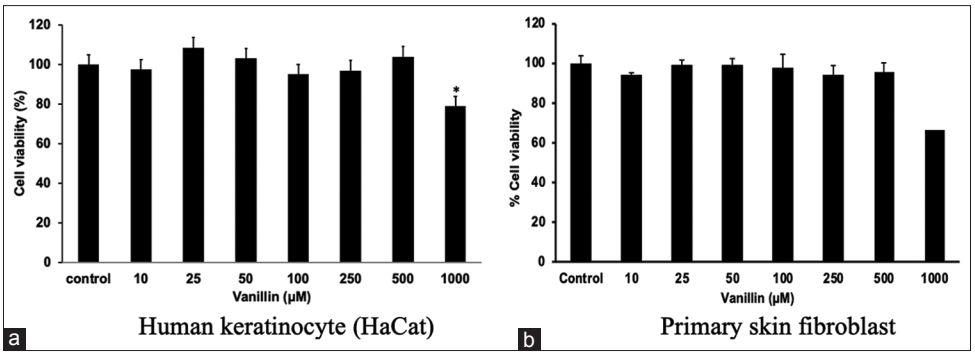

Figure 1: Cell viability percentages of (a) HaCaT and (b) primary skin fibroblasts after 24 h of incubation with various vanillin concentrations. HaCaT: Human keratinocytes

Vanillin-induced apoptosis cell death

The safe concentrations of vanillin at 10, 25, and 50 μM, indicated by MTT assays, were determined the potential to induce apoptotic cell death to validate the safety of utilizing vanillin in the following step. HaCaT keratinocytes and skin fibroblasts were fluorescence double stained with AO and PI, with the results depicted in Figure 2a and b. Following a 24-h incubation with various vanillin concentrations, both HaCaT keratinocyte [Figure 2a] and skin fibroblast [Figure 2b] cells exhibited the same intact green fluorescence as untreated control cells.

Vanillin's potential to protect cells from reactive oxygen species

HaCaT keratinocyte cell lines or skin fibroblast cells were treated with 10, 25, or 50 μM vanillin for 24 h before being challenged with ROS (750 μM H_2O_2), and the proportion of cells that survived after being exposed to H_2O_2 was determined.

The susceptibilities of HaCaT and fibroblast cells to ROS-induced cell death were different. According to the data presented in Figure 3a and b, 750 μM H_2O_2 induced approximately 46% and 81% cell survival in HaCaT and primary fibroblast cells, respectively. The HaCaT cell survival ratio was significantly increased ($P < 0.05$) when cells were treated with 50 μM vanillin for 24 h before being exposed to 750 μM H_2O_2. This demonstrates that vanillin is able to protect cells from the harmful effects of reactive oxygen. However, vanillin did not show a significant cytoprotective effect on fibroblast cell survival ($P > 0.05$).

Vanillin-induced cell migration

As epithelial and dermal wound healing model cells, HaCaT keratinocytes and dermal fibroblasts were examined. HaCaT keratinocytes and primary skin fibroblast cell lines were scraped before being exposed to different concentrations of vanillin. Cell migration was observed in 24 h, as shown in Figure 4a and b. Consideration of Figure 4a and relative migration levels in Figure 5a revealed that vanillin was significantly effective in promoting HaCaT cell migration, with the

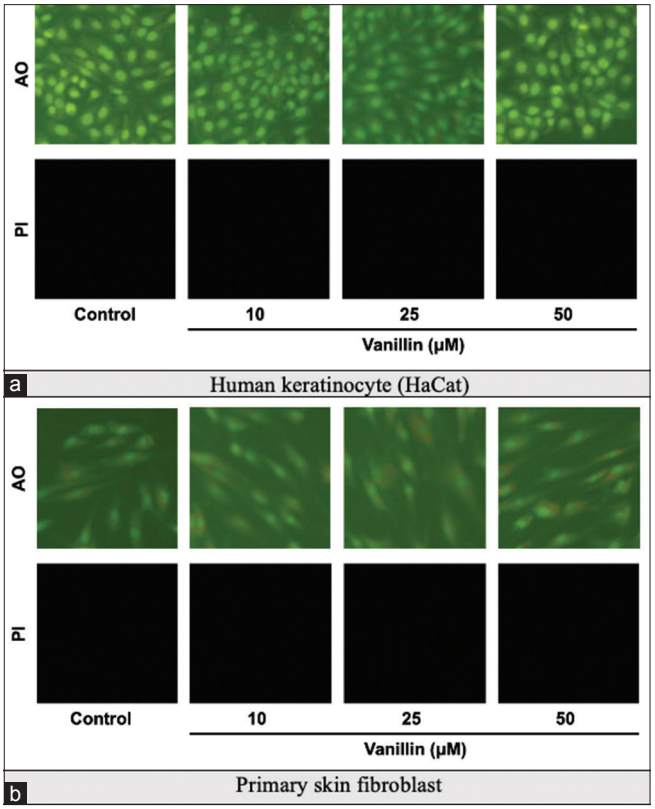

Figure 2: Fluorescence imaging of (a) HaCaT and (b) primary skin fibroblasts stained with AO/PI following exposure to various concentrations of vanillin. HaCaT: Human keratinocytes. AO: Acridine orange/PI: Propidium iodide

gap being filled after 16 h and significantly higher relative migration levels achieved when 25 or 50 μM of vanillin were applied. The gap was, however, entirely filled in all studies after 24 h of exposure. For human primary fibroblasts, the gap was enclosed for longer than 24 h for all tested experiments [Figure 4b]. The relative migration level of the vanillin-treated fibroblast (50 μM) [Figure 5b], however, showed that it migrated significantly more than the untreated control fibroblast cell. In conclusion, 50 μM vanillin could accelerate cell migration, which is advantageous for the healing process of wounds.

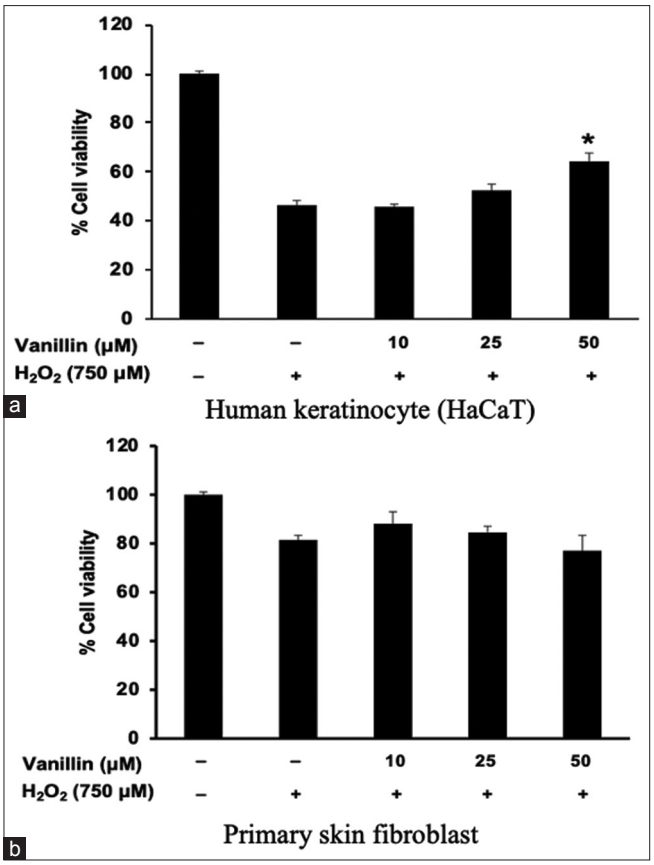

Figure 3: Viability percentage of (a) HaCaT and (b) primary skin fibroblast after exposure to vanillin at various concentrations for 24 h followed by 750 μM hydrogen peroxide. The symbol * denotes a statistically significant difference between the tested sample and the control ($P = 0.05$). HaCaT: Human keratinocytes, H_2O_2: Hydrogen peroxide

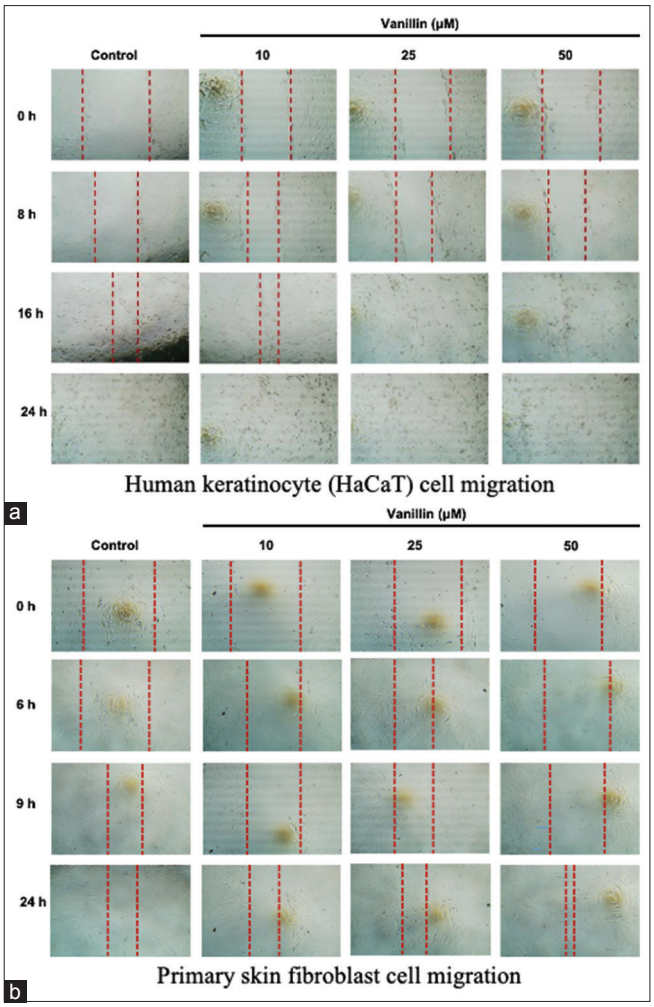

Figure 4: (a) HaCaT and (b) primary skin fibroblast cell migration when exposed to vanillin in the concentration range of 10–50 μM for 24 h. HaCaT: Human keratinocytes

DISCUSSION

Wound healing is a biologically complex process that can be separated into four distinct phases: coagulation, immune response and inflammation, proliferation, and remodeling.[1-4] However, if there is excessive ROS present in the wound, oxidative stress is created, which harms cells and hinders recovery. Therefore, employing antioxidants to reduce ROS levels in injured tissue to nontoxic levels could improve the healing process.[1,4]

Vanillin, derived from *V. planifolia*, is a flavoring component used in food, drink, and pharmaceutical products. In this study, the application of vanillin to protect skin cells from injury induced by ROS-like H_2O_2 and its ability to promote wound healing were the purposes. Vanillin at a concentration of 10–500 μM was safe for both HaCaT keratinocytes and fibroblast cells. In addition, the cell characteristics seen after AO/PI double staining supported vanillin's safety. At the tested concentration, 10–50 μM, no morphological changes, membrane blebbing, chromatic condensation, or a change in the fluorescent cells' color from green to orange were seen, indicating that no early apoptosis had taken place. PI staining did not show any redness in any of the conditions tested, indicating that there were no necrosis cells present. This indicates that, at any of the measured concentrations, vanillin did not result in necrosis or apoptotic cell death. This establishes that vanillin is safe and appropriate for additional testing at doses of 10, 25, and 50 μM.

Vanillin was evaluated for its effectiveness in protecting the HaCaT keratinocyte cell or primary fibroblast cell against ROS-induced cell death. The tested cell was treated with 10, 25, or 50 μM vanillin for 24 h before being challenged with 750 μM H_2O_2. The ROS level required to threaten the cell should be carefully examined because, whereas low levels of ROS protect tissues from infection and promote efficient wound healing, high levels of ROS cause oxidative stress, which can damage cells. For H_2O_2, a concentration between 100 and 250 μM is thought to be appropriate for typical wounds, although 500 μM or above could injure cells.[1] As

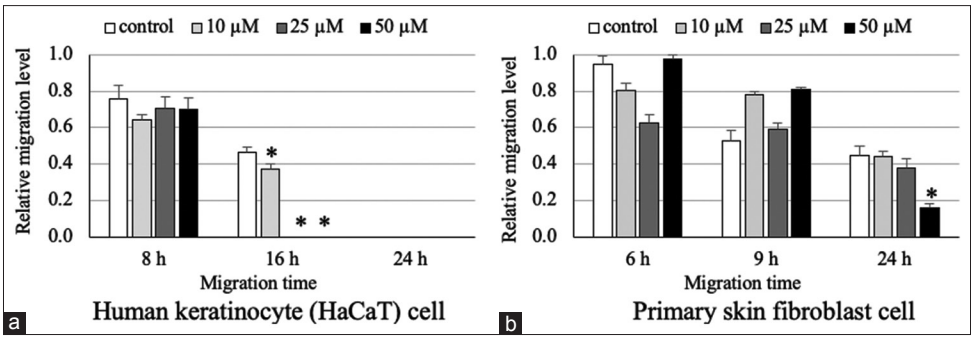

Figure 5: Relative cell migration level of (a) HaCaT and (b) primary skin fibroblast when exposed to vanillin in the concentration range 10–50 µM for 24 h. The symbol * denotes a statistically significant difference between the tested sample and the control ($P = 0.05$). HaCaT: Human keratinocytes

a result, to threaten the cell in this experiment, 750 µM of H_2O_2 was used to produce oxidative stress. Considering the data presented in Figure 3a, the HaCaT keratinocyte cells' survival ratio was significantly increased ($P < 0.05$) when cells were treated for 24 h with 50 µM vanillin before being exposed to 750 µM H_2O_2. This demonstrates that vanillin is able to protect HaCaT cells from the harmful effects of ROS. However, because H_2O_2 at the tested concentration (750 µM) did not harm primary fibroblast cells, the survival of the cells was >80%; hence, the cytoprotective impact of vanillin on this cell did not demonstrate any significant difference ($P > 0.05$) in cell survival [Figure 3b]. The strong antioxidant activity of vanillin to scavenge the free radical to a level below the hazardous level could be responsible for vanillin's protective impact against ROS-induced cell death.

Because cell migration is a crucial step in the healing process for wounds,[10] it was investigated to determine whether vanillin could enhance cell migration. The signaling processes leading to the activation of migration may be influenced by the induction of HaCaT keratinocytes. The results depicted in Figures 4a and 5a demonstrated that vanillin increased the migration of HaCaT keratinocyte cells. The gap in the vanillin-treated HaCaT cell was filled after 16 h, whereas it took more than 24 h for the untreated control. Thus, vanillin at 25 or 50 µM significantly improved cell migration, as seen by the relative migration level of vanillin-treated HaCaT cells compared to untreated control samples. An increase in keratinocyte migration induced by vanillin is helpful for initiating wound closure. For human primary fibroblasts, the gap was enclosed longer than 24 h for all treated conditions [Figure 4b]. However, the relative migration level of the vanillin-treated fibroblast (50 µM) showed that it migrated significantly more than the untreated control fibroblast cell [Figure 5b]. In conclusion, 50 µM vanillin could accelerate cell migration, which is advantageous for the healing process of wounds. Vanillin's positive properties on cell migration stimulation could be used in wound treatment formulations such as the hydrogel dressing with polyvinyl alcohol, chitosan, and vanillin developed by Xiong *et al*.[11] Vanillin thereby stimulates cell migration and speeds up the healing process of wounds, in addition to protecting cells from oxidative stress.

CONCLUSIONS

Vanillin has potent antioxidant properties and is safe to be used on keratinocytes and fibroblast cells found in the skin. The powerful antioxidant properties of vanillin are able to protect cells from oxidative stress and ROS-induced cell death. In addition, vanillin has the potential to promote cell migration. Therefore, it is expected that vanillin's antioxidant and wound-healing capabilities may promote the development of wound dressings or future wound-healing applications.

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Conflicts of interest

There are no conflicts of interest.

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