**Phyllanthus acidus** (L.) Skeels and *Rhinacanthus nasutus* (L.) Kurz leaf extracts suppress melanogenesis in normal human epidermal melanocytes and reconstitutive skin culture

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**ABSTRACT**

**Objective:** To determine the effect of extracts from *Phyllanthus acidus* (P. acidus) (L.) Skeels and *Rhinacanthus nasutus* (R. nasutus) (L.) Kurz leaves on melanogenesis and the underlying mechanism in normal human epidermal melanocytes (NHEM) and a reconstitutive skin model.

**Methods:** NHEM and a reconstitutive skin model were stimulated with ethanol extracts of *P. acidus* (L.) Skeels and *R. nasutus* (L.) Kurz leaves. mRNA expression of microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and dopachrome tautomerase (DCT) were examined by real-time PCR. The melanin content in NHEM was also measured. Moreover, protein levels of tyrosinase were determined using western blot analysis.

**Results:** In NHEM and the reconstitutive skin model, ethanol extracts from *P. acidus* (at 12.5 and 25.0 μg/mL) and *R. nasutus* (at 6.25 and 12.50 μg/mL) significantly diminished mRNA expression of MITF, TYR, TYRP1 and DCT in a concentration-dependent manner. *P. acidus* and *R. nasutus* extracts also reduced the amount of melanin in α-MSH-stimulated NHEM. Moreover, *P. acidus* and *R. nasutus* extracts markedly suppressed tyrosinase at the translational level in the reconstitutive skin model.

**Conclusions:** *P. acidus* and *R. nasutus* extracts significantly reduced melanogenesis in NHEM and the reconstitutive skin model, suggesting that *P. acidus* and *R. nasutus* extracts can inhibit melanin synthesis through downregulation of MITF, TYR, TYRP1 and DCT. Therefore, the ethanol extracts of *P. acidus* and *R. nasutus* contain compounds that have the potential for development as a skin lightening agent for the treatment of hyperpigmentation disorder or melasma.

1. Introduction

Melasma are gray-brown symmetrical patches in the sun-exposed areas of the facial skin[1]. In Southeast Asian countries with Fitzpatrick skin types III - V, control of melasma is particularly challenging, and melasma is frequently related to a physiological impact[2]. The effect of ultraviolet radiation (UVR) on melanin...
The methanol extract of Rhinacanthus nasutus (L.) Kurz contains alkaloids, anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25]. In a recent study, the anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25]. In a recent study, the anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25]. In a recent study, the anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25]. In a recent study, the anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25]. In a recent study, the anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25]. In a recent study, the anthraquinones, flavonoids (quercetin, rutin), saponins, triterpenoids, naphthoquinones (Rhinacanthin-A, -B, -C, -D, -E, -F, -G, -N, -M, -O, -Q), carotenoids, and polyphenols.[21-25].
human skin, was reconstituted with NHEM, NHEK, and human dermal fibroblasts as previously described[35,36]. Briefly, a collagen type 1a matrix containing fibroblasts (3.5×10^5 cells/well) was placed on a transwell clear polycarbonate membrane with a 0.4 μm pore size and the membrane was immersed into DMEM medium, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FCS in the well of a 6-well plate[37]. The plate was cultured at 37 °C in a 5% CO₂ incubator for 2 d. Then, the plate was incubated with fresh culture medium and incubated for 3 d. On day 7, mixed epidermal cells (4.2×10^5 cells) were added onto the plate at a melanocyte to keratinocytes ratio of 1: 2.5 (1.2×10^5 melanocytes and 3×10^5 keratinocytes) and cells were cultured in keratinocyte culture medium (HuMedia-KG2) for 2 d in a 5% CO₂ incubator. Subsequently the reconstitutive reconstructed skin model was treated with 0.5 mM Ca^2+ (day 9), 1.0 mM Ca^2+ (day 11) and 1.5 mM Ca^2+ (day 13 and 15) in keratinocyte culture medium. On day 17, DMEM medium/high glucose supplemented with 10% FCS was used to replace the medium and cells were incubated for 3 d in a 5% CO₂ incubator. On day 20, the upper layer of plate (the reconstituted skin compartment) was exposed to air and lower layer was incubated with DMEM medium/high glucose and 10% FCS for 3 d. The culture medium in the lower layer was changed every day. On day 27, the reconstitutive skin model was used for other experiments.

2.4. Cell viability assay for NHEM

Cell viability was established through the MTT assay essentially as described by others[38]. Briefly, NHEM were seeded at a concentration of 3×10^5 cells/well and incubated for 24 h. After 24 h, the cells were incubated with 1 μM of α-MSH medium containing DMSO as a control or with each ethanol extract at doses of 3.125-100.000 μg/mL for 48 h. Then, the cells were incubated with 10 μL of yellow tetrazole for 4 h at 37 °C. Solubilizing agent (100 μL/well) was added into each well and incubated overnight at 37 °C after which the absorbance of formazan was measured at 550 nm using a microplate reader (Molecular Devices, USA). Values are expressed as a percentage of control group (no treatment).

2.5. Real-time quantitative RT–PCR for MITF, TYR, TYRP1, DCT mRNA

NHEM were seeded at a density of 3×10^5 cells/well and incubated for 24 h in a 5% CO₂ incubator. After 24 h, the cells were incubated with 1 μM of α-MSH with each ethanol extract for 48 h[38]. Extraction of total RNA was performed with ISOGEN reagent. The reconstitutive skin model was treated with each ethanol extract for 7 d. The medium containing ethanol extract was changed every 2 d. After treatment, the medium in each well was removed after which the reconstructed skin model was crushed by a Cryo-press (Microtec, Japan) and the resultant samples were transferred into a new tube. Total protein was extracted by adding 1 mL of RIPA buffer and protein concentrations in sample were determined by a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) or a rabbit polyclonal anti-GAPDH antibody (1: 50) (Thermo Fisher Scientific). Western blot analysis was performed as previously described[38]. Samples with equal protein content (20 μg/sample) were run on a 10% SDS-PAGE and proteins were transferred onto a PVDF membrane. The PVDF membranes were blocked with 5% BSA. After blocking, the membranes were incubated with a mouse monoclonal anti-tyrosinase antibody (1: 50) (Thermo Fisher Scientific, Waltham, MA) or a rabbit polyclonal anti-GAPDH antibody (1: 50) (Thermo Fisher Scientific, Waltham, MA). The reconstitutive skin model was treated with extract for 7 d, after which the culture medium was discarded and cells were crushed using a Cryo-press (Microtec, Japan) and the resultant samples were transferred into a new tube. Total protein was extracted by adding 1 mL of RIPA buffer and protein concentrations in sample were determined by a BCA protein assay kit (Thermo Fisher Scientific). Western blot analysis was performed as previously described[38]. Samples with equal protein content (20 μg/sample) were run on a 10% SDS-PAGE and proteins were transferred onto a PVDF membrane. The PVDF membranes were blocked with 5% BSA. After blocking, the membranes were incubated with a mouse monoclonal anti-tyrosinase antibody (1: 50) (Thermo Fisher Scientific, Waltham, MA) or a rabbit polyclonal anti-GAPDH antibody (1: 50) (Thermo Fisher Scientific, Waltham, MA). The reconstitutive skin model was treated with extract for 7 d, after which the culture medium was discarded and cells were crushed using a Cryo-press (Microtec, Japan) and the resultant samples were transferred into a new tube. Total protein was extracted by adding 1 mL of RIPA buffer and protein concentrations in sample were determined by a BCA protein assay kit (Thermo Fisher Scientific). Western blot analysis was performed as previously described[38]. Samples with equal protein content (20 μg/sample) were run on a 10% SDS-PAGE and proteins were transferred onto a PVDF membrane. The PVDF membranes were blocked with 5% BSA. After blocking, the membranes were incubated with a mouse monoclonal anti-tyrosinase antibody (1: 50) (Thermo Fisher Scientific, Waltham, MA) or a rabbit polyclonal anti-GAPDH antibody (1: 50) (Thermo Fisher Scientific, Waltham, MA).
of cell viability were determined by the MTT assay. Low concentrations (A) and 6.25 and 12.5 μg/mL of extracts, NHEM were treated with different concentrations (3.125 to 100.000 μg/mL) of each extract and 1 μM of α-MSH for 48 h and cell viability were determined by the MTT assay. Low concentrations of P. acidus extract (3.125-25.000 μg/mL) did not affect NHEM cell viability (Figure 1B). Therefore, we used the two highest concentrations of 3.125-12.500 μg/mL also did not affect NHEM treated cells.

2.8. Statistical analysis

Results are shown as the mean±SEM of three independent experiments. Statistical analysis was undertaken by ANOVA with Dunnett’s post-hoc test using SPSS version 20.0. P<0.05 indicated statistical significance.

3. Results

3.1. Effect of P. acidus and R. nasutus extracts on viability of NHEM

To determine the potential toxicity of P. acidus and R. nasutus extracts, NHEM were treated with different concentrations (3.125 to 100.000 μg/mL) of each extract and 1 μM of α-MSH for 48 h and cell viability were determined by the MTT assay. Low concentrations of P. acidus extract (3.125-25.000 μg/mL) did not affect NHEM cell viability though higher concentrations (50.000-100.000 μg/mL) significantly suppressed viability (Figure 1A). The R. nasutus extract between concentrations of 3.125-12.500 μg/mL also did not affect NHEM cell viability (Figure 1B). Therefore, we used the two highest non-cytotoxic concentrations; 12.50 and 25.00 μg/mL of P. acidus and 6.25 and 12.50 μg/mL of R. nasutus, in further experiments.

3.2. P. acidus and R. nasutus extracts attenuate MITF, TYR, TYRP1 and DCT mRNA induced by α-MSH in NHEM

We examined whether P. acidus and R. nasutus extracts affected the transcription of melanogenic enzymes such as MITF, TYR, TYRP1 and DCT. α-MSH increased mRNA expression of these melanogenic enzymes, and P. acidus and R. nasutus extracts significantly suppressed the α-MSH-dependent increase in MITF, TYR and DCT expression in a concentration-dependent manner (Figure 2A, 2B and 2D). P. acidus extract also significantly reduced TYRP1, while R. nasutus extract at the concentration of 12.5 μg/mL but not 6.25 μg/mL decreased TYRP1 (Figure 2C). These results suggest that the P. acidus and R. nasutus extracts modify melanogenesis enhanced by α-MSH through suppression of MITF, TYR, TYRP1 and DCT expression.
3.3. *P. acidus* and *R. nasutus* extracts reduce melanin contents in NHEM stimulated by α-MSH

To determine the effects of *P. acidus* and *R. nasutus* extracts on melanin synthesis, we stimulated NHEM with 1 μM of α-MSH for 48 h together with each ethanol extract. As shown in Figure 3, α-MSH markedly enhanced the melanin content in NHEM by about 18% when compared with control cells (without α-MSH treatment). The *P. acidus* extract at a dose of 25 μg/mL, and the *R. nasutus* extract at a dose of 12.5 μg/mL significantly diminished the amount of melanin as compared to the α-MSH-treated cells. These results indicate that *P. acidus* and *R. nasutus* extracts exert an anti-melanogenic effect on NHEM.

![Figure 3. Effects of PA and RN extracts on melanin synthesis in NHEM.](image)

Percentage values of treated cells relative to control cells are shown. Data are the mean±SEM from three independent experiments. *p<0.05, **p<0.01 compared with control group.

3.4. *P. acidus* and *R. nasutus* extracts decrease melanogenic gene expressions in the reconstitutive skin model

To gain further understanding of the effects of *P. acidus* and *R. nasutus* extracts on melanogenic mRNA expression in human skin, we employed a reconstitutive skin model. The reconstitutive skin model has an epidermal layer consisting of NHEK and NHEM on top of a dermal layer containing dermal human dermal fibroblasts in a collagen matrix, mimicking normal human skin[35,36]. The reconstitutive skin model was treated with each extract for 7 d, and mRNA levels of melanogenic enzymes were examined by real-time PCR analysis. As shown in Figure 4A-4D, *P. acidus* (at 25.0 μg/mL) and *R. nasutus* (at 12.5 μg/mL) significantly suppressed mRNA levels of MITF, TYR, TYRP1 and DCT when compared with untreated control. It is noteworthy that the suppression of melanogenetic gene by *P. acidus* and *R. nasutus* extracts was more than kojic acid (at 125 μg/mL). We also confirmed that *P. acidus* and *R. nasutus* extracts significantly decreased tyrosinase protein expression compared to the control model (Figure 5). Thus, in agreement with the anti-melanogenic effect of *P. acidus* and *R. nasutus* in monoculture of NHEM, we observed *P. acidus* and *R. nasutus* extracts decreased the transcriptional and translational levels of melanogenic enzymes in the reconstitutive skin culture model.

![Figure 5. Effects of PA and RN extracts on the expression of tyrosinase protein in the reconstitutive skin model.](image)

Data are the mean±SEM from three independent experiments. *p<0.05, **p<0.01 compared with control without extract treatment. KA 125: kojic acid at 125 μg/mL.

![Figure 4. Effects of PA and RN extracts on mRNA levels of melanogenic enzymes in the reconstitutive skin model.](image)

(A) MITF, (B) TYR, (C) TYRP1, and (D) DCT were examined by qPCR, using GAPDH as an internal control. *p<0.05, **p<0.01, ***p<0.001 compared with α-MSH treated cells. KA 125: kojic acid at 125 μg/mL.
4. Discussion

Natural drugs from plants are a popular developmental route because of many advantages, such as fewer side effects, being less expensive and being based on a long history of traditional use[44]. In this study, we sought to determine if herbal extracts have the potential to regulate melanogenic gene expression. We observed the inhibitory effects of P. acidus and R. nasutus extracts on the melanogenic enzymes mRNA expression in medium pigmented NHEM stimulated by α-MSH. We also used a reconstitutive skin model which is similar to human skin structure containing epidermis and dermis layers. This model allowed us to examine the ability of P. acidus and R. nasutus extracts to permeate through the epidermal layer and to have idea if P. acidus and R. nasutus extracts exert inhibitory effects on melanogenesis in human skin[45]. We observed that P. acidus and R. nasutus extracts significantly decreased tyrosinase protein expression when compared with the control model, and P. acidus and R. nasutus extracts suppressed melanogenetic genes more than kojic acid (at 125 μg/mL). P. acidus and R. nasutus extracts inhibited melanogenesis through suppression of melanogenetic enzymes in α-MSH-stimulated NHEM and in the reconstitutive skin model.

DNA damage in keratinocytes is results from UVR exposure, and this results in the up-regulation of the p53 protein[46]. The p53 protein promotes the activation of proopiomelanocortin, which is further cleaved to generate α-MSH[47]. Keratinocytes secrete α-MSH which binds to MC1R on melanocytes[48,49]. MC1R stimulation by α-MSH induces an increased cAMP within the melanocytes which upregulate the transcription of MITF through the CRE binding protein[50]. Binding of MITF to the M box sequences in the gene promoters activates the transcription of TYR, TYRP1 and DCT genes. Our previous study found that P. acidus and R. nasutus extracts inhibit melanogenesis through inhibiting mushroom tyrosinase activity by about 43% and 65%, respectively[33]. In this study, P. acidus and R. nasutus extracts significantly reduced MITF, TYR, TYRP1 and DCT at the transcriptional level in α-MSH-induced NHEM. P. acidus (at 25 μg/mL) and R. nasutus (at 12.5 μg/mL) extracts significantly suppressed melanin content without affecting NHEM cell viability. Given that UVR increases α-MSH production from keratinocytes, our data indicates that P. acidus and R. nasutus extracts inhibit melanin synthesis through downregulation of MITF, TYR, TYRP1 and DCT in α-MSH-stimulated NHEM and in the reconstitutive skin model.

Our previous study[33] showed that P. acidus and R. nasutus extracts have phenolic compounds corresponding to approximately 50 and 17 mg gallic acid equivalent/g dry weight. The flavonoid content in the P. acidus extract was around 11 mg quercetin equivalents/g dry weight, while that of the R. nasutus extract was around 9 mg quercetin equivalents/g dry weight. The reactive oxygen species (ROS) scavenging activities of the two extracts were examined by an ABTS assay, and it was shown that P. acidus and R. nasutus extracts had ROS scavenging activities of around 99% and 56%, respectively. These antioxidant properties are important for regulating melanogenesis and skin treatments[51]. Human skin cells are normally exposed to ROS and oxidative stress as a result of exposure to UVR[52]. So, P. acidus and R. nasutus extracts may have phytochemical compounds that scavenge ROS generated as a consequence of UVR exposure. Our results suggest that P. acidus and R. nasutus extracts have phytochemical compounds which decrease melanin synthesis through the suppression of melanogenetic genes in α-MSH-stimulated NHEM and in the reconstitutive skin model. Thus, P. acidus and R. nasutus extracts seem to have the potential for development as ingredient for anti-melasma agents or skin whitening agents. In this study, we further suggest the exact compounds of P. acidus and R. nasutus that have the anti-melanogenic effects.

In conclusion, the present study showed that ethanol extracts of P. acidus and R. nasutus suppressed cellular melanin content in α-MSH-stimulated NHEM by decreasing mRNA expression of MITF, TYR, TYRP1, DCT. P. acidus and R. nasutus extracts also suppressed melanogenic enzymes, especially tyrosinase expression at the transcriptional and translational levels in the reconstitutive skin model. Therefore, this study suggests that ethanol extracts of P. acidus and R. nasutus inhibit melanogenesis through the suppression of MITF, TYR, TYRP1 and DCT. These extracts have the potential to be ingredients of skin whitening agents against hyperpigmentation by UVR. In future studies, we aim to isolate the bioactive compounds of P. acidus and R. nasutus extracts using high performance liquid chromatography. We will also develop these extracts as a nanoemulsion cream for treating hyperpigmentation. The limitation of this study is the use of these extracts in model systems, and the P. acidus and R. nasutus extracts should be investigated for toxicity and anti-melanogenic effects on human skin.

Conflicts of interest statement

The authors declare that there was no conflict of interest.

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