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Pilea symmeria: A natural ally in antioxidant defence and wound repair in mice

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ABSTRACT

Introduction: *Pilea symmeria,* traditionally used for wound healing in Mizoram, was evaluated for its antioxidant activity and wound healing ability using a mouse model.

Methods: Mice were inflicted with excision and incision wounds. They were divided into four groups of six: Group I (negative control) animals were treated with simple ointment base (SO); group II (positive control) with povidone-iodine (PVI) 5%; groups III and IV with 5% and 10% extract ointment (PSEE) prepared using polyethylene glycol (PEG), respectively. The ointment was applied topically on the wound for 16 days. The rate of wound contraction and breaking strength of the incision wound was recorded. The antioxidant status of the excision wound tissue was assessed by measuring the levels of reduced glutathione (GSH), glutathione-s-transferase (GST), superoxide dismutase (SOD), and malondialdehyde (MDA). A histological examination was also conducted to support the results.

Results: When compared with the SO-treated group, PVI 5%, PSEE 5%, and PSEE 10% treated groups showed a significant ($P<0.001$) increase in antioxidant markers (GSH, GST, SOD) and a decrease in MDA levels. The result also showed a significant ($P<0.001$) increase in the rate of wound contraction and a shortened period of epithelialization, which was confirmed by histopathology results. Moreover, the wound-breaking strength of incision wounds was significantly increased in the PVI 5%, PSEE 5%, and PSEE 10% treated groups ($P<0.001$) compared with the SO-treated group.

Conclusion: These findings validate the traditional use of *P. symmeria* as a wound-healing enhancer, supporting its potential as a therapeutic agent for wound management.

Implication for health policy/practice/research/medical education:
The antioxidant and wound-healing properties of the ethanolic extract of *Pilea symmeria* were shown in a mouse model. This may have implications for the use of this plant in the production of new medications for the management of wounds and various types of ailments affected by environmental stress.

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Introduction

Wounds have various phases, including hemostasis, inflammation, proliferative, maturation, and remodeling. Immediately following an injury, platelets aggregate to prevent blood loss (hemostasis phase). During the inflammatory phase, immune cells are drawn to the wound site and secrete proinflammatory cytokines. During the proliferative phase, matrix deposition and collagen synthesis occur, resulting in the formation of granulation tissue (1). Fibroblasts are drawn to the wound edge, proliferate, and promote keratinocyte migration and proliferation. Fibroblasts then produce matrix proteins like collagen to form the extracellular matrix (ECM). Fibroblasts eventually develop into myofibroblasts, which boost collagen production. The collagen production causes the wound to constrict and shrink in size. During the last phase, the temporal ECM is transformed into a mature scar (1). If wounds are not treated immediately, they can lead to severe infections or complications (2). Although there are many modern drugs used nowadays to cure wounds, many of these medications have side effects that can have many damaging effects on the

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body. People, especially from rural areas, still rely on traditional medicinal herbs that are less harsh and more effective (3,4). It is of immense importance to make use of these traditional remedies, which can be less costly and relatively safe (5). It is essential to have a scientific study of these plants and know their exact nature, which will help in using them more effectively (6).

Free radicals are molecules that have unpaired electrons and are highly detrimental to the body (7). Our body produces antioxidants that can neutralize these free radicals. However, due to health issues and the overproduction of free radicals, there are sometimes insufficient antioxidants to neutralize them. Therefore, we sometimes need to take antioxidants from an external source (8). Various studies have shown that plants' healing benefits are mostly due to their antioxidant capabilities (9). *Pilea symmeria* is a plant often found around river banks and moist areas. In Mizoram, its leaf paste is widely used to treat wounds (10). Although the therapeutic properties of this plant are not well documented in the literature, information about its traditional usage is obtained from elders in the community. This research aimed to provide scientific validation of its application in wound healing while also serving as a useful record of this plant and the first detailed account of its therapeutic qualities. We hope that scientific proof of its medicinal properties will help advance the usage of this plant in the development of safer herbal medications in the future.

Materials and Methods

Plant collection and extraction

Pilea symmeria was collected from Tanhril, Aizawl district, Mizoram. The plant was identified and authenticated at the Natural History Museum Mizoram, Mizoram University, with the accession number NHMM-P/000161. The leaves were washed and shade-dried in a clean and hygienic room. The dried leaves were then ground to powdered form using an electrical grinder. The powdered leaves were subjected to 72 hours of cold maceration using chloroform, ethanol, and aqueous as solvents.

Animals

Healthy Swiss albino mice of either sex, eight to ten weeks old, weighing 30-35 g, were obtained from North Eastern Hill University (NEHU). The animals were kept for acclimatization at Animal House, Department of Zoology, Mizoram University, for at least one week before the experiment. They were kept in a clean polypropylene cage with a temperature of 22 ± 3 °C and a 12-hour light-dark cycle; they were provided food and water ad libitum.

Ointment preparation

A simple ointment base was made using polyethylene glycol 400 (PEG-400) and polyethylene glycol 4000 (PEG-4000) (20 g each) and melted in a water bath at 70-75 °C. The mixture was stirred continuously until congealed; the extract was added and stirred at 40 °C to make 5% and 10% extract ointment (11).

Acute dermal toxicity

An acute dermal toxicity test was performed according to the OECD guideline no. 402. Three animals each of healthy female Swiss albino mice (30-35 g) were used for toxicity study. The animals were housed individually in a cage and acclimatized to the laboratory conditions for five days before the test. Before the study, the dorsal back of the mouse was shaved. First, a limit test dose of 2000 mg/kg of the 10% extract ointment was applied uniformly on the shaved area of one mouse each from the group, no death or skin irritation was observed within 24 hours, and then two additional mice each were treated with the same dose of the extract ointment. At the end of the exposure, the test substance was removed and the animals were observed for the development of any skin irritations daily. Animals were observed with special attention given during the first 2 to 6 hours of exposure, and daily thereafter for 14 days. No deaths or signs of skin irritations were observed at the end of the study.

Processing of wound tissue

The wound tissue was excised at day 16 and immediately used to make 5% (w/v) tissue homogenate using 1xPBS. The homogenates were centrifuged at 10,000 rpm at 4 ˚C for 30 minutes. The supernatant was collected and stored at -80 ˚C until further use. The protein content of the supernatant was determined following the method of Lowry et al (12).

Glutathione (GSH) assay

GSH content was measured using the standard method (13). GSH was tested by reacting it with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), resulting in a molecule that absorbed at 412 nm (Ellman's method). Each test tube contained 2 ml of 0.6 mM DTNB in 0.2 M sodium phosphate, pH 8.0, 100 µL of the sample, and 0.2 M phosphate buffer to give a final volume of 3 ml. The reference cuvette contained 100 µL of 5% trichloroacetic acid instead of the sample solution. GSH concentration was then calculated from the standard graph and expressed in µmol/mg protein.

Glutathione-S-transferase (GST) assay

GSH was measured following the method of Beutler (14) with minor modifications. To 100 µL of 0.1M phosphate buffer (pH 6.5), 20 µL of 20 mM CDNB and 1.76 mL of distilled water were added. The mixture was incubated at 37 °C for 10 minutes. Then, 60 µL 20mM GSH and tissue homogenate were added to the mixture. For the blank, distilled water was added instead of the tissue homogenate. The absorbance was then measured at 340 nm with a 1-minute interval. The GST activity was then calculated using the following formula:

GSH activity = (OD of test – OD of blank/9.6 × volume of test sample) × 1000

where OD is the optical density of the tested sample.

Superoxide dismutase (SOD) assay

SOD activity was tested following the method of Sun et al (15) with slight modifications. A sample of 10 µL was mixed with 15 µL distilled water, 25 µL phenazine methosulfate (PMS), 75 µL nitro-blue tetrazolium (NBT), and 50 µL nicotinamide adenine dinucleotide (NADH). The mixture was incubated for 90 seconds at 30 ºC. Then, 250 µL of acetic acid and 1000 µL of butanol were added to the mixture. The mixture was vortexed for 2-3 seconds and let stand for 10 minutes. It was then centrifuged for 30 seconds. For blank, distilled water was added instead of the tissue homogenate. The absorbance was then taken at 560 nm. The enzyme activity was expressed in unit/mg protein.

Lipid peroxidation assay

Lipid peroxidation activity was tested using the method of Ohkawa et al (16) with slight modifications. To 125 µL of the tissue homogenate, 125 µL of 15% TCA was added and centrifuged at 10 000 rpm for 10 minutes. The supernatant was taken out and kept in a clean and dry test tube. Twice the amount of the present thiobarbituric acid (0.8%) solution was added to the test sample. Test tubes were then kept in a 95 ºC water bath for 45 minutes and the formation of colored complexes was observed. For the blank, distilled water was added instead of the tissue homogenate. After cooling, the absorbance was read at 532 nm.

Grouping of animals

The animals were randomly divided into four groups of 6 animals each. Group I animals (negative control group) were treated with the vehicle of the simple ointment (SO). Group II animals (positive control group) were treated with povidone-iodine (PVI) 5%. Group III and IV animals were treated with 5% and 10% (w/w) of *P. symmeria* ethanol extract ointment (PSEE), respectively.

Excision wound

Mice were inflicted with an excision wound as described by Morton and Malone (17). Animals were anesthetized before and during the creation of the wounds, with intraperitoneal ketamine + xylazine (50 mg/kg). After anesthesia, the hair was removed by shaving the dorsal back of the mice. Ethanol (70%) was used as an antiseptic for the shaved region before making the wound. An excision wound was created by removing a full thickness of the skin 200 mm^2 circular area from a predetermined shaved area on the back of each animal. Each mouse was kept in a separate cage after the creation of the wound. All vehicles were applied once daily till the epithelisation day. The wound closure rate was assessed by tracing the wound on days 0, 4, 8, 12, 16, and 21 post-wounding using transparent paper and a permanent marker. The wound area recorded was used to calculate the percentage of wound contraction.

% Wound closure = (Wound area on 1st day − Wound area on day (n)) × 100/Wound area on 1st day

Where n is the number of days.

The period of epithelialization measurement

The period of epithelialization was calculated as the days required for the dead tissue remnants to fall off without leaving any open wounds (18).

Incision wound

An incision wound was created following the standard method of Ehrlich and Hunt (19). Mice in each group were anaesthetized as mentioned earlier. An incision of about 3 cm was made on the paravertebral midline on the back of the mice. After the incision, the parted skin was stitched together with black thread at 0.5 cm intervals. The sutures were removed on day 8, and the tensile strength of the healed wound was measured on day 10 using a water flow system. The weight of water collected in the container was recorded and taken as a measure of the wound-breaking strength in grams.

Histological analysis

On day 16, the wound tissue was removed and fixed in 10% formalin. Histopathological examination was carried out following the procedure of Drury and Wallington (20). The prepared section was stained using hematoxylin and eosin (H&E), mounted with xylene, and carefully examined under a microscope for the evaluation of any histopathological changes.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey multiple comparison of means. The values were expressed as mean ± SEM (n = 6). The analysis was carried out using SPSS (online) and the graphs were plotted using GraphPad prism (online). The value $P < 0.05$ was considered to be statistically significant.

Results

Antioxidant levels

The extent of wound healing progression was assessed by estimating the levels of GSH, GST, SOD, and malondialdehyde (MDA). The elevated levels of these biochemical parameters directly reflect alterations in the wound tissue. Topical treatments caused a significant increase in the level of GSH, GST, and SOD and a significant decrease in the MDA level in animals treated with PVI 5%, PSEE 5%, and PSEE 10% compared with the SO-treated group (Figure 1).

Excision wound

The data for wound contraction is given in Table 1. The wound size started reducing from day 4. The positive control (PVI 5% treated group) showed somewhat similar activity as the 10% PSEE ointment treated group. On day 16, the 5% PSEE ointment-treated group, the 10% PSEE ointment-treated group, and the PVI 5% treated group showed 97.63%, 98.84%, and 99.25% of wound healing, respectively, when compared with the SO-treated group,

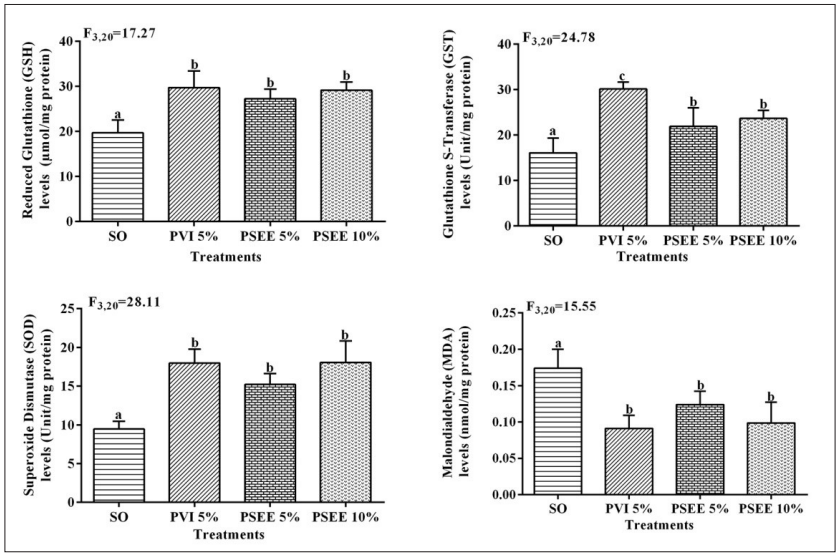

Figure 1. Effect of topical application of simple ointment (SO), povidone-iodine (PVI), and *Pilea symmeria* ethanol extract (PSEE) on the antioxidant levels of excision wound in mice. Different letters (a, b, c) indicate statistical variations between the groups ($P < 0.001$).

which showed only 54.48% of wound healing. On day 21, all the extract and PVI 5% treated animal groups showed complete healing, while the SO-treated group showed 96.35% wound healing. Photographs provided further evidence for the above-mentioned results (Figure 2).

Period of epithelialization

Compared to the SO-treated group, the positive control group that received PVI 5% had the shortest duration of epithelialization, followed by the PSEE 10% and PSEE 5% ointment-treated groups (Table 1). The photographs of the rate of wound contraction on different days of various treatments are presented in Figure 2.

A significant increase in wound tensile strength was observed in the animals treated with PVI 5% (361.68 ± 10.58), PSEE 5% (373.53 ± 8.90), and PSEE 10% (325.01 ± 6.16) when compared with the SO-treated group (270.41 ± 9.62) (Figure 3).

Histology

In histological examinations, the animals receiving the 10% PSEE ointment exhibited a significant improvement in wound healing, as evidenced by the abundance of collagen deposition and fewer blood vessels, as well as the presence of mature epidermis that was thin, well-organized, and comparable to the epidermis of normal skin. The animals receiving PVI 5% also showed a thin layer of mature epidermis with fewer fibroblasts, evidenced by wound healing. The results were more or less the same as the PSEE 10% treated group. Animals receiving PSEE 5% showed thicker epidermis, indicating incomplete healing, and a high level of inflammatory cell infiltration, suggesting a delayed rate of wound healing. Animals receiving only the SO exhibited thicker epidermis, the presence of more blood vessels, and lesser collagen deposition, indicating a slower pace of wound healing (Figure 4).

Discussion

Many medicinal plants are reported to have natural antioxidants that can help neutralize or eliminate reactive oxygen species (21). These reactive species, if not eliminated, can cause various complications in the

Table 1. Effect of topical application of simple ointment (SO), povidone-iodine (PVI), and *Pilea symmeria* ethanol extract (PSEE) on wound diameter in mice

Days	Wound diameter in groups (mm²)			
	SO	PVI 5%	PSEE 5%	PSEE 10%
Day 0	195.83 ± 1.54	200.67 ± 0.42	197.50 ± 1.05	200.50 ± 1.40
Day 4	192.67 ± 1.76 (1.62%)	185.67 ± 0.84 (7.47%)^{ac}	192.50 ± 1.18 (2.53%)^{b}	191.67 ± 1.28 (4.23%)^{abc}
Day 8	178.17 ± 1.45 (9.00%)	154.33 ± 0.42 (23.08%)^{ac}	164.33 ± 1.05 (16.78%)^{ab}	164.00 ± 3.21 (18.22%)^{ab}
Day 12	136.33 ± 0.67 (30.37%)	100.16 ± 1.97 (50.08%)^{ac}	113.66 ± 3.89 (42.45%)^{ab}	109.00 ± 3.08 (45.63%)^{a}
Day 16	89.00 ± 3.44 (54.48%)	1.50 ± 0.56 (99.25%)^{a}	4.83 ± 0.60 (97.63%)a	2.33 ± 0.33 (98.84%)^{a}
Day 21	7.16 ± 0.70 (96.35%)	0.00 ± 0.00 (100%)^{a}	0.00 ± 0.00 (100%)^{a}	0.00 ± 0.00 (100%)^{a}
Period of epithelialization (day)	19.00 ± 0.45	14.00 ± 0.26^{ac}	17.00 ± 0.45^{ab}	15.00 ± 0.52^{ac}

Data within parenthesis indicate wound healing/closure time (%); [a] $P < 0.001$ compared with SO; [b] $P < 0.001$ compared with PVI 5%; [c] $P < 0.001$ compared with PSEE 5% ($P < 0.001$).

Figure 2. Photographic representation of the rate of wound contraction on different days of treatment. Abbreviations: SO: simple ointment; PVI: povidone-iodine; PSEE: *Pilea symmeria* ethanol extract.

body. Antioxidants, like GSH, play an important role in protecting cells from oxidative stress (22). GSH, a detoxification enzyme, catalyzes the conjugation of electrophilic substrate to GSH (23). SOD is reported to form the first line of defense against injury mediated by reactive oxygen species (24). These proteins catalyze the dismutation of superoxide anion radical (O_2^-) into molecular oxygen and hydrogen peroxide, reducing the concentration of O_2^-, which may harm cells when presenting in excess (24-26).

Lipid peroxidation is the process by which oxidants, such as free radicals, target the lipid membrane's polyunsaturated fatty acids, directly damaging the membrane and producing a variety of oxidized byproducts (27,28). During lipid peroxidation, MDA is formed through the decomposition of hydrogen peroxide; the level of MDA is an important indicator to check the extent of progression for several diseases (29). Results from this study showed a marked increase in the level of antioxidants and enzymes like GSH, GST, and SOD level. A decrease in the level of lipid peroxidation in the animals treated with the plant extract ointment also supported the plant's ability to scavenge the radicals.

Wounds are a complex process involving several events, including cellular and biochemical processes (30). Phytochemicals play a significant role in wound healing (4). Excision and incision wounds are common experimental models used for studying cell or tissue regeneration and skin-breaking strength (31). Animals

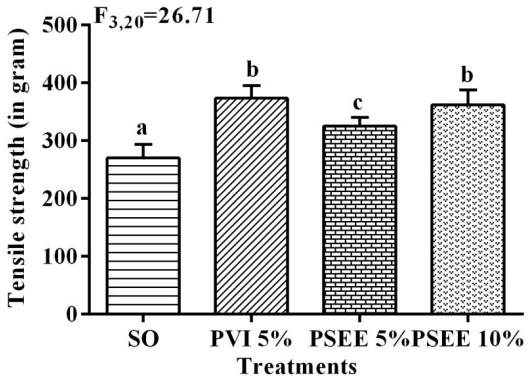

Figure 3. Effect of topical application of simple ointment (SO), povidone-iodine (PVI), and *Pilea symmeria* ethanol extract (PSEE) on mice's wound incision. Different letters (a, b, c) indicate significant variation among groups ($P < 0.001$).

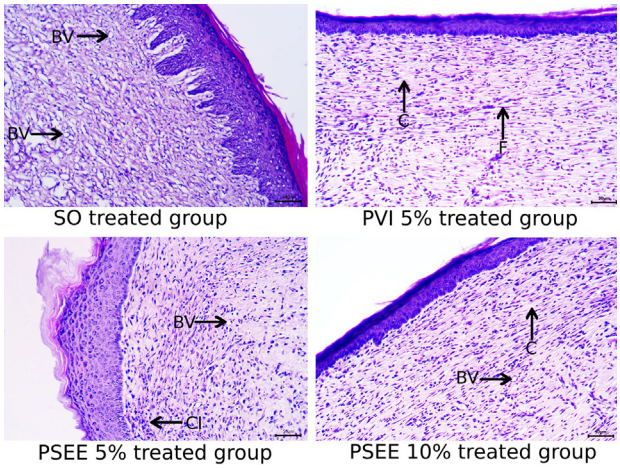

Figure 4. Histological slides showing the effect of topical application of simple ointment (SO), povidone-iodine (PVI), and *Pilea symmeria* ethanol extract (PSEE) on the excision wound model in mice on day 16 of the experiment. Cellular changes are pointed with arrows. Abbreviations: BV: blood vessel; CI: cellular infiltration; C: collagen; F: fibroblast.

treated with *P. symmeria* extract ointment showed a marked increase in wound healing, characterized by an increase in wound contraction and a shorter epithelialization period. Although the 5% extract ointment showed a significant increase in wound contraction rate when compared with the SO-treated group, it was at a slower pace when compared with the PVI 5% and PSEE 10% treated groups. The positive control group that received PVI 5% and PSEE 10% showed a marked increase in the rate of wound healing and was comparable with each other.

In the incision wound, the extract-treated wound had a higher wound-breaking strength compared with the negative control. The result of the excision wound was further supported by the histological examination that showed enhanced proliferation of cells, an increase in collagen deposition, and the formation of new blood vessels, which was a sign of proper wound healing (32) and faster epidermal remodeling in the group receiving the extract ointment. Another study on the wound-healing efficacy of traditional medicinal plants produced findings comparable to our results (32-34). However, slight changes in the rate of healing may be attributed to the animal's health, diet, breed, and environment, as well as variances in plant content and ointment concentrations. Even in the present experiment, animals receiving the same treatment showed slight variance in the rate of wound healing among their groups, suggesting that the variables listed above might influence the rate of wound healing. Although our research identified several phytochemicals present in *P. symmeria* extract that might be responsible for antioxidant and wound-healing properties, a more in-depth research is still required to identify the entire phytochemical composition of the extract and ascertain which phytochemical has the most impact on wound-healing, how these compounds interact with the healing process, how they interfere with the gene involved in the wound healing pathway, and whether this plant contains other compounds that may have significant biological activities beyond wound healing.

Conclusion

Results of this study demonstrated that there was a significant increase in the level of antioxidants and a decrease in lipid peroxidation, an increase in wound contraction, a reduced period of epithelialization, an increase in wound breaking strength, and faster dermal and epidermal regeneration in the treated animal wound. Pharmacological interactions and wound-healing processes may vary across species. Therefore, research using animal models alone may not be sufficient, and if possible, human trials should be conducted before suggesting its probable applicability for therapeutic use.

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Conflict of interests

The authors declare that there are no conflicts of interest.

Ethical considerations

The use of animals in this experiment was approved by the Institutional Animal Ethics Committee (IAEC), Mizoram University, Mizoram, India (the approval code: MZU/IAEC/2020/07).

Authors' contribution

Conceptualization: Lalremtluangi Rokhum, Lalhmingliani Esther.
Data curation: Lalremtluangi Rokhum, Lalthansangi Chinzah.
Formal analysis: Lalremtluangi Rokhum, Lalthansangi Chinzah, Mathipi Vabeiryureilai.
Funding acquisition: Lalremtluangi Rokhum, Lalhmingliani Esther
Investigation: Lalremtluangi Rokhum, Lalthansangi Chinzah, Mathipi Vabeiryureilai.
Methodology: Lalremtluangi Rokhum, Lalthansangi Chinzah, Lalhmingliani Esther.
Project administration: Lalhmingliani Esther, Mathipi Vabeiryureilai.
Resources: Lalremtluangi Rokhum, Lalthansangi Chinzah.
Software: Lalremtluangi Rokhum, Mathipi Vabeiryureilai.
Supervision: Lalhmingliani Esther, Mathipi Vabeiryureilai.
Validation: Lalremtluangi Rokhum, Lalthansangi Chinzah.
Visualization: Lalremtluangi Rokhum, Lalhmingliani Esther.
Writing–original draft: Lalremtluangi Rokhum, Lalthansangi Chinzah.
Writing–review & editing: Lalremtluangi Rokhum, Lalthansangi Chinzah, Lalhmingliani Esther, Mathipi Vabeiryureilai.

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