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Comparative analysis of bioactive compounds and anti-inflammatory effects via COX-2 inhibitory activity in forest-derived and tissue-cultured extracts of umbrella moss (*Rhodobryum giganteum* (Schwägr.) Par.)

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ABSTRACT: *Rhodobryum giganteum* (umbrella moss) is a traditional Chinese medicinal plant primarily used for treating heart diseases through anti-inflammatory mechanisms. Its key bioactive compounds are 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid. Since umbrella moss grows in humid mountain regions, overharvesting wild populations risks extinction and contamination with heavy metals. To address this, tissue culture techniques were developed to propagate the moss sustainably. This study compares the bioactive compounds content and anti-inflammatory efficacy via cyclooxygenase-2 (COX-2) inhibitory activity of forest-derived and tissue-cultured *R. giganteum* extracts. Both samples were extracted using 60% ethanol and analyzed by high-performance liquid chromatography for active compounds, followed by enzyme-linked immunosorbent assay for COX-2 inhibition. The tissue-cultured moss had much higher amounts of 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid than the natural forest moss. Specifically, it contained about 15 times more 7,8-dihydroxycoumarin (6.16 ± 1.25 μg/ml vs. 0.41 ± 0.03 μg/ml) and nearly 5 times more *p*-hydroxycinnamic acid (17.63 ± 0.47 μg/ml vs. 3.69 ± 0.06 μg/ml). These results demonstrate that tissue culture not only supports sustainable propagation but also enhances bioactive compound production and therapeutic efficacy. This preliminary *in vitro* study provides a foundation for developing pharmaceutical products from *R. giganteum* while conserving natural populations. Further cell-based assays and *in vivo* studies are necessary to evaluate the therapeutic potential.

KEYWORDS: *Rhodobryum giganteum*, umbrella moss, *p*-hydroxycinnamic acid, 7,8-dihydroxycoumarin, human cyclooxygenase-2

INTRODUCTION

Inflammation is a vital defense mechanism in humans, serving as the immune system's response to injuries and pathogens [1]. However, prolonged or excessive inflammation can lead to chronic tissue damage, cancer, organ failure, and even fatal outcomes. Among the critical pathways driving inflammation is the cyclooxygenase (COX) pathway, which involves two enzyme isoforms: COX-1 and COX-2. COX-1 is a constitutive isoform that supports homeostatic functions under normal conditions. In contrast, COX-2 is an inducible enzyme that plays a pivotal role in inflammatory responses. Upon stimulation, arachidonic acid (AA) is released from cell membranes and converted by COX-2 into prostaglandin E_2 (PGE_2), a key mediator of inflammation that causes symptoms such as pain, redness, and swelling. Consequently, the inhibition of COX-2 and PGE_2 synthesis is a primary strategy for managing inflammation [2].

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly employed for this purpose; examples include ibuprofen, aspirin, indomethacin, and diclofenac. However, their broad effects on both COX-1 and COX-2 often result in adverse side effects such as gastrointestinal ulcers and bleeding. COX-2-specific NSAIDs, including celecoxib and rofecoxib, offer greater efficacy but are associated with risks such as nephrotoxicity, hypertension, and cardiovascular complications [3]. Given these challenges, medicinal plants with natural anti-inflammatory properties offer promising alternatives, as they target inflammatory pathways with fewer side effects.

Rhodobryum giganteum (Schwägr.) Par., commonly known as umbrella moss (Fig. 1), is a traditional medicinal plant found in Japan, Korea, China, and Southeast Asia, including Thailand. The plant has demonstrated various therapeutic effects, including cardiovascular protection, anti-inflammatory activity, and sedative and aphrodisiac properties [4–7]. Phytochemical studies have identified key bioactive compounds in *R. giganteum*, including *p*-hydroxycinnamic acid and 7,8-dihydroxycoumarin, which exhibit significant cardiovascular and anti-inflammatory benefits [6].

In Thailand, *R. giganteum* grows in high-humidity,

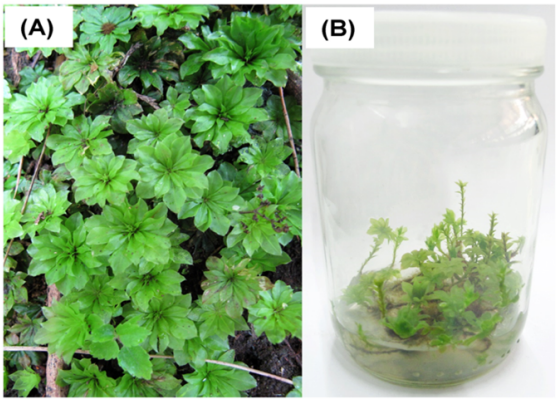

Fig. 1 Morphology of umbrella moss (*R. giganteum* (Schwägr.) Par.); (A) natural habitat, (B) tissue-cultured condition.

low-temperature mountainous regions at elevations exceeding 1,500 m, such as Doi Suthep-Pui National Park, Doi Inthanon National Park, and Doi Luang Chiang Dao [8]. However, harvesting wild populations for medicinal use poses risks of overexploitation and extinction. Additionally, as a poikilohydric plant, *R. giganteum* absorbs heavy metals such as arsenic, cadmium, chromium, lead, mercury, etc. directly into its cells [9], raising concerns about contamination.

To address these issues, tissue culture techniques have been developed to propagate *R. giganteum* sustainably, reducing the risk of extinction and heavy metal contamination [10]. This study investigates the efficacy of tissue-cultured moss by comparing its bioactive compounds content and anti-inflammatory activity via COX-2 inhibition to that of the wild-collected moss. Extracts were analyzed using high-performance liquid chromatography (HPLC) and evaluated for their ability to inhibit COX-2 and PGE_2 synthesis. These findings aim to highlight the potential of tissue culture as a sustainable approach to harnessing the medicinal properties of *R. giganteum*.

MATERIALS AND METHODS

Plant preparation and extraction

R. giganteum plants were collected from Doi Suthep-Pui National Park, Chiang Mai Province, Northern Thailand, at 18°49′54″ N and 98°53′16″ E on October 15, 2021. The permission of sample collection has been approved by the Department of National Parks, Wildlife and Plant Conservation of Thailand, Thai permission license No. TS.0907.4/29044. Naturally, the *R. giganteum* grown on the trunk base of the Himalayan cypress, *Cupressus torulosa* D.Don ex Lamb. (Cupressaceae), in planted pine areas near the summit of Doi Pui mountain at 1,680 m elevation. The general pH of tree bark of Himalayan cypress was 5.7–5.9. Half of specimens were acclimated in the growth chamber at temperature of 22 ± 2 °C, light intensity (PPFD) of approximately 110 µmol/m^2/s, 16 h light/8 h dark photoperiod, and 80% relative humidity [11]. At approximately 4 weeks, 10 new young shoots (3 mm long pieces) were selected and sterilized for 1 min using 1% bleach solution (Haiter™, 6% NaOCl), then rinsed 3 times using sterilized deionized water (by adding 0.1% bleach solution) for 1 min each. Individual sterilized shoots were then cultured using the optimum condition of tissue culture technique in sterile agar medium 1/4 MH (modified hydroponic medium), 1% sucrose, pH 5.8, 4.5% agar, and 1 ml/l bleach solution [11]. All the plants were grown in the same growth chamber as above for 16 weeks.

The fresh moss samples from both natural habitat and tissue culture (30 g each) were washed, air-dried under the shade, and then macerated with 60% ethanol in a ratio of 1:40 w/v on a shaker at room temperature for 18 h. The macerated solution was filtered using No. 1 Whatman filter papers and then concentrated using a rotary evaporator and dried on water bath for 24 h at 80 °C. The percent yields were calculated, and the crude extracts were stored at −20 °C until use. The extract yields were calculated using the following equation: Extraction yield (%) = Weight of extract obtained × 100/Weight of the fresh sample.

High Performance Liquid Chromatography (HPLC)

Chemical and reagents

The reference standards 7,8-dihydroxycoumarin, *p*-hydroxycinnamic acid, and celecoxib were obtained from Merck KGaA, Darmstadt, Germany. Methanol and acetonitrile of analytical reagent (AR) and HPLC grades were purchased from RCI Labscan, Bangkok, Thailand. The Human COX-2 ELISA Kit was obtained from Cayman Chemical, Ann Arbor, MI, USA. The HPLC system (Agilent 1200 series, Agilent Technologies, Waldbronn, Germany) was equipped with a Sepax C18 column (4.6 mm × 250 mm, 5 µm particle size; Sepax Technologies, Newark, DE, USA). All other chemicals and reagents, unless otherwise specified, were analytical grade supplied by Merck KGaA, Darmstadt, Germany.

Linearity

Linearity was determined by preparation of various concentrations of two standards, 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid, dissolved in methanol (AR) at the concentration range of 7.8125–500 µg/ml derived from two-fold dilution. Calibration curves were plotted as the peak area versus the corresponding nominal analyte concentration. To obtain calibration curve equations and determination coefficients, the linear regression model was applied. The method is defined as linear

over the selected working range if it fits to a linear regression model giving a determination coefficient (R^2) value of greater than 0.9999.

Precision

Precision was evaluated by calculating the coefficient of variation (CV%) from mosses extracts at the concentration of 20 mg/ml. Intraday and Interday precisions were evaluated using three replicates over three non-consecutive days.

Accuracy

Accuracy was determined by analyzing the percent recovery of each compound at different concentration levels. Each moss extracts were prepared at 20 mg/ml, and three concentrations of each standard (0.1, 0.2, and 0.4 mg/ml) were added, with the results expressed as % recovery.

Sensitivity

Sensitivity was established by considering the standard deviation and slope of the calibration curve for determining the limit of detection (LOD) and limit of quantification (LOQ). These were calculated using the formulas $3.3\alpha/S$ and $10\alpha/S$, respectively, where α represents the standard deviation of the y-intercept and S signifies the slope of the calibration curve.

Quantitative analysis of active compounds in the extracts

HPLC analysis was performed on an Agilent 1200 series Variable Wavelength Detector (G1314D) instrument with a Sepax C18 column particle size of 5 µm (4.6 mm × 250 mm). The isocratic mobile phase system consisted of solvent A, B, and C in a ratio of 65:25:10, respectively, where solvent A was 0.1% orthophosphoric acid in water, solvent B was 0.1% orthophosphoric acid in methanol (HPLC), and solvent C was 0.1% orthophosphoric acid in acetonitrile (HPLC). The flow rate was 1.0 ml/min, the temperature was 30 °C, and the extracts at 10 mg/ml injection volume was 20 µl. The monitoring wavelength was 310 nm. The identification of each compound was based on the retention time comparing with authentic samples using Agilent ChemStation software.

COX-2 (human) inhibitor screening assay

The 0.5, 2.5 and 5 mg/ml of the extracts and both active compounds were investigated for anti-inflammatory activity using Human COX-2 ELISA Kit. Celecoxib (known COX-2 inhibitor) was used as positive control. This assay directly measures $PGF_{2\alpha}$ reduction of COX-derived PGH_2 produced in the COX reaction. The prostanoid product was quantified via ELISA using an antiserum specific to $PGF_{2\alpha}$. The % COX-2 inhibition values were then calculated.

Statistical analysis

The results were indicated as mean ± standard deviation (S.D.) from three replications ($n = 3$). The statistical analysis was performed using RStudio ver.4.2.1 [12]. Independent-sample t-test was performed at a significance level of 0.05 for two-treatment comparison. One-way ANOVA and Duncan's new multiple range test (DMRT) were evaluated at a significance level of 0.05 for multiple comparisons of means.

RESULTS AND DISCUSSION

Percent yields and the content of bioactive compounds in umbrella moss extracts

Plants synthesize secondary metabolites to adapt and respond to various biotic and abiotic stressors. These metabolites are influenced by environmental factors, including biological, physical, and chemical conditions. Variations in environmental conditions result in differences in the production of these compounds. Previous study demonstrated that *Hymenocallis littoralis* grown in controlled cultivation, with appropriate plant growth regulators, could produce higher quantity of lycorine compared to those found in nature, highlighting the potential for cultivated plants to yield bioactive compounds similar to or greater than wild counterparts [13]. Environmental conditions profoundly influence secondary metabolite accumulation in plants. Variations in temperature, humidity, light intensity, and altitude can regulate phenolic and flavonoid biosynthesis, as demonstrated in the study of *Hippophae rhamnoides*, where leaf flavonoids varied significantly across environmental gradients in northern China [14]. Similarly, cultivation under controlled conditions can enhance metabolite yield compared to natural habitats.

In this study, HPLC was employed to quantify the active compounds 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid in extracts of *R. giganteum* from both forest and tissue-cultured sources. The validated HPLC method showed linearity for 7,8-dihydroxycoumarin ($Y = 59.76X + 122.85$, $R^2 = 0.9996$) and *p*-hydroxycinnamic acid ($Y = 83.356X - 21.28$, $R^2 = 1.000$) over a concentration range of 7.8125–500 µg/ml (Fig. 2). LOD and LOQ for 7,8-dihydroxycoumarin were 0.05 µg/ml and 0.10 µg/ml, respectively, while for *p*-hydroxycinnamic acid were 0.025 µg/ml and 0.05 µg/ml, respectively. High recovery values (98.70–99.98%) and low relative standard deviations (intraday: 1.17–1.87%, interday: 1.64–1.76%) indicated the method's precision, accuracy, and repeatability (Table 1).

Analysis of the extracts revealed that tissue-cultured moss consistently produced higher quantities of the active compounds than forest-derived samples in the same concentration of extracts. Retention times for standards 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid were 4.183 and 6.086 min, respectively (Fig. 3A–C). Tissue-cultured moss

Table 1 The validated parameters of the HPLC system in this study.

Compound	R_T* (min)	R^2	Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Accuracy Added (µg/ml)	Accuracy % Recovery*	% RSD Intraday	% RSD Interday
7,8-dihydroxycoumarin	4.047 ± 0.15	0.9996	7.8125–500	0.05	0.10	100	99.57 ± 0.58	1.87	1.76
						200	98.70 ± 0.75		
						400	99.98 ± 0.89		
p-hydroxycinnamic acid	6.027 ± 0.03	1.000	7.8125–500	0.025	0.05	100	98.75 ± 0.87	1.17	1.64
						200	99.57 ± 0.43		
						400	99.87 ± 0.67		

* Data shows mean ± SD of triplicates.

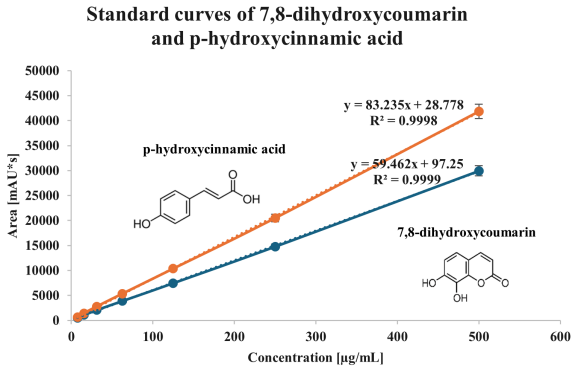

Fig. 2 HPLC standard curves for bioactive compounds, 7,8-dihydroxycoumarin (blue) and *p*-hydroxycinnamic acid (orange) dissolved in methanol.

extracts contained 6.16 ± 1.25 µg/ml of 7,8-dihydroxycoumarin and 17.63 ± 0.47 µg/ml of *p*-hydroxycinnamic acid, compared to 0.41 ± 0.03 µg/ml and 3.69 ± 0.06 µg/ml in forest-derived samples (Fig. 3D). These concentrations correspond to percent yields of 0.03% for 7,8-dihydroxycoumarin and 0.12% for *p*-hydroxycinnamic acid in tissue-cultured moss, compared to 0.002% and 0.02% in forest moss, respectively. This indicates that tissue-cultured samples contain approximately five times more 7,8-dihydroxycoumarin and fourteen times more *p*-hydroxycinnamic acid than forest-derived samples.

In vitro tissue culture provides a sterile, controlled environment independent of geographical or seasonal limitations, using nutrient-rich culture media to support their growth and development. This ensures consistent growth conditions, reducing variability in metabolite production and enabling year-round cultivation. In addition, stress induction such as abiotic stressors (e.g., UV light, drought, salinity) and elicitors (e.g., methyl jasmonate, fungal extracts) can be applied to mimic natural defense responses, stimulating plants to synthesize higher levels of secondary metabolites as protective mechanisms. Many studies support the potential of tissue culture techniques for the greater production of phenolic compounds [15]. Tissue culture also avoids overharvesting endangered medicinal plants by providing a sustainable alternative for metabolite production, particularly for slow-growing or low-yield species [15]. Previous studies have emphasized that *in vitro* culture systems can be strategically optimized to enhance the biosynthesis of bioactive compounds through the manipulation of growth regulators, pre-treatment strategies, or the application of elicitors. For example, Wongmaneeroj et al [15] investigated *Clinacanthus nutans* ("Phaya Yo") and demonstrated that varying concentrations of cytokinins and auxins, particularly thidiazuron and 1-Naphthaleneacetic acid, profoundly affected both biomass accumulation and the concentration of phenolic and flavonoid metabolites. Their results confirmed that specific combinations of plant growth regulators can serve as biochemical factors to activate secondary metabolic pathways, directly increasing the yield of pharmacologically relevant compounds. Similarly, Choonong et al [16] established that pre-treatment of *Eurycoma harmandiana* adventitious roots using physical drying and solvent conditioning enhanced the recovery of quassinoids and phenolic constituents, leading to a substantial improvement in anti-inflammatory activity as measured by suppression of COX-2 and nitric-oxide-mediated responses. This study highlighted that controlled stress induction prior to extraction can act as a metabolic trigger, enriching the chemical profile of plant materials for therapeutic use. In addition, Xiao et al [17] revealed that blue-light irradiation in *Amaranthus tricolor* seedlings altered morphological development, specifically inhibiting hypocotyl elongation, while simultaneously promoting the accumulation of anthocyanins and other stress-responsive metabolites. This finding underscores how light-quality manipulation can function as a non-chemical elicitor to enhance metabolite biosynthesis, providing a sustainable and precise tool for improving plant secondary-metabolite output under controlled conditions. Collectively, these studies demonstrate that environmental and hormonal modulation in *in vitro* systems can mimic natural stress responses and significantly amplify the production of valuable secondary metabolites. The present study's observation that tissue-cultured *R. giganteum* accumulated

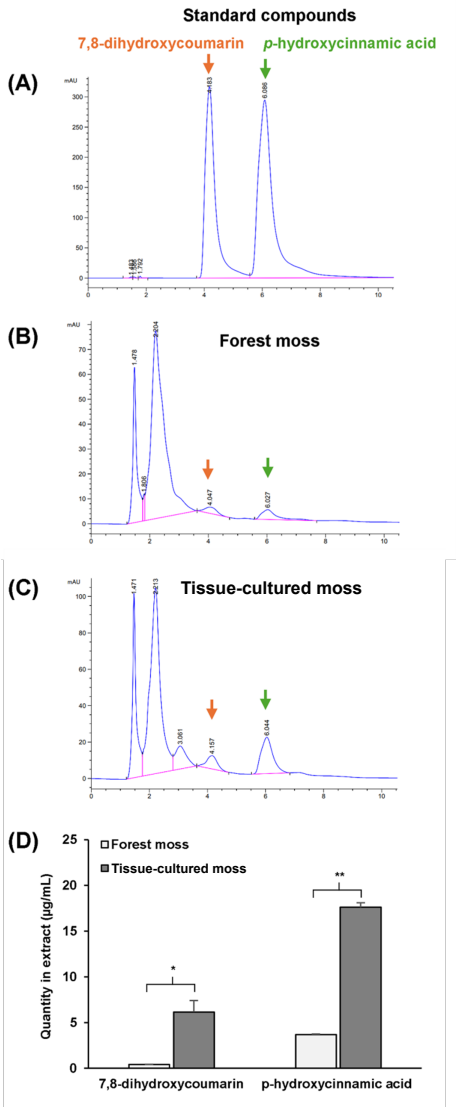

Fig. 3 Quantification of bioactive compounds using HPLC; HPLC profile of (A) standard compounds, (B) forest moss extract, and (C) tissue-cultured moss extract; (D) quantity of bioactive component in the moss extracts. Bar graphs show mean ± SD of triplicates. The quantity of each compound in both mosses is significantly different at $^{*}p < 0.05$ and $^{**}p < 0.01$ by independent-sample t-test.

substantially higher levels of 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid is therefore consistent with these reports. It supports the concept that carefully controlled *in vitro* propagation is not merely a conservation tool but also a scalable biotechnological platform capable of producing high-quality bioactive compounds for pharmaceutical development [18].

These findings suggest that tissue culture not only provides a sustainable method for propagating *R. giganteum* but also enhances the production of its

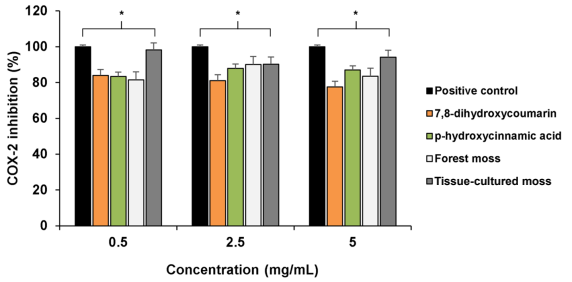

Fig. 4 The percent inhibition of COX-2 of two standard compounds and two types of moss extracts. Celecoxib (known COX-2 inhibitor) was used as a positive control. Data shows mean ± SD of triplicates. Treatments in the same concentration were significantly different at $^{*}p < 0.05$ by DMRT test.

bioactive compounds, offering potential advantages for pharmaceutical applications. Although this study only evaluated the effect of two well-known bioactive compounds, the complete profiles of bioactive compounds from both moss extracts should be further investigated to identify other potentially important compounds with anti-inflammatory properties.

Anti-inflammatory activity evaluated via COX-2 inhibitor screening assay

COX, also known as prostaglandin-endoperoxide synthase, is a crucial enzyme in the biosynthesis of prostaglandins (PGs), which mediate inflammatory responses. Proinflammatory PGs are associated with redness, pain, fever, and swelling, as well as the pathophysiology of various conditions, including stroke, cancer, allergies, asthma, arthritis, and Alzheimer's disease. COX enzymes exist in two isoforms; COX-1 and COX-2 that catalyze the transformation of arachidonic acid into PGs. While these isoforms share a common function, they differ significantly in their tissue distribution and roles in health and disease [19].

In this study, the anti-inflammatory potential of *R. giganteum* extracts was evaluated using a COX-2 (human) inhibitor screening assay. The performance of moss extracts was compared with standard phenolic compounds: 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid, which are also key bioactive components in *Lantana camara* methanolic extracts, a well-documented anti-inflammatory agent. For context, *L. camara* extract achieved 91.87% COX-2 inhibition, with an IC_{50} value of 4.06 ± 0.42 µg/ml [20].

R. giganteum extracts from both tissue-cultured and forest-derived sources demonstrated potent COX-2 inhibitory activity at concentrations of 0.5, 2.5, and 5 mg/ml (Fig. 4). The inhibitory efficacy ranged from 77.50% to 98.18%, with tissue-cultured extracts consistently outperforming their forest counterparts. Notably, the tissue culture extract exhibited the highest

COX-2 inhibition (98.18%), surpassing the activity of two standard compounds and the extract from forest moss at the same concentration. Furthermore, the data revealed that inhibitory activity did not vary significantly across the tested concentration range, indicating that a concentration as low as 0.5 mg/ml is sufficient to achieve significant COX-2 inhibition.

These findings highlight the promising potential of *R. giganteum*, particularly tissue-cultured moss, as a source of anti-inflammatory agents. However, this study has certain limitations, as it only examined COX-2 inhibition *in vitro*. Future research should involve testing the extracts in living cells to evaluate cytotoxicity and investigate the expression of inflammation-related genes, including metabolomic profiling and broader COX-2-relevant phytochemical screening. Such analyses are essential for advancing the development of natural extract-based anti-inflammatory therapeutics.

CONCLUSION

This study highlights tissue culture as a sustainable and innovative strategy to enhance the biosynthesis of anti-inflammatory metabolites in *Rhodobryum giganteum*. The tissue-cultured moss exhibited substantially higher levels of 7,8-dihydroxycoumarin and *p*-hydroxycinnamic acid—approximately five- and fourteen-fold greater than natural samples—and achieved 98.18% COX-2 inhibition. These results validate *in vitro* propagation as an eco-friendly and scalable platform for producing high-quality bioactive compounds and advancing the development of natural anti-inflammatory therapeutics.

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