Effect of *Vitis gracilis* Wall. administration on maximal swimming exercise apoptosis via cytochrome c in rat lung cells

**[Efecto de la administración de Vitis gracilis Wall. sobre la apoptosis del ejercicio máximo de natación via citocromo c en células pulmonares de rata]**

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**Abstract**

**Context:** The high mortality rate in people with lung disease indicates that this disorder requires immediate attention. It is established that excessive swimming can reduce the ability of lung cells to function and cause respiratory problems. *Vitis gracilis* Wall. is a traditional medicinal plant used by the Karo people of North Sumatra to improve stamina.

**Aims:** To analyze apoptosis via cytochrome c in serum and lung tissue after *V. gracilis* administration.

**Methods:** Rats that experienced excessive physical activity received six different treatments: two groups as negative and positive control groups, one vitamin C control group, and three groups orally administered with ethanolic extract of *V. gracilis* leaves at different doses. Immunohistochemistry, ELISAs, and TUNEL assays were used to assess study parameters.

**Results:** There were significant differences (p<0.05) in the expression of cytochrome c and apoptotic cells in the lung. Excessive swimming can increase TNF-α levels and decrease interleukin-10 (IL-10) in rats, but administration of *V. gracilis* produced nearly identical results to the positive control group and was not significantly different to the vitamin C group, indicating that *V. gracilis* was effective in reducing TNF-α levels and increasing IL-10.

**Conclusions:** Excessive swimming in rats can increase cytochrome c expression and apoptosis, whereas *V. gracilis* can reduce apoptosis that occurs via the intrinsic pathway through cytochrome c expression in the lungs.

**Keywords:** apoptosis; cytochrome c; lung; plant extract; *Vitis gracilis*.

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**Resumen**

**Contexto:** La elevada tasa de mortalidad en personas con enfermedades pulmonares indica que este trastorno requiere atención inmediata. Está demostrado que la natación excesiva puede reducir la capacidad de funcionamiento de las células pulmonares y causar problemas respiratorios. *Vitis gracilis* Wall. es una planta medicinal tradicional utilizada por el pueblo karo del norte de Sumatra para mejorar la resistencia.

**Objetivos:** Analizar la apoptosis vía citocromo c en suero y tejido pulmonar tras la administración de *V. gracilis*.

**Métodos:** Ratas que experimentaron una actividad física excesiva recibieron seis tratamientos diferentes: dos grupos como control negativo y positivo, un grupo control de vitamina C y tres grupos administrados por vía oral con un extracto etanólico de hojas de *V. gracilis* a diferentes dosis. Se utilizaron pruebas inmunohistoquímicas, ELISAs y TUNEL para evaluar los parámetros del estudio.

**Resultados:** Hubo diferencias significativas (p<0.05) en la expresión de citocromo c y células apoptóticas en el pulmón. La natación excesiva puede aumentar los niveles de TNF-α y disminuir la interleucina-10 (IL-10) en ratas, pero la administración de *V. gracilis* produjo resultados casi idénticos al grupo de control positivo y no fue significativamente diferente al grupo de vitamina C, lo que indica que *V. gracilis* fue eficaz para reducir los niveles de TNF-α y aumentar la IL-10.

**Conclusiones:** La natación excesiva en ratas puede aumentar la expresión del citocromo c y la apoptosis, mientras que *V. gracilis* puede reducir la apoptosis que se produce por la vía intrínseca a través de la expresión del citocromo c en los pulmones.

**Palabras Clave:** apoptosis; citocromo c; pulmón; extracto vegetal; *Vitis gracilis*. 

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INTRODUCTION

The Karo people of North Sumatra use a traditional medicinal plant called *Vitis gracilis* Wall. or “gagatan harimau”. This plant is a synonym of the species *Ampelocissus gracilis* (Wall.) Planch. (*Vitaceae* family), which is also effective for increasing stamina (Wasnis et al., 2022a). The results of phytochemical screening revealed the presence of glycosides, alkaloids, saponins, tannins, and flavonoids in the leaves of *V. gracilis*. Flavonoids, glycosides, phenols, amino acids, and organic acids are a few of the chemical elements of *V. gracilis* that can be used to make medicinal compounds (Wasnis et al., 2022b; Yamakawa et al., 1983). According to the Karo people, Sumatran tigers eat this plant to boost their endurance when hunting prey. The Karo people also use this herb as a traditional tonic to increase endurance, increase libido during intercourse, and speed up childbirth (Aththorick and Berutu, 2018).

The high mortality rate for people with lung disease indicates that this type of disorder requires immediate attention (Zanchi et al., 2010). This is due to the general public’s lack of knowledge about lung health. Excessive physical activity is harmful to lung tissue. However, excessive exercise increases proinflammatory cytokines like TNF-α while decreasing IL-10 (Calegari et al., 2017). Excessive exercise also raises malondialdehyde (MDA), a marker of lipid peroxidation in lung tissue. The functional properties of the lung define the histological structure (Zanchi et al., 2010). Excessive exercise can cause deformation of the respiratory portion, which develops from the ciliated cuboidal epithelium to the squamous epithelium (Beeler-Marfisi et al., 2020). Exercise alone, especially with maximum effort, can result in pulmonary edema. In several recorded cases, unilateral pulmonary edema developed while swimming, indicating an underlying hemodynamic cause (Calegari et al., 2017). This potentially fatal condition occurs when blood fluid leaks abnormally from the small vessels (pulmonary capillaries) of the lung into the alveoli (Beeler-Marfisi et al., 2020; Calegari et al., 2017).

Oxidative stress, such as when the body engages in excessive physical activity, can lead to lung apoptosis, resulting in irreparable cell damage (Thimmulappa et al., 2020). Apart from excessive exercise activities, some causes of cell apoptosis are extreme temperatures, hypoxia, exposure to chemicals, immune reactions, infectious agents, ischemia, and radiation (Kruk et al., 2019). Apoptosis has two intrinsic and extrinsic pathways, often occurring in molecular processes and resulting in caspase activation (Kruk et al., 2020). Activation of caspase proteins is a prerequisite for apoptosis. In the intrinsic pathway, in particular, cysteine proteases and cytochrome c carry out the catalytic process for initiating the pathway in response to oxidative stress (Thimmulappa et al., 2020). It has been demonstrated that the administration of 100 mg/kg body weight (BW) *V. gracilis* improves and decreases inflammation in lung tissue. Less lung cell apoptosis occurs at higher doses of *V. gracilis* (Wasnis et al., 2022b).

This study aimed to look into the histological changes caused by treatment with *V. gracilis* leaves, as well as the associated changes of apoptosis via cytochrome c expression in lung tissues and changes in TNF-α and IL-10 levels in serum from overexercising rats. Our findings are relevant for further developing *V. gracilis* as a potential lung protectant.

MATERIAL AND METHODS

Chemicals and reagents

The chemicals and reagents used were IL-10 monoclonal antibodies (JES5-16E3, eBioscience™, ThermoFisher Scientific, United States). TNF alpha rat ELISA kit (#KRC3011, ThermoFisher Scientific, United States), anti-cytochrome c antibody (#ab218312, Abcam, United States), Click-i™ TUNEL assay IHC detection kit (C10625, ThermoFisher Scientific, United States). Ethanol p.a., distilled water, ethanol (96%), glacial acetic acid and sodium tripolyphosphate were from Merck (United States). DAB (#94524-3, 3'-diaminobenzidine, Sisco Research Laboratories Pvt. Ltd., India).

Vegetal material

The *V. gracilis* plants were collected from the Tangkahan area and were prepared in two villages, Sei Serdang and Namo Sialang, in Batang Serangan District, Northern Sumatra Province, Indonesia (position coordinates X = 395928.28 and Y = 408308.48). This plant has been identified by the plant Systematics Laboratory herbarium team at the University of Sumatera Utara (USU) with Voucher Number 185/MEDA/2020, verifying that this plant is *Vitis gracilis* Wall.

Preparation

The leaves and twigs of *V. gracilis* were separated and then cleaned of adhering dust particles, dried for seven days at room temperature, and then smoothed.

Micro-colloidal *V. gracilis* preparation

The particle size of the extract was adjusted using High Energy Milling (HEM), (HEM-E3D, PT Nano-
tech Herbal, Indonesia) with a ball milling mass of 1:20 and milling times of 3, 6, or 9 h (Situmorang et al., 2023). Samples of *V. gracilis* and alumina milling balls were weighed in advance and transferred into the milling vial in the appropriate ratio. The HEM machine was operated using a specific time variation pattern for each run of 10 min on and then 1 h off. The milling time was applied repeatedly until the specified accumulated time was reached.

**Scanning Electron Microscopy (SEM) of micro-colloidal *V. gracilis***

Micro-colloidal *V. gracilis* was observed using SEM (JSM-6390A, Tokyo, Japan) with 5000× magnification. Particle size analysis (PSA) (SALD-MS23, LabX, Japan) was performed using a water diluent.

**Extraction**

50 g of *V. gracilis* was macerated in 2 L of 96% technical ethanol at room temperature for 24 h. The maceration products were filtered with a vacuum pump and a Buchner funnel. Using the same procedure, the residue from the filtered plants was macerated for twice as long as the first step. After being concentrated in a rotary evaporator at 38°C (N-1200 BS Series Evaporator, EYELA, China), the concentrated ethanol extract was dried for 8 h to create a solid ethanol extract using a freeze dryer (CHRIST Alpha 1-2 LDplus). Micro-colloidal extracts of *V. gracilis* were prepared at three dose levels: 100, 125, and 150 mg/kg BW (Midoen et al., 2023).

**Animals**

This study used 36 male Wistar rats supplied by the Biology Laboratory at Universitas Sumatera Utara. Male rats weighed 180–200 g and were 10–15 weeks old. Six groups of male rats were formed, each containing six animals. Experimental animals were acclimatized in animal houses at the Animal Physiology Laboratory in USU’s Biology study program. The houses, which were pre-cleaned by irradiation, were maintained on a 12 h dark/12 h light cycle, with humidity levels of 35–60% for 2 weeks. The male rats were given unrestricted access to water, maize, and pellets. For the experiments, the rats were placed into a 40 cm by 30 cm plastic container. The study was carried out with the approval of the USU FMIPA Medan’s Health Research Ethics Committee (No.0908/KEPH-FMIPA/2022).

**Study design**

A Completely Randomized Design (CRD) was used in these studies. As a form of physical exercise, the rats were subjected to excessive swimming. Swimming took place five times per week at a pace of 7–15 meters per minute over the course of a 30-day training program (i.e., a duration of one month). During 13 training days, the average time required to find the hidden platform – the escape latency – was calculated. The studies were conducted in six treatment groups: G- was a negative control, G+ contained rats that underwent excessive swimming activities, and GVtc represented rats that swam excessively and, for comparison, were administered 0.2 mg/kg BW vitamin C. The G100, G125, and G150 groups contained rats that swam excessively and were given 100, 125, or 150 mg/kg BW *V. gracilis*, respectively. Following euthanasia with ketamine, the lungs were extracted for dissection and analysis using the TUNEL assay and immunohistochemistry, and the blood was taken for ELISA testing.

**Measuring the levels of IL-10 and TNF-α**

Quantitative analysis of IL-10 and TNF-α levels in serum was performed using the TNF-α and IL-10 ELISA kits (ThermoFisher Scientific, United States) with three replicates, as directed by the manufacturer. For the separation of serum and plasma, a 1.5 mL tube sample was placed into an EDTA tube. The samples were then placed in EDTA-free microcentrifuge tubes and centrifuged (DTS-6A (2) low-speed centrifuge, Tianjin, China) to separate the serum and plasma. After that, the wash solution was prepared, and the plate was washed four times with the wash solution. Then 100 μL of the diluted enzyme was added to each well, and the plate was wrapped in foil and placed in the dark. The plate was then incubated with 100 μL TMB Substrate Solution (chromogen substrate) for 10 minutes at room temperature in a dark place. After incubation, 100 μL of stop solution was added to each well, and then the ELISA reader (Well Reader-Elisa Reader, R-Biopharm, Germany) was used to measure the absorbance values at a wavelength of 450 nm.

**Immunohistochemistry of cytochrome c**

Lung tissue was made into paraffin blocks and then stained using immunohistochemistry techniques to examine the histological differences that occur with changes in cytochrome c expression following administration of *V. gracilis* leaves. Lungs fixed in formalin were immersed in xylol for 15 min. After 5 min each in 96% and 70% pure alcohol, the tissues were washed with distilled water (Ilyas et al., 2022a; Irianti et al., 2020). The hematoxylin stain was dripped onto the slide and left for 5 min. The slide was then rinsed with distilled water for three minutes and stained with eosin for 1 min (Irianti et al., 2020). To improve the results, slides containing lung organs were dried with graded alcohol, namely 70, 96, and 100%, and then soaked in xylol (Ilyas et al., 2022b). To reduce endogenous peroxidase activity, 5 μm thick paraffin-
embedded lung organ slices were deparaffinized and treated for 30 min with 1% H2O2 in methanol. 0.01 M Tris-buffered saline (TBS pH 7.4) was then used to wash the slides (Situmorang et al., 2023). Affinity antigen-purified cytochrome c antibodies and polyclonal antibodies were applied to tissue slices (Simanullang et al., 2022a; 2022b).

**Tunel assay**

Paraffin-embedded lung tissue was sliced using a microtome 4–5 µm thick. Slides containing lung tissue were rehydrated in graded ethanol before washing for 5 min in 0.85% NaCl and PBS, with the addition of proteinase K (20 mg/mL) at room temperature for 15 min. The rTdT reaction mixture was added to the slides for the final labeling reaction at 37°C for 1 h. The lung tissue was immersed in a buffer solution to stop the rTdT enzyme reaction (room temperature) and was then washed with PBS for 5 min. Hydrogen peroxide 0.3% in PBS inhibited endogenous peroxidases. Streptavidin-HRP solution was applied to the tissue, and the chromogenic substrate DAB was applied. Each slide was cleaned three times with 100% xylene after being dehydrated with ethanol for 5 min (Manurung et al., 2021; Situmorang et al., 2021a). The slides were observed using an AxioCam ERC 5s microscope (Germany).

**Statistical analysis**

Evaluation of data using the mean ± standard deviation (SD) was carried out by ANOVA and Duncan Posthoc tests using the SPSS 25 program. For categorical (ordinal) or numerical data that were not normally distributed, the Kruskal-Wallis and Mann-Whitney tests were used.

**RESULTS**

**Scanning Electron Microscopy (SEM) of *V. gracilis* preparations**

Microscopic examination at 40× magnification revealed *V. gracilis*-like black crystals, and SEM examination at 5000× magnification revealed that it resembled a shadow (Fig. 1). The *V. gracilis* nanoherbs had an average diameter of 344.62 µm according to PSA results with water diluent.

**Effect of *V. gracilis* administration on TNF-α levels in excessively swimming rats**

TNF-α levels could be raised by excessive swimming. The administration of *V. gracilis* resulted in a significant difference in TNF-α levels in the rat model of excessive exercise (p<0.05) compared to the positive control group, as shown in Fig. 2. According to the ANOVA analysis, the dose of 150 mg/kg BW (the highest dose) produced the lowest TNF-α levels, but rats dosed with 100 and 125 mg/kg BW exhibited high TNF-α levels, which when compared to the positive control were insignificant (p>0.05). A 150 mg/kg BW dose produced nearly identical results to the positive control group and did not differ significantly from the vitamin C (0.02 mg/kg) group.
Effect of *V. gracilis* administration on IL-10 levels in excessively swimming rats

The administration of *V. gracilis* resulted in a significant difference in IL-10 levels in the rat model of excessive exercise (p<0.05) compared to the untreated control group. According to ANOVA analysis, the highest dose of 150 mg/kg BW had the same high IL-10 levels as the positive control and vitamin C groups. However, when the doses of 100 mg/kg BW and 125 mg/kg BW were compared to the positive control, they showed lower levels of IL-10, although this observation was not statistically significant. As shown in Fig. 3, the highest dose (150 mg/kg BW) had the same effectiveness in increasing IL-10 levels as vitamin C (0.02 mg/kg) administration.

Histological changes of cytochrome c on lungs tissue after *V. gracilis* administration

The administration of *V. gracilis* resulted in a significant difference in cytochrome c expression in the overexercising rat model (p<0.05) (Fig. 4). The highest dose of 150 mg/kg BW had the same cytochrome c expression score as the vitamin C group, according to statistical analysis. The 125 mg/kg BW dose produced the same results as the control group. The lung histology showed that giving overworked rats *V. gracilis* can reduce cytochrome c expression. In the histology of excessively swimming rats, it can be seen that the alveolar areas experience widening and stretching, affecting the interstitial lung tissue and even decreasing lung function. Such structural changes can impact the mechanism of alveolar gas exchange in the lungs. There was reduced inflammation at the lower doses, 100–125 mg/kg BW, but alveolar membrane cells were largely unnuccleated, with surrounding endothelial cells missing. At a 150 mg/kg BW dosage, the alveolar membrane appeared structured, with normal nucleation, and lined with normal endothelial cells. As shown in Fig. 5, the highest dose (150 mg/kg BW) was as effective as vitamin C (0.02 mg/kg) administration at mitigating structural impairment of the lung.

Histological changes of apoptotic lungs tissue after *V. gracilis* administration

The administration of *V. gracilis* resulted in a significant difference in the level of apoptotic markers (as determined by the TUNEL assay) in the overexercising rat model (p<0.05) (Fig. 6). The highest dose, 150 mg/kg BW, produced a TUNEL expression score indicating the same level of apoptosis as the vitamin C (0.02 mg/kg) group, according to statistical analysis. The extent of apoptosis was the same for 100 and 125 mg/kg BW doses. Although it was not pronounced, the lowest dose (100 mg/kg BW) showed reduced apoptosis, occurring mainly in the alveolar sacs and parts of the interalveolar septum. Increased apoptosis correlated with the severity of inflammation. However, when the dose was increased, the balance between the alveoli was loosened, and apoptosis began to decrease in the 125 mg/kg BW group. Cell stretching persisted in the 150 mg/kg BW group but was less pronounced than in the lower dose and was accompanied by a sharp drop in apoptosis. The
highest dose of *V. gracilis* in rats could reduce apoptosis, as shown in the lung histology results (Fig. 7).

**DISCUSSION**

The average diameter of the *V. gracilis* particles was 344.62 µm, according to the PSA analysis (Fig. 1). We have downsized this plant extract to nano or micro-colloidal size to improve its effectiveness. Micro-colloidal refers to particles with a size exceeding 100 µm. Plant nano- and micro-colloidal particles have improved pharmacological activity and dispersion in tissue macrophages, delivery, and protection against physical and chemical deterioration. This particle size favors solubility, bioavailability, stability, and decreased toxicity (Müller et al., 2011). Nanoparticle materials have many benefits due to their small size and physicochemical characteristics. Some of these features can be modified by regulating size, chemical composition, surface, and particle contact (Müller et al., 2011). Based on the outcomes of PSA tests, the distribution of the average particle size in a sample can indicate the state of the sample as a whole (Rhyn-
er et al., 2006). Changing to a smaller size can result in a larger surface area, thereby promoting adhesion and increasing the spread (Situmorang et al., 2021b). Such size changes in the preparations of plants such as *V. gracilis* provide superior properties that other types of materials do not have, namely a much larger surface area to volume ratio under normal conditions (Situmorang et al., 2021b). These surface properties are critical for drug delivery to receptors or target masses within the cell (Müller et al., 2011; Situmorang et al., 2021b).

TNF-α is a cytokine that rises immediately after tissue injury, including in the lungs. Excessive swimming raises TNF-α levels in rats. *V. gracilis* administration produced nearly identical results to the positive control group and did not significantly differ from the comparison group or the vitamin C group, indicating that it effectively lowered TNF-α levels.

Asthma, sarcoidosis, acute respiratory distress syndrome, and pulmonary interstitial fibrosis are a few inflammatory lung illnesses that involve increased TNF-α levels (Bohr et al., 2017). Tumor necrosis factor receptor (TNFR) family members from at least 29 different species are known to participate in the complicated chain of biological events of TNF-α signaling (Lee et al., 2021). This cytokine family has key roles in organogenesis and has advantageous and protective effects on innate immunity, hematopoiesis, and other biological processes under physiological homeostatic conditions (I-Ta and Chuen-Mao, 2012). TNFR superfamily members also involve cell proliferation, survival, and apoptosis signaling mechanisms (Bohr et al., 2017). The development of many diseases, including lung ailments, is largely dependent on inflammatory and prooxidative responses, both of which are exacerbated by excess TNF-α (Lee et al., 2021).

**Figure 7.** Histology change of lung on apoptosis after *V. gracilis* administration.

| G-: negative control; G+: Swim rats; G.Vit.C: Swim rats + Vitamin C 0.02 mg/kg BW; G100: Swim rats + *V. gracilis* 100 mg/kg BW; G125: Swim rats + *V. gracilis* 125 mg/kg BW; G150: Swim rats + *V. gracilis* 150 mg/kg BW. Yellow arrows: apoptotic cells (40×). |
Vitamin C can reduce IL-10 levels in the lungs during excessive swimming. Vitamin C can regulate the inflammatory status by reducing IL-6 and hs-CRP in obese patients with hypertension and/or diabetes (Laurer et al., 2021). Daily oral administration of vitamin C may also provide potential health benefits by modulating inflammatory and anti-inflammatory gene expression profiles (Fesahat et al., 2022; Laurer et al., 2021). IL-10 is known as a cytokine inhibitor (Dolch et al., 2019). IL-10 functions as an anti-inflammatory cytokine during infection, inhibiting the production of several pro-inflammatory cytokines, and inhibiting the role of macrophages and dendritic cells in assisting T-cell activation, so it is immunosuppressive and reduces the likelihood of tissue damage brought on by an overactive immune response (Wong et al., 2020). Th2 cells, CD4+ T cell subsets including Th1 and Th17, B cells, neutrophils, macrophages, and some dendritic cell subsets all produce IL-10 (Dolch et al., 2019). IL-10 can inhibit Th1 cytokine production by preventing myeloid cells, like macrophages and dendritic cells, from activating Th1 cells. IL-10 is also described as having anti-inflammatory and antioxidant characteristics (Wong et al., 2020). It has been demonstrated that the anti-inflammatory antioxidant IL-10 interacts with oxidant-related pathways, which regulate inflammatory processes (Wong et al., 2020).

Excessive swimming in rats can increase cytochrome c expression and apoptosis, whereas *V. gracilis* can reduce apoptosis in the lung. Two signaling cascades mediate apoptosis: intrinsic and extrinsic apoptotic pathways. The extrinsic pathway is due to extracellular signaling via death receptors, while the intrinsic pathway is caused by damage to cellular homeostasis. Cells undergo intrinsic apoptosis as a result of mitochondrial outer membrane permeabilization (MOMP) activates cytochrome c (Redza-Dutordoir and Averill-Bates, 2016). MOMP causes proapoptotic elements to become trapped in the cytoplasm of the mitochondrial intermembranous space, including cytochrome c and the second mitochondria-derived activator of caspases (SMAC) (Krüger et al., 2009). Caspase 9 is activated by the binding of cytochrome c to apoptotic protease activating factor 1 (APAF1), and SMAC prevents caspase activation by blocking the cytoplasmic inhibition of apoptotic protein (Ilyas et al., 2021). Administration of *V. gracilis* at 150 mg/kg BW can improve lung histology while reducing inflammation and apoptosis by cytochrome c via the intrinsic pathway. This effect may be due to changes in the size of the *V. gracilis* preparations and the presence of plant antioxidants such as glycosides, alkaloids, saponins, tannins, and flavonoids (Wasnis et al., 2022a). Therefore, this plant effectively reduces inflammatory and apoptotic cells through the intrinsic pathway by suppressing cytochrome c to regenerate lung cells caused by excessive swimming activity.

**CONCLUSION**

Excessive swimming can increase TNF-α levels and decrease IL-10 in rats, but administration of *Vitis gracilis* Wall. was successful in reducing TNF-α levels and increasing IL-10. Excessive swimming in rats can increase cytochrome c expression and apoptosis, whereas *Vitis gracilis* Wall. can decrease apoptosis via the intrinsic pathway through the histological expression of cytochrome c.

**CONFLICT OF INTEREST**

The authors declare no conflicts of interest.

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