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Effect of *Angelica keiskei* leaves extract against overtraining-related oxidative stress and hormonal changes in male mice

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ABSTRACT

Introduction: Overtraining may accelerate the aging process due to the accumulation of oxidative damage and a progressive decline in hormone synthesis and action. The extract of *Angelica keiskei* leaves contains high secondary metabolites with remarkable antioxidant capacity. This study aimed to investigate the effect of *A. keiskei* leaves extract against overtraining-related oxidative stress and hormonal changes in male mice.

Methods: In this experimental experiment thirty male BALB/c mice were randomly divided into five equal-sized groups. The negative control (NC) group received only 1 mL of water by orogastric gavage/day as a placebo. The overtrained (OT) group received 1 mL of placebo orally and a forced swimming regimen. The treatment groups received 20 (T20), 50 (T50), or 100 (T100) mg/kg BW/day of *A. keiskei* leaves extract and a forced swimming regimen. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), cortisol, insulin-like growth factor 1 (IGF-1), and testosterone were measured before and after treatment for 28 days using the ELISA method.

Results: The OT group experienced depletion in SOD ($P<0.001$), IGF-1 ($P<0.01$), and testosterone ($P<0.05$) levels, as well as elevation in MDA ($P=0.001$) and cortisol ($P<0.001$) levels. The administration of *A. keiskei* leaves extract in the T100 group significantly increased SOD ($P<0.001$), reduced MDA ($P<0.001$), decreased cortisol ($P<0.001$), elevated IGF-1 ($P<0.001$), and improved testosterone ($P<0.001$) compared to the OT group.

Conclusion: The present findings clearly indicate that *A. keiskei* extract mitigates oxidative stress and hormonal dysregulation associated with overtraining, suggesting its potential as an anti-aging and adaptogenic supplement.

Implication for health policy/practice/research/medical education:
The findings have significant implications for developing pharmacological strategies to support endocrine and metabolic health in both physically active individuals and aging populations. Further research is warranted to elucidate the underlying molecular mechanisms, determine optimal dosing regimens, and assess the long-term safety of *A. keiskei* treatment.

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Introduction

One of the most extensively studied contributors to the aging process is the accumulation of oxidative damage driven by elevated levels of reactive oxygen species (ROS). While ROS are routinely produced as by-products of normal cellular metabolism, an imbalance favoring ROS over the body's antioxidant defenses results in oxidative stress, which ultimately leads to cellular damage and senescence (1). Although regular, moderate-intensity exercise induces low levels of ROS that play beneficial roles in cell signaling, high-intensity or exhaustive exercise significantly increases ROS production, potentially disrupting tissue homeostasis, particularly in skeletal muscle (2). During vigorous physical activity, oxygen consumption can rise by 100- to 200-fold, thereby accelerating ROS generation and initiating a cascade

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of oxidative chain reactions (3). The extent of ROS accumulation depends on the type, intensity, and duration of exercise. Low levels of ROS may serve as second messengers to promote growth factor signaling and adaptive responses (4); however, overtraining surpasses this adaptive threshold. It results in elevated levels of lipid peroxidation products such as malondialdehyde (MDA), along with a marked depletion of endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), collectively indicating the onset of oxidative stress (5). We previously reported that overtraining increases serum protease activity and accelerates cellular senescence, further supporting its pro-aging effects (6).

In parallel with oxidative stress, aging is also characterized by a gradual decline in hormonal synthesis, bioavailability, and receptor sensitivity, all of which contribute to reduced physiological resilience and increased susceptibility to chronic diseases (7). Key hormones affected by aging include cortisol, insulin-like growth factor 1 (IGF-1), and testosterone, each of which serves as a relevant biomarker of age-related endocrine dysfunction (8,9). It is well documented that aging is associated with elevated cortisol levels (10), reduced circulating IGF-1 (11), and diminished free testosterone (12). Similar hormonal profiles have been observed in states of chronic overtraining, indicating that excessive physical exertion may phenocopy certain endocrine aspects of aging (13).

Antioxidant supplementation has been proposed as a potential strategy to mitigate oxidative damage associated with overtraining. *Angelica keiskei* (commonly known as Ashitaba), a medicinal plant with high antioxidant capacity, has demonstrated broad pharmacological effects, including antiviral, antibacterial (14), anti-diabetic (15), anti-hyperlipidemic (16), and anti-thrombotic (17) properties. Our previous studies have also shown its efficacy in alleviating depressive symptoms (18) and protecting against neurodegeneration (19). Despite its wide-ranging benefits, the specific role of *A. keiskei* in counteracting oxidative stress and hormonal dysregulation due to overtraining remains underexplored. Therefore, this study aimed to evaluate the therapeutic potential of *A. keiskei* leaves extract in modulating oxidative stress markers and endocrine parameters in an overtrained mouse model.

Materials and Methods

Preparation of *A. keiskei* leaves extract

Fresh leaves of *Angelica keiskei* were obtained from Ashitaba Farm, Trawas, Kabupaten Mojokerto, East Java, Indonesia (postal code: 61375). The plant specimen was authenticated by the Research Center for Biology, Indonesian Institute of Sciences (specimen no. 1982/IPH.1.01/If.08/VI/2020). Upon collection, the leaves were thoroughly rinsed with clean water to remove debris. The extraction process followed a previously established protocol (18). Briefly, the leaves were air-dried in the shade, ground into a coarse powder, and sieved through a 40-mesh filter. The resulting powder was macerated in 5 liters of 70% ethanol for 48 hours at room temperature. After filtration, the solvent was evaporated under reduced pressure using a rotary evaporator. The concentrated ethanol extract was then stored at –20 °C until use.

Animals and experimental protocols

Thirty healthy male BALB/c mice (3 months old, body weight 25–30 g) were obtained from iRATco Veterinary Laboratory, Bogor, Indonesia. Mice were housed under standard laboratory conditions (12 hours light–dark cycle, ambient temperature 22–24 °C, relative humidity 45%–65%) with ad libitum access to food and water. Following a 7-day acclimatization period, animals were randomly assigned into five groups (n = 6 per group). The negative control (NC) group received only 1 mL of water by orogastric gavage/day as a placebo. The overtrained (OT) group received 1 mL of placebo orally and a forced swimming regimen. The treatment groups received 20 mg/kg BW/day (T20), 50 mg/kg body weight (BW)/day (T50), or 100 mg/kg BW/day (T100) of *A. keiskei* leaves extract and a forced swimming regimen. The overtraining regimen consisted of forced swimming sessions lasting approximately 60 minutes, or until the mice exhibited signs of exhaustion (defined by loss of coordinated swimming and submersion for >10 seconds) (6). This procedure was repeated daily for 28 consecutive days. Swimming was conducted in a tank measuring 80 cm (L) × 50 cm (W) × 90 cm (H), filled to a depth of 75 cm with water maintained at 33–35 °C.

The biochemical analysis

Blood samples were collected under anesthesia (ketamine 100 mg/kg and xylazine 20 mg/kg, intraperitoneally) via the medial canthus using 100 µL heparinized capillary tubes (Jorgensen Laboratories, Loveland, CO, USA). Serum was separated by centrifugation at 1000 × g for 10 minutes at 4 °C. Serum levels of SOD (Cat. No. E2608Mo, BT. Lab), MDA (RE10165, Reed Biotech), cortisol (EA0010Ge, BT. Lab), IGF-1 (ab108874, Abcam), and testosterone (ab285350, Abcam) were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to compare differences among groups. Pre- and post-treatment comparisons within groups were performed using paired sample t-tests with Bonferroni correction. Statistical significance was set at $P < 0.05$.

Results

Effect of *A. keiskei* leaves extract on oxidative stress markers

At baseline, no significant differences in SOD or MDA levels were observed among the experimental groups ($P>0.05$). After 28 days of intervention, the OT group exhibited a significant reduction in SOD levels compared to the NC group ($P<0.001$), consistent with exercise-induced oxidative stress. Administration of *A. keiskei* extract at doses of 50 mg/kg (T50) and 100 mg/kg (T100) significantly restored SOD levels relative to the OT group ($P<0.001$ for both), while the 20 mg/kg dose (T20) did not result in a significant change ($P>0.05$) (Figure 1A). MDA levels, indicative of lipid peroxidation, were significantly elevated in the OT group compared to the NC group ($P<0.001$). Treatment with *A. keiskei* extract at all tested doses (T20, T50, and T100) led to a significant reduction in MDA levels compared to the OT group ($P<0.001$ for all) (Figure 1B). These findings may support the hypothesis that overtraining induces oxidative stress, as evidenced by SOD depletion and MDA accumulation, and that *A. keiskei* extract ameliorated these effects in a dose-dependent manner.

Effect of *A. keiskei* leaves extract on age-related hormones

No significant differences in serum cortisol, IGF-1, and testosterone levels were observed among groups at baseline ($P>0.05$). Following 28 days of overtraining, the OT group displayed significantly elevated cortisol levels ($P<0.001$), along with decreased IGF-1 ($P<0.01$) and testosterone levels ($P<0.001$) compared to the NC group. Treatment with *A. keiskei* extract at all doses (20, 50, and 100 mg/kg BW/day) significantly reduced cortisol levels compared to the OT group (Figure 2A). In contrast, a significant elevation in IGF-1 levels was observed only in the T100 group ($P<0.001$ vs. OT), with no significant changes in the T20 or T50 groups (Figure 2B). Testosterone levels were significantly improved in both the T50 and T100 groups ($P<0.001$ vs. OT), whereas the T20 group did not show a statistically significant effect ($P>0.05$) (Figure 2C). These results indicate that overtraining

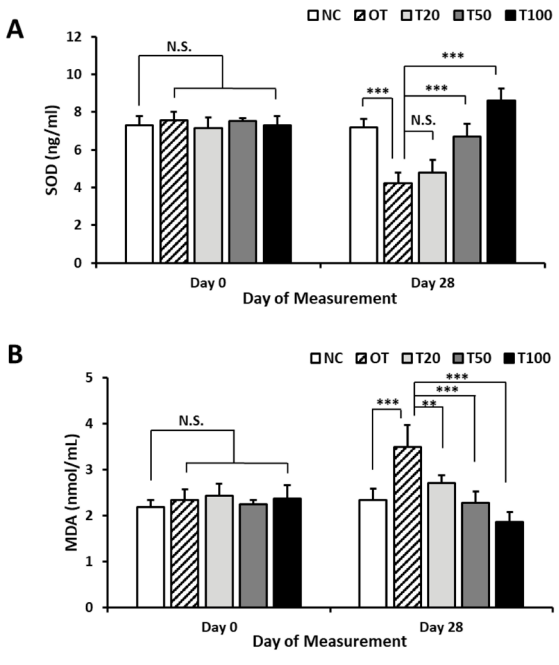

Figure 1. Effect of *Angelica keiskei* leaves extract on oxidative stress markers (mean ± SD, *n*=6). (A) Total SOD and (B) total MDA of the NC, OT, T20, T50, and T100 groups. $^{**}P<0.01$, $^{***}P<0.001$; One-way ANOVA followed by Tukey's post-hoc test. IGF-1: insulin-like growth factor-1; SD: standard deviation, NC: negative control group, OT: overtrained group; T20, T50, and T100: treatment groups of 20, 50, and 100 mg/kg BW/day of *A. keiskei* leaves extract, respectively.

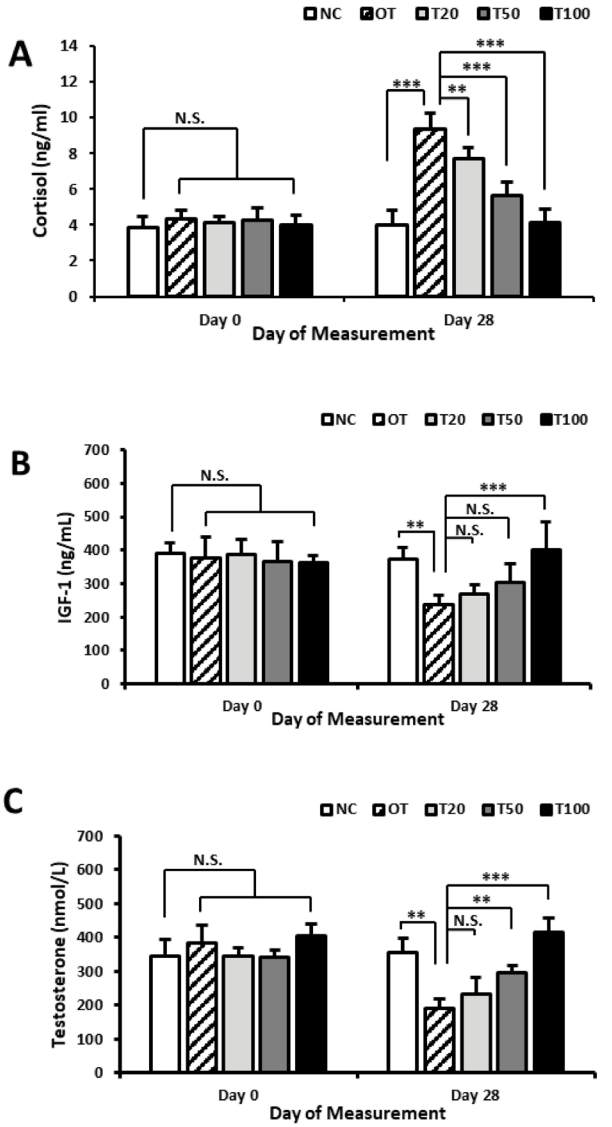

Figure 2. Effect of *Angelica keiskei* leaves extract on age-related hormones (mean ± SD, *n*=6). (A) Total cortisol, (B) total IGF-1, and (C) total testosterone of the NC, OT, T20, T50, and T100 groups. $^{**}P<0.01$, $^{***}P<0.001$; One-way ANOVA followed by Tukey's post-hoc test. IGF-1: insulin-like growth factor-1; SD: standard deviation, NC: negative control group, OT: overtrained group; T20, T50, and T100: treatment groups of 20, 50, and 100 mg/kg BW/day of *A. keiskei* leaves extract, respectively.

induces characteristic hormonal alterations associated with accelerated aging, including hypercortisolemia and suppression of anabolic hormones. Treatment with *A. keiskei* extract attenuated these hormonal disturbances in a dose–dependent manner.

Discussion

The present study demonstrated that 28 days of exhaustive training in mice induced a state of oxidative stress and hormonal dysregulation, the hallmark features of accelerated aging. These effects were characterized by a decrease in the antioxidant enzyme SOD, an increase in the lipid peroxidation marker MDA, and alterations in age-related hormones, including elevated cortisol and reduced IGF-1 and testosterone. Importantly, these changes were significantly attenuated following treatment with *A. keiskei* leaves extract, indicating its protective role in counteracting overtraining-induced physiological decline.

Overtraining is a well-recognized contributor to oxidative stress and aging-related pathologies (20-22). While moderate physical activity is known to delay aging by enhancing telomere stability (22), promoting stem cell function (23), and supporting endocrine balance (24), excessive training has been shown to exert detrimental effects. Animal models of overtraining have demonstrated metabolic and molecular profiles that closely resemble natural aging (6,25), potentially increasing the risk of chronic diseases such as cardiovascular, neurodegenerative, and neoplastic disorders (26-28).

The protective effects observed in this study are likely attributable to the rich phytochemical content of *A. keiskei* extract. Our previous analyses of the same extract revealed high concentrations of flavonoids (11 523.66 mg/g), polyphenols (3100.41 mg/g), and tannins (10 569.44 mg/g), along with a potent antioxidant activity (IC_{50}: 80.16 mg/L) (18). Flavonoids are known to act as direct radical scavengers and indirect modulators of endogenous antioxidant systems, including SOD (29). The observed prevention of SOD depletion and MDA accumulation in the treated groups supports the hypothesis that *A. keiskei* mitigates oxidative damage and preserves cellular integrity. These results are consistent with previous studies showing that flavonoid-rich plant extracts reduce lipid peroxidation and enhance antioxidant defenses in both in vitro and in vivo models (30).

Mechanistically, the antioxidant effects of *A. keiskei* are multifactorial. Its bioactive constituents are known to regulate key signaling pathways involved in oxidative defense, inflammation, and apoptosis (31). The extract may exert its effects through direct ROS scavenging or by activating endogenous antioxidant enzymes such as SOD (32). Additionally, its anti-inflammatory properties may further contribute to the observed protective outcomes, given the close interplay between oxidative stress and inflammation (33). While the current study did not assess markers such as Nrf2, cytokines, or heat-shock proteins, these pathways warrant further investigation to fully elucidate the extract's mechanism of action.

Beyond its antioxidant properties, *A. keiskei* also exhibited regulatory effects on endocrine function, a central component in the aging process. Overtraining is known to activate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in elevated cortisol levels and suppression of anabolic hormones like IGF-1 and testosterone (13). These hormonal disturbances can accelerate tissue degeneration and mirror age-related endocrine decline (34). Consistent with these findings, our study demonstrated significant increases in cortisol and reductions in IGF-1 and testosterone in overtrained mice—effects that were reversed by *A. keiskei* extract treatment.

Cortisol, a well-established marker of physiological stress, is typically elevated under chronic overtraining conditions and contributes to muscle catabolism, immune dysfunction, and metabolic imbalances (35-37). The ability of *A. keiskei* to reduce cortisol levels in this context suggests its adaptogenic potential, likely through modulation of the HPA axis. This is supported by previous research demonstrating the antidepressant and neuroprotective effects of *A. keiskei* in stress-related models (18,19).

IGF-1 plays a critical role in muscle repair and maintenance. Its decline is commonly associated with sarcopenia and impaired recovery capacity (38). The suppression of IGF-1 in overtrained animals observed here mirrors the findings in elite athletes undergoing chronic physical stress (39). Restoration of IGF-1 levels by *A. keiskei* suggests a potential for enhancing anabolic signaling. A recent study in *Drosophila* demonstrated that *A. keiskei* could extend lifespan via the insulin/IGF-1 pathway (40), though further in vivo validation in mammals is required. To our knowledge, this study is the first to report the role of *A. keiskei* extract in modulating IGF-1 in a mammalian overtraining model.

Testosterone, another critical anabolic hormone, is sensitive to overtraining-induced suppression of the hypothalamic-pituitary-gonadal (HPG) axis (41). Our findings confirmed that testosterone levels significantly declined following exhaustive training, a change that was mitigated by medium and high doses of *A. keiskei* extract. This protective effect may result from the extract's ability to preserve Leydig cell function or enhance androgen biosynthesis under stress conditions. Supporting this, prior studies have shown that *A. keiskei* protects testicular tissue from heat-induced damage (42).

To assess the dose-dependent efficacy of *A. keiskei*, mice were treated with 20, 50, or 100 mg/kg BW/day of the ethanolic extract. These doses were selected based on established human-to-animal dose conversions,

toxicological data, and prior preclinical findings. The recommended upper intake for humans is approximately 4 g/d of *A. keiskei* dry matter, equivalent to 66.7 mg/kg/d in a 60 kg individual. Using body surface area conversion, this translates to roughly 667 mg/kg/d in mice (43). Toxicological evaluations have confirmed the absence of genotoxic effects at doses up to 300 mg/kg in rodents (44). In preliminary experiments, doses exceeding 100 mg/kg/d did not yield additional improvements in oxidative stress markers. Thus, 100 mg/kg/d was designated as the upper test dose, while 20 and 50 mg/kg/d were included to evaluate minimum effective concentrations and potential dose-response relationships.

Interestingly, our results revealed a dose-dependent yet partially paradoxical pattern in the response to *A. keiskei* extract. While both the 50 mg/kg and 100 mg/kg doses significantly increased SOD levels and reduced MDA, the lowest dose of 20 mg/kg did not improve SOD but still resulted in a significant reduction in MDA levels. This suggests that the 20 mg/kg dose may exert lipid peroxidation-inhibitory effects independently of robust SOD activation, potentially through direct radical scavenging or modulation of other antioxidant systems not measured in this study. Conversely, the 100 mg/kg dose restored both SOD and hormonal levels most effectively but did not produce proportionally greater effects than the 50 mg/kg dose in all parameters. This plateau effect at higher concentrations may reflect a ceiling in the biological response or a shift in the mechanism of action at supraphysiologic doses. These findings suggest that *A. keiskei* extract may exert distinct effects at different concentration thresholds, involving both enzymatic and non-enzymatic antioxidant mechanisms. Such differential dose effects underscore the importance of determining optimal dosing regimens and avoiding assumptions of linear dose-response relationships. Further studies investigating broader molecular targets and pharmacodynamic profiles are needed to clarify these divergent effects and inform appropriate therapeutic windows for potential clinical use.

Our study primarily focused on evaluating the effects of *A. keiskei* leaves extract on specific biomarkers such as oxidative stress markers (SOD and MDA) and age-related hormones (cortisol, IGF-1, and testosterone). However, we recognize that the absence of direct molecular data, such as measurements of oxidative stress response regulators (Nrf2), inflammatory cytokines levels, and markers of the HPA axis, limits our ability to fully elucidate the underlying mechanisms of action. By pursuing these avenues, future research can provide a more detailed understanding of the molecular actions of *A. keiskei* leaves extract, thereby enhancing its therapeutic potential and informing clinical applications. While our findings provide strong evidence for the protective role of *A. keiskei* leaves extract in mitigating overtraining-induced hormonal disturbances, further studies are warranted to explore its long-term benefits and clinical applications, particularly in population that are susceptible to overtraining such as athletes. Investigating dose-response relationships, bioavailability, and potential synergistic effects with other recovery-enhancing compounds will be essential for optimizing its therapeutic use. Additionally, exploring its effects in human subjects engaged in high-intensity training could offer valuable insights into its potential as a natural performance-enhancing and recovery-promoting agent.

Conclusion

This study demonstrated that *A. keiskei* leaf extract effectively attenuated oxidative stress and hormonal dysregulation induced by overtraining in male mice. These findings support the potential of *A. keiskei* as a natural therapeutic agent with anti-aging and adaptogenic properties. By reversing elevations in cortisol and preserving IGF-1 and testosterone levels, *A. keiskei* may help mitigate the physiological consequences of excessive physical stress. Further clinical studies are warranted to validate these effects in humans, particularly in populations vulnerable to overtraining.

Authors' contribution

Conceptualization: Ferbian Milas Siswanto.
Data curation: Putu Suwarastra Andarisuta.
Formal analysis: Ferbian Milas Siswanto.
Funding acquisition: Ferbian Milas Siswanto.
Investigation: Putu Suwarastra Andarisuta, Ferbian Milas Siswanto.
Supervision: Ferbian Milas Siswanto.
Validation: Ferbian Milas Siswanto.
Visualization: Ferbian Milas Siswanto.
Writing–original draft: Putu Suwarastra Andarisuta.
Writing–review & editing: Ferbian Milas Siswanto.

Conflict of interests

The authors declare no conflicts of interest relevant to the content of this article.

Ethical considerations

All animal care and experimental procedures were conducted in accordance with the guidelines set by the Animal Research Ethics Board of School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia (Ethical code number: 23/05/KEP-FKIKUAJ/2025).

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