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*Original Research Article*

# Immunostimulatory Activity of *Costus speciosus, Enhydra fluctuans, Kalanchoe pinnata,* and *Panicum auritum* in Mice Exposed to Dengue Virus Antigen

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ABSTRACT

*Tawa nan ampek* (comprising the leaf of *Costus speciosus;* aerial parts of *Enhydra fluctuans*, *Kalanchoe pinnata*, and *Panicum auritum*) are traditional medicinal plants from West Sumatra, Indonesia, that have been traditionally used to alleviate inflammation and boost immunity. The study aimed to evaluate the immunostimulatory activity of the ethanol extracts of *tawa nan ampek* plants on total and differential leukocyte counts. Fifty-five white male mice (*Mus musculus*) were divided into eleven groups of 5 each: groups 1-2 (negative and positive controls, 0.5 % sodium CMC), group 3 (standard comparator, Stimuno at a dosage of 6.5 mg/kg BW), groups 4-11 (extracts of *tawa nan ampek* plants at a dosage of 200 and 500 mg/kg BW for each plant component). The extracts were orally administered once daily for 7 days, while the dengue virus vaccine (Qdenga®) was injected subcutaneously at 0.0013 mL. On the 8$^{th}$ day, blood was withdrawn from the carotid artery in mice for haematological analysis. The data were statistically analysed using one-way ANOVA followed by DMRT. The results showed that total and differential leukocyte counts were significantly increased by *Costus speciosus* leaf extract ($p<0.05$) compared with all groups (extract, standard comparator, and control groups). The highest immunostimulatory effect was observed with the 500 mg/kg BW dosage of *Costus speciosus* leaf extract, as exhibited by improvements in total and differential leukocyte counts. The study provides the first *in vivo* evidence that *tawa nan ampek* plants, particularly *Costus speciosus*, are natural immunostimulants and promising targets for future cellular and molecular investigation.

***Keywords:*** Dengue Virus (DENV), Ethanol Extract, Immunostimulatory, Leukocyte, *Tawa Nan Ampek.*

## Introduction

Dengue fever is caused by infection with the dengue virus, a member of the family Flaviviridae, which comprises four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. These serotypes are transmitted through the bites of mosquitoes *Aedes aegypti* and *Aedes albopictus*.[1] Dengue fever is a rapidly increasing disease, particularly prevalent in Southeast Asia (Indonesia, Bangladesh, Nepal, and Thailand), Africa (Burkina Faso, Chad, Ethiopia, Guinea, Niger, Cape Verde, Benin and Togo), the Americas (Costa Rica, Brazil, Mexico, Guatemala, Panama, and Honduras), and the Western Pacific (Singapore, Australia, Cambodia, Viet Nam, and Malaysia),[2] a global health challenge for people in subtropical and tropical regions.[3] According to the World Health Organisation (WHO), in 2024, there were 14.2 million confirmed cases of dengue fever, resulting in 10,554 deaths, placing dengue fever among diseases with a mortality burden that continues to increase annually. WHO has classified dengue fever as a level 3 emergency, reflecting its international urgency.[4]

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Epidemiologically, dengue infection is estimated to affect approximately 390 million people annually, with around 96 million experiencing clinical symptoms.[5] The dengue prevalence study calculated that 3.9 billion people in 128 countries are at risk of dengue infection, with the Asia-Pacific region accounting for approximately 70% of the global caseload.[6] Indonesia is among the Asian countries with the highest number of dengue cases, putting the country in a crucial position in global efforts to control the disease.[4] However, there is no specific antiviral agent that is effective against the dengue virus;[7] therefore, treatment still relies on supportive therapy (fluid therapy (oral rehydration, intravenous crystalloid, and colloid), symptom management (antipyretic), clinical monitoring (haematocrit, platelet, and electrolyte), management of complications), and preventive efforts such as sanitation and hygiene.[8] Considering the scale of the dengue outbreak, the potential for spread, and the complexity of factors affecting global transmission, the risk remains relatively high. Thus, dengue fever remains a serious threat to public health and the economy.[9] The immune system plays a vital role in protecting the body against dengue virus infection,[10] with leukocytes, the main circulating components of the immune system, mediating the immune response.[11] The immunomodulatory approach, which involves using certain compounds to modify the immune system, aims at improving the immune system function. This approach has a mechanism of action that includes upregulation (immunostimulant) and downregulation (immunosuppressant), tailored to physiological needs.[12] An immunomodulatory approach may be a viable option in the management of dengue infection, offering potential to boost the body's resistance, given the current lack of specific antiviral therapies.[13] Immunomodulators can be sourced from synthetic, biological, or natural compounds. Natural material sources of immunomodulators play an essential role, offering advantages such as relatively high safety, minimal side effects, lower production costs, and multicomponent active compounds.[13] Indonesia, a megadiverse country, boasts over 30,000 plant species. Indonesia ranks third in the world for biodiversity, with more than 7,000 species of medicinal plants.[14] *Tawa nan ampek*

are four traditional medicinal plants from West Sumatra in Indonesia,[15] which have been empirically used to treat various diseases, including boosting immunity.[16] The four plant species include *Costus speciosus* leaf (crepe ginger), Costaceae; *Enhydra fluctuans* aerial part (marsh herb), Asteraceae;[17] *Kalanchoe pinnata* aerial part (air plants), Crassulaceae,[18] and *Panicum auritum* aerial part (West Indian marsh grass), Poaceae.[19]

*Costus speciosus* is native to Southeast Asia and the Indian subcontinent but is now widely distributed across tropical and subtropical regions due to its adaptability and ornamental value. It has been recorded in Sri Lanka, India, Thailand, Myanmar, Indonesia, Malaysia, and the Philippines. The species is a perennial rhizomatous herb characterised by erect, unbranched, succulent stems that form dense clumps and typically grow 1.5-3 m tall. It's simple, ovate, elliptic leaves are arranged spirally in a helical pattern, with a smooth, glossy, dark green upper surface and lighter venation beneath. The plant produces conspicuous terminal inflorescences, a distinctive feature of the species.[20]

*Enhydra fluctuans* is widely distributed across tropical Asia and Africa, including all Southeast Asian countries. The plant has fleshy, branched stems that extend beyond 30 cm and root at the lower nodes, with a slightly hairy surface. Its sessile, linear-oblong leaves measure 3-5 cm, tapering to pointed or blunt apices, are typically truncate at the base, and are finely toothed along the margins. The solitary, sessile flower heads arise in the leaf axils and are less than 1 cm in diameter, bearing white to greenish white florets. The outer involucral bracts are ovate (1-1.2 cm long), with smaller inner bracts, and the achenes are enclosed by rigid receptacular scales.[17]

*Kalanchoe pinnata* is widely distributed across tropical regions, including parts of Asia, Australia, New Zealand, the West Indies, Macaronesia, the Mascarenes, the Galapagos Islands, Melanesia, Polynesia, and Hawaii, where it is often regarded as native. Macroscopically, the leaves are oblong to elliptic with crenate or serrated margins, an asymmetric base, and reticulate venation. The petiole is relatively long, the leaf surface is glabrous, with a dark green upper epidermis and a lighter lower epidermis, and the leaf emits a characteristic odour.[18]

*Panicum auritum* is a perennial grass widely distributed across South and Southeast Asia. The plant has glabrous, ascending culms up to 1.5 m tall that root from the basal nodes. Leaves are linear, 11-35 cm long, with a subcordate base, entire margins, and an acuminate apex; both surfaces are glabrous with a few basal cilia. The sheaths are glabrous with eciliate or partially ciliate margins, and the ligule is short and membranous. The inflorescence is a dense, contracted panicle with suberect, minutely hispid branches. Spikelets are solitary, oblong-lanceolate, and purplish green, each containing two florets.[19]

Several studies have reported the pharmacological activities of *Costus speciosus* which has identified phenolic compounds, specifically caffeic acid, chlorogenic acid, and cinnamic acid from the aqueous extract, which has activated the type I interferon pathway by inducing the phosphorylation of Signal Transducer and Activator of Transcription 1 (STAT1), Interferon Regulatory Factor 3 (IRF3), TANK-Binding Kinase 1, Inhibitor of Nuclear Factor Kappa-B Alpha (IκB-α), and p65, which may be potentially inhibited viral replication.[21] The n-butanol fraction has shown immunostimulatory activity by increasing IgG levels and humoral immune responses.[22] The anti-inflammatory activity of *Costus speciosus*, primarily associated with its sesquiterpene lactone, has been demonstrated through the inhibition of the production of pro-inflammatory cytokines, including Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Tumour Necrosis Factor-alpha (TNF-α), Prostaglandin E2 (PGE2), and Cyclooxygenase-2 (COX-2).[23] The methanol extract has potent antioxidant activity with an $IC_{50}$ of 14.26±0.88 µg/mL.[24]

The ethanol extract of *Kalanchoe pinnata* has shown selective anticancer effects by inducing reactive oxygen species (ROS), caspase activation, and chromatin fragmentation, [25] it has been indicated to have antiviral activity, proven to inhibit herpes simplex virus type 1-2 (HSV 1-2), and vaccinia virus.[26] It's flavonoids that inhibit acute and chronic inflammation by blocking the arachidonic acid pathway.[27] In contrast, no evidence currently supports the immunostimulatory effects of *Enhydra fluctuans* and *Panicum auritum*.

Based on the description above, the study aimed to evaluate the immunostimulatory activity of ethanol extracts from *tawa nan ampek* plants on total and differential leukocyte counts in white male mice exposed to dengue virus antigen. The research is expected to fill a knowledge gap, provide opportunities to develop natural-material-based immunostimulant candidates, and yield pharmacological data to serve as a basis for future studies.

## Materials and Methods

*Materials*

The materials used were 70% ethanol, distilled water, filter paper, microtip, Ethylenediaminetetraacetic Acid (EDTA), sodium chloride, sodium carboxy methyl cellulose, immersion oil, Stimuno suspension (Dexamedica®, Palembang, Indonesia), Giemsa dye (Merck®, Darmstadt, Germany), Turk reagent (Merck®, Darmstadt, Germany), Qdenga vaccine (Takeda Pharmaceutical®, Singen, Germany).

*Sample preparation and identification*

The plants used were *Costus speciosus* leaves, *Kalanchoe pinnata* aerial parts, *Enhydra fluctuans* aerial parts, and *Panicum auritum* aerial parts obtained in Air Manis Hill, Padang Selatan District, Padang City, West Sumatra, Indonesia (0°58'42.1" S 100°22'05.3" E) on January 5, 2025. The plant sample was identified and validated by Dr. Nurainas of Andalas University Herbarium (ANDA), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang City, West Sumatra, Indonesia, with specimen numbers 673/K-ID/ANDA/I/2025 (*Kalanchoe pinnata*), 674/K-ID/ANDA/I/2025 (*Costus speciosus*), 675/K-ID/ANDA/I/2025 (*Panicum auritum*), 676/K-ID/ANDA/I/2025 (*Enhydra fluctuans*).

*Extract preparation*

About 1 kg each of fresh *tawa nan ampek* plants was harvested, dried, and finely crushed to yield a fine sample of 445 g of *Costus speciosus* leaves, 250 g of *Enhydra fluctuans* aerial parts, 105 g of *Kalanchoe pinnata* aerial parts, and 300 g of *Panicum auritum* aerial parts. Extracts were prepared from the fine powder of *Costus speciosus* leaf, *Kalanchoe pinnata* aerial part, *Enhydra fluctuans* aerial part, and *Panicum auritum* aerial part, by macerating using a ratio of 1:10 (w/v) fine sample to 70% ethanol, meaning 1 g of sample was extracted with 10 mL of 70% ethanol. This process was carried out in a dark-coloured glass container, soaked in 70% ethanol for the first 6 hours with occasional stirring, then allowed to stand for 18 hours per plant. Each macerate was collected and evaporated using a rotary evaporator until a thick extract was generated. After generating the thick extract, the percentage yield was determined.[28] The % yield was calculated using the formula:

$$\text{Yield (\%)} = \frac{\textit{The weight of extract generated (g)}}{\textit{The weight of fine sample (g)}}\ x100\% \qquad (1)$$

*Preparation of experimental animals*

The experimental animals were obtained from the Animal House at the Faculty of Pharmacy, Universitas Andalas. The animals used were healthy white male mice (*Mus musculus*) aged 2-3 months, with a body weight of 20-30 grams, for a total of 55 animals. These animals were acclimated to the laboratory environment for seven days before the experiment commenced. During acclimatisation, animals were provided with standard food and *ad libitum* access to drinking water. Ethical approval for animal testing was obtained from the Ethics Committee of the Faculty of Pharmacy, Universitas Andalas, Padang, West Sumatra, with approval number 42/UN16.10.D.KEPK-FF/2025.

*Grouping experimental animals*

Fifty-five (55) white male mice were divided into eleven (11) groups, each group consisting of 5 mice.

Group I : Negative control (0.5% sodium CMC suspension)
Group II : Positive control (Dengue virus vaccine, 0.5% sodium CMC suspension)
Group III : Standard comparator (Dengue virus vaccine and Stimuno suspension at a dosage of 6.5 mg/kg BW)
Group IV : Dengue virus vaccine and *Costus speciosus* leaf ethanol extract at a dosage of 200 mg/kg BW

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Group V : Dengue virus vaccine and *Costus speciosus* leaf ethanol extract at a dosage of 500 mg/kg BW
Group VI : Dengue virus vaccine and *Enhydra fluctuans* aerial part ethanol extract at a dosage of 200 mg/kg BW
Group VII : Dengue virus vaccine and *Enhydra fluctuans* aerial part ethanol extract at a dosage of 500 mg/kg BW
Group VIII : Dengue virus vaccine and *Kalanchoe pinnata* aerial part ethanol extract at a dosage of 200 mg/kg BW
Group IX : Dengue virus vaccine and *Kalanchoe pinnata* aerial part ethanol extract at a dosage of 500 mg/kg BW
Group X : Dengue virus vaccine and *Panicum auritum* aerial part ethanol extract at a dosage of 200 mg/kg BW
Group XI : Dengue virus vaccine and *Panicum auritum* aerial part ethanol extract at a dosage of 500 mg/kg BW
The treatment was administered orally with 0.5% sodium CMC suspension as the control, stimuno suspension at a dosage of 6.5 mg/kg BW as the standard comparator, and ethanol extracts of *tawa nan ampek* plants at a frequency of once daily for seven days, following stimulation with dengue virus antigen Qdenga Vaccine (Takeda Pharmaceutical®) at a dosage of 0.0013 mL subcutaneously administered the day before treatment. Qdenga, a live attenuated dengue virus strain, was used as the antigenic stimulus in this study. Its composition enables it to serve as a safe and controlled antigenic challenge, making it suitable for evaluating immune responses in mice. On the 8$^{th}$ day, mice were sacrificed by cervical dislocation, and 1.0-1.5 mL of blood was collected via the carotid artery into Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) for haematological analysis.

*Calculation of total leukocyte counts*
Fresh blood treated with Ethylenediaminetetraacetic Acid (EDTA) anticoagulant was sucked using a leukocyte pipette to the 0.5$^{th}$ mark, then the Turk solution was added to the 11$^{th}$ mark. The mixture was shaken for 3 minutes using the tool from inside the pipette. The first 1-2 drops were discarded, and one drop was added to the haemocytometer counting chamber. The mixture was left for 2 minutes to allow the leukocytes to settle. The number of leukocytes in the four corner boxes of the counting chamber was counted under a microscope at 10x magnification.[29]

$$\text{Total leukocyte counts } (/\mu L) = \text{Leukocyte counts x } \frac{20}{0.4} \qquad (2)$$

Description:
Dilution factor: 20
Counting chamber size: 0.4

*Percentage of differential leukocyte counts*
One drop of fresh blood was applied to a glass slide and flattened with another glass slide to obtain a homogeneous layer of blood (blood smear), which was then dried. Then, the blood smear was fixed with 1mL of methanol, coating the entire smear, and left for 5 minutes. Furthermore, the preparations were stained with a diluted 1mL Giemsa solution and left for 20 minutes. The preparations were washed with distilled water and dried, then lightly dabbed with immersion oil and viewed under a microscope. Observations were made under a microscope at 100× magnification to count differential leukocyte cells.[29]

$$\text{Differential leukocyte counts } (\%) = \frac{n}{t} x100\% \qquad (3)$$

Description:
n: Differential leukocyte cells (basophils/eosinophils/neutrophils/ lymphocytes/monocytes)
t: Total leukocyte counts

*Statistical Analysis*
The data obtained from the measurement of total and differential leukocyte counts were expressed as mean ± standard deviation (SD) and analysed statistically by one-way ANOVA, followed by Duncan's Multiple Range Test (DMRT) ($p<0.05$) using software IBM SPSS Statistics version 25.

## Results and Discussion

The maceration produced thick ethanol extracts from *Costus speciosus* leaves, *Kalanchoe pinnata* aerial parts, *Enhydra fluctuans* aerial parts, and *Panicum auritum* aerial parts, with yields of 12.26%, 15.70%, 7.30% and 25.64% respectively, as shown in Table 1. An *in vivo* study examined the immunostimulatory activity of several *tawa nan ampek* plant extracts in white male mice exposed to dengue virus antigens on total and differential leukocyte counts. The microscope and hemacytometer were used to observe the counts. The morphology of leukocyte cells is shown in Figure 1, and their differential morphology is shown in Figure 2.

**Table 1:** Weights of fine sample (g), extract (g), and extract yield (%) of *tawa nan ampek* plants

| Plant | Weight of fine sample (g) | Weight of extract (g) | Yield (%) |
|---|---|---|---|
| *Costus speciosus* | 445 | 54.57 | 12.26 |
| *Enhydra fluctuans* | 250 | 39.26 | 15.70 |
| *Kalanchoe pinnata* | 105 | 7.66 | 7.30 |
| *Panicum auritum* | 300 | 64.10 | 25.64 |

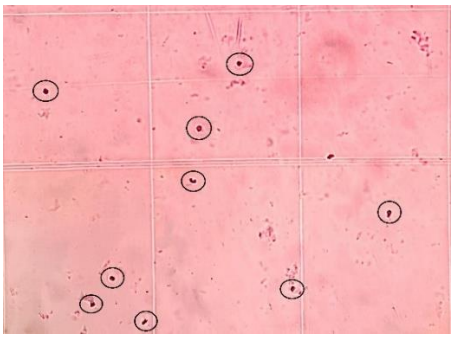
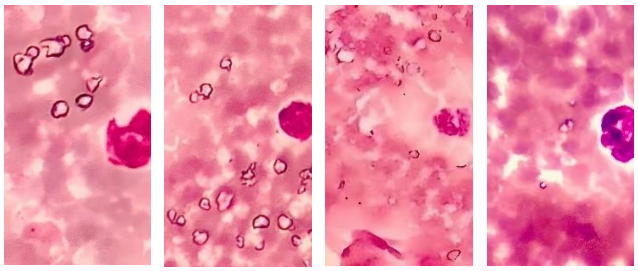


**Figure 1:** Leukocyte counts observed using a microscope at 10x magnification



A B C D

**Figure 2:** Differential leukocyte counts observed using a microscope at 100x magnification (Description: (A) Monocytes, (B) Lymphocytes, (C) Rod neutrophils, (D) Segment neutrophils)

One-way ANOVA statistical analysis revealed that total leukocyte counts were significantly increased by the ethanol extract of *tawa nan ampek* plants at both dosages ($p<0.05$) compared to both controls (negative and positive). Duncan's Multiple Range Test (DMRT) demonstrated that the 500 mg/kg BW dosage of *Costus speciosus* leaf extract exhibited the highest immunostimulatory activity ($p<0.05$) compared to all groups (extracts, stimuno, and control groups). Generally, the administration of ethanol extract of *tawa nan ampek* plants increased total leukocyte counts compared to the control group. The average total leukocyte counts (cells/µL) in the negative control (0.5% sodium CMC), positive control (0.5% sodium CMC), standard comparator (Stimuno suspension 6.5 mg/kg BW), *Costus speciosus* leaf

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extract (200 and 500 mg/kg BW), *Enhydra fluctuans* aerial part extract (200 and 500 mg/kg BW), *Kalanchoe pinnata* aerial part extract (200 and 500 mg/kg BW), aerial part extract *Panicum auritum* (200 and 500 mg/kg BW) respectively are 4,600 cells/µL ± 203.10; 6,330 cells/µL ± 286.36; 10,560 cells/µL ± 326.73; 10,100 cells/µL ± 176.78; 11,020 cells/µL ± 241.35; 8,670 cells/µL ± 286.36; 9,640 cells/µL ± 531.98; 7,900 cells/µL ±544.29; 8,040 cells/µL ± 451.94; 6,930 cells/µL ± 632.06; and 6,970 cells/µL ± 605.81 (Table 2). Under normal physiological conditions, the total leukocyte count in mice ranges from 2,000 to 10,000 cells/µL.[30]

Leukocytes are essential blood components that play a crucial role in the body's defence against viruses.[31] The increase ($p<0.05$) in leukocytes in the treatment group, particularly at the 500 mg/kg BW dosage of *Costus speciosus* leaf extract (11,020 cells/µL ± 241.35), suggests an immunostimulatory effect on the immune system. The immunostimulatory activity of *Costus speciosus* may be attributed to its saponins, which have been reported to stimulate cytokine production, including interleukins and interferons, thereby enhancing immunostimulatory activity.[32] An advantage of using saponin-based adjuvants is their ability to modulate all components of the immune system and increase antibody production at low dosages.[23]

**Table 2:** Total leukocyte counts of the ethanol extract of *tawa nan ampek* plants

| Group | Mean±SD (cells/µL) |
|---|---|
| Negative control (0.5% sodium CMC) | 4,600±203.10 |
| Positive control (0.5% sodium CMC) | 6,330±286.36 |
| Standard comparator (Stimuno 6.5 mg/kgBW) | 10,560±326.73 |
| *Costus speciosus* 200 mg/kgBW | 10,100±176.78* |
| *Costus speciosus* 500 mg/kgBW | 11,020±241.35* |
| *Enhydra fluctuans* 200 mg/kgBW | 8,670±286.36* |
| *Enhydra fluctuans* 500 mg/kgBW | 9,640±531.98* |
| *Kalanchoe pinnata* 200 mg/kgBW | 7,900±544.29* |
| *Kalanchoe pinnata* 500 mg/kgBW | 8,040±451.94* |
| *Panicum auritum* 200 mg/kgBW | 6,930±632.06* |
| *Panicum auritum* 500 mg/kgBW | 6,970±605.81* |

*Values are expressed as mean ± SD (n=5). $p < 0.05$ compared with the control group**.**

One-way ANOVA statistical analysis revealed that the percentage of monocytes was significantly increased by both dosages of the ethanol extract of *tawa nan ampek* plants ($p<0.05$) compared with the negative control. Duncan's Multiple Range Test (DMRT) showed that the 500 mg/kg BW dosage of *Costus speciosus* leaf extract exhibited the highest immunostimulatory activity ($p<0.05$) compared to all groups (extracts, stimuno, and control groups). The average monocytes percentage in the negative control (0.5% sodium CMC), positive control (0.5% sodium CMC), standar comparator (Stimuno 6.5 mg/kg BW), *Costus speciosus* leaf extract (200 and 500 mg/kg BW), *Enhydra fluctuans* aerial part extract (200 and 500 mg/kg BW), *Kalanchoe pinnata* aerial part extract (200 and 500 mg/kg BW) and *Panicum auritum* aerial part extract (200 and 500 mg/kg BW) were 2.8% ± 0.84, 5.6% ± 1.14, 6.2% ± 1.48, 5% ± 1.58, 7.4% ± 1.14, 3% ± 0.71, 6.6% ± 1.14, 3.8% ± 0.84, 4.6% ± 1.14, 3.4% ± 1.14, and 4% ± 0.71, respectively (Table 3). In normal physiological conditions, the percentage of monocytes in mice ranges from 2% to 6%.[30]

**Table 3:** Differential leukocyte counts of the ethanol extract of *tawa nan ampek* plants

| Group | Differential leukocyte counts Mean±SD (%) | | |
|---|---|---|---|
| | **Monocytes** | **Neutrophils** | **Lymphocytes** |
| Negative Control | 2.8±0.84 | 20.4±1.82 | 50.2±6.30 |
| Positive Control | 5.6±1.14 | 26.2±3.56 | 64.8±3.83 |
| Stimuno suspension 6.5 mg/kg BW | 6.2±1.48 | 28±3.94 | 70.4±2.07 |
| *Costus speciosus* 200 mg/kg BW | 5±1.58* | 24±2.24* | 59.4±6.19* |
| *Costus speciosus* 500 mg/kg BW | 7.4±1.14* | 31.4±3.05* | 73.8±3.27* |
| *Enhydra fluctuans* 200 mg/kg BW | 3±0.71* | 20.6±1.14* | 51.4±1.95* |
| *Enhydra fluctuans* 500 mg/kg BW | 6.6±1.14* | 29.8±0.84* | 69.6±4.39* |
| *Kalanchoe pinnata* 200 mg/kg BW | 3.8±0.84* | 21±2.45* | 53.6±1.52* |
| *Kalanchoe pinnata* 500 mg/kg BW | 4.6±1.14* | 22.6±2.30* | 58.8±5.93* |
| *Panicum auritum* 200 mg/kg BW | 3.4±1.14* | 21.8±1.92* | 55±3.16* |
| *Panicum auritum* 500 mg/kg BW | 4±0.71* | 21.4±2.70* | 54.4±5.94* |

*Values are expressed as mean ± SD (n=5). $p < 0.05$ compared with the control group**.**

The increase in the percentage of monocytes in this study indicates an immunostimulatory effect on the innate immune pathway by the chemical compounds in the extract, which is relevant in the early response to dengue virus infection. Monocytes are a large leukocyte subtype that plays a vital role in phagocytosis and antigen presentation to sensitised lymphocytes. Monocytes migrate to tissues, damage sites, or areas of infection, where they mature into macrophages. Monocytes, along with macrophages and tissue neutrophils, are the primary cells involved in first-line defence against pathogenic organisms or foreign cells.[31]

One-way ANOVA statistical analysis revealed that the percentage of neutrophils was significantly increased by both dosages of the ethanol extract of *tawa nan ampek* plants ($p<0.05$) compared with the negative control. Duncan's Multiple Range Test (DMRT) revealed that the 500 mg/kg BW dosage of *Costus speciosus* leaf extract exhibited the highest immunostimulatory activity ($p<0.05$) compared to all groups (extracts, stimuno, and control groups), as illustrated in Figure 5. The average percentage of neutrophils in the negative control (0.5% sodium CMC), positive control (0.5% sodium CMC), standard comparator (Stimuno 6.5 mg/kg BW), *Costus speciosus* leaf extract (200 and 500 mg/kg BW), *Enhydra fluctuans* aerial part extract (200 and 500 mg/kg BW), *Kalanchoe pinnata* aerial part extract (200 and 500 mg/kg BW), and *Panicum auritum* aerial part extract (200 and 500 mg/kg BW) were 20.4% ± 1.82, 26.2% ± 3.56, 28% ± 3.94, 24% ± 2.24, 31.4% ± 3.05, 20.6% ±1.14, 29.8% ± 0.84, 21% ± 2.45, 22.6% ± 2.30, 21.8% ± 1.92, and 21.4% ± 2.70, respectively (Table 3). In normal physiological conditions, the percentage of monocytes in mice ranges from 20% to 30%.[30]

Neutrophils are the primary gatekeepers of the innate immune system, which sends signals to other cells to mount responses to pathogens that cause disease. The increase in neutrophil percentage in this study reflects the body's initial response to foreign agents, in which neutrophils, as the first-line effector cells in the immune system, play a crucial role in phagocytosis, the release of proinflammatory mediators, and the formation of neutrophil extracellular traps (NETs).[33] Chemical compounds content of *Costus speciosus*, especially flavonoids such as quercetin,[34] It is thought to contribute to the stimulation of granulocyte-colony stimulating factor (G-CSF) production needed to accelerate neutrophil differentiation in the bone marrow, while providing antioxidant protection against oxidative burst tissue damage.[35]

Conversely, the One-way ANOVA statistical analysis revealed that the percentage of lymphocytes was significantly increased by both dosages of the ethanol extract of *tawa nan ampek* plants ($p<0.05$) compared with the negative control. Duncan's Multiple Range Test (DMRT) revealed that the 500 mg/kg BW dosage of *Costus speciosus* leaf extract exhibited the highest immunostimulatory activity ($p<0.05$) compared to

all groups (extracts, stimuno, and control groups). The average percentage of lymphocytes in the negative control (0.5% sodium CMC), positive control (0.5% sodium CMC), standard comparator (Stimuno 6.5 mg/kg BW), *Costus speciosus* leaf extract (200 and 500 mg/kg BW), *Enhydra fluctuans* aerial part extract (200 and 500 mg/kg BW) *Kalanchoe pinnata* aerial part extract (200 and 500 mg/kg BW), and *Panicum auritum* aerial part extract (200 and 500 mg/kg BW) were 50.2% ± 6.30, 64.8% ± 3.83, 70.4% ± 2.07, 59.4% ± 6.19, 73.8% ± 3.27, 51.4% ± 1.95, 69.6% ± 4.39, 53.6% ± 1.52, 58.8% ± 5.93, 55% ± 3.16, and 54.4% ± 5.94, respectively (Table 3). In normal physiological conditions, the percentage of monocytes in mice ranges from 70% to 75%.[30]

An increase in lymphocyte percentage suggests that *Costus speciosus* leaf extract can stimulate a specific immune response. Lymphocyte activation typically occurs after a phase of increased neutrophil counts, which marks the transition from the innate to the adaptive immune response. This happens during times of stress and severe infection.[36]

The results indicated that the ethanol extract of *Costus speciosus* exhibited the highest immunostimulatory activity at a dosage of 500 mg/kg BW ($p<0.05$) compared with all groups (extract, stimuno, and control groups), as evidenced by increases in total and differential leukocyte counts. This study provided the first *in vivo* evidence that *tawa nan ampek* plants, particularly *Costus speciosus*, are potential natural immunostimulants for dengue therapy and candidates for further cellular and molecular evaluation.

## Conclusion

Administration of the ethanol extract of *tawa nan ampek* plants produced a significant improvement in total and differential leukocyte counts ($p<0.05$). The ethanol extract of *Costus speciosus* leaf at a dosage of 500 mg/kg BW provided the highest immunostimulatory effect, as indicated by the significant increase in total and differential leukocyte counts, surpassing the standard comparator Stimuno at a dosage of 6.5 mg/kg BW and the positive control group exposed to dengue virus antigen.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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