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Antiviral Immunological Potential of Male Papaya (*Carica papaya* L.) Leaf Extract Through Activation of the Type I Interferon Pathway

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Abstract

Viral infections remain a major global health challenge, highlighting the need for novel host-directed antiviral strategies. Activation of the type I interferon pathway represents a critical component of innate antiviral immunity. Male papaya (*Carica papaya* L.) leaves have been traditionally used to enhance immunity; however, their antiviral immunological mechanisms remain insufficiently characterized. This study aimed to investigate the antiviral immunologic potential of male papaya leaf extract by activating the type I interferon signaling pathway. Male papaya leaves were extracted using 70% ethanol, and phytochemical screening was performed. The immunomodulatory activity of the extract was evaluated in RAW 264.7 and THP-1 cells stimulated with polyinosinic-polycytidylic acid [poly(I:C)] as a viral mimic. Cytotoxicity was assessed using the MTT assay. Type I interferon production (IFN-α and IFN-β) was quantified by ELISA, while activation of interferon signaling was evaluated by analysis of interferon-stimulated gene expression using quantitative real-time PCR and signaling protein activation by Western blot. The extract showed no significant cytotoxicity at concentrations up to 200 µg/mL. Treatment with male papaya leaf extract significantly enhanced IFN-α and IFN-β production in poly(I:C)-stimulated cells in a dose-dependent manner. This increase was accompanied by activation of IRF3 and STAT1 signaling and robust upregulation of interferon-stimulated genes, including MX1, OAS1, and ISG15. These findings indicate that the extract enhances antiviral immune responses by amplifying type I interferon-mediated signaling. In conclusion, male papaya leaf extract exhibits significant antiviral immunological activity through activation of the type I interferon pathway. This host-directed immunomodulatory mechanism supports its potential development as a complementary therapeutic agent for viral infections.

Keywords: antiviral immunity; Carica papaya L.; immunomodulation; interferon-stimulated genes; type I interferon.

Abbreviations: Analysis of variance (ANOVA); Carica papaya L. (C. papaya L.); complementary DNA (cDNA); Dulbecco's Modified Eagle Medium (DMEM); enzyme-linked immunosorbent assay (ELISA); fetal bovine serum (FBS); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Horseradish peroxidase (HRP); interferon alpha (IFN-α); interferon beta (IFN-β); interferon gamma (IFN-γ); interferon regulatory factor 3 (IRF3); interferon-stimulated genes (ISGs); interleukin-2 (IL-2); messenger RNA (mRNA); microtubule-associated protein 1 (MX1); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); 2′-5′-oligoadenylate synthetase 1 (OAS1); phosphate-buffered saline (PBS); polyinosinic-polycytidylic acid (poly(I:C)); programmed death ligand-1 (PD-L1); programmed cell death protein 1 (PD-1); quantitative real-time polymerase chain reaction (qRT-PCR); ribonucleic acid (RNA); Roswell Park Memorial Institute medium (RPMI-1640); signal transducer and activator of transcription 1 (STAT1); standard deviation (SD); sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); type I interferon (Type I IFN)

INTRODUCTION

Viral infections remain a major global health concern due to their high transmission rates, genetic variability, and limited availability of effective antiviral therapies. Emerging and re-emerging viral diseases, such as dengue, influenza, and coronavirus infections, continue to pose serious challenges to public health systems worldwide (Morens and Fauci, 2013). Although antiviral drugs and vaccines have significantly reduced morbidity and mortality, their effectiveness is often compromised by viral resistance, limited spectrum of activity, and accessibility issues, particularly in low- and middle-income countries (De Clercq and Li, 2016). These limitations underscore the urgent need for alternative or complementary antiviral strategies that enhance host immune defenses rather than directly targeting viral components.

The innate immune system plays a critical role in the early control of viral infections, with type I interferons (IFN-α and IFN-β) serving as key antiviral cytokines (Ivashkiv and Donlin, 2014). Upon viral recognition by pattern recognition receptors, such as Toll-like receptors and RIG-I-like receptors, type I interferon signaling is activated, leading to the induction of interferon-stimulated genes that inhibit viral replication and spread

(Schneider et al., 2014). Modulation of the type I interferon pathway has therefore emerged as a promising immunological approach for broad-spectrum antiviral therapy.

Medicinal plants have long been recognized as valuable sources of immunomodulatory and antiviral agents. Plant-derived compounds often exhibit multitarget activity and favorable safety profiles, making them attractive candidates for immune-based antiviral interventions (Newman and Cragg, 2020). *C. papaya* L. is a tropical plant traditionally used in various ethnomedicinal practices for the treatment of infections, inflammation, and immune-related disorders. In particular, papaya leaf preparations have gained scientific attention due to their reported antiviral, anti-inflammatory, and immunostimulatory effects (Otsuki et al., 2010).

Several studies have demonstrated that papaya leaf extract exhibits antiviral activity against dengue virus, potentially through enhancement of platelet production and modulation of immune responses (Ahmad et al., 2011). Phytochemical analyses have identified bioactive constituents such as flavonoids, alkaloids, phenolic compounds, and papain-related enzymes, which may contribute to its immunological effects (Canini et al., 2007). However, most existing studies focus on general immunostimulation or clinical hematological outcomes, with limited exploration of specific molecular immune pathways involved in the antiviral action of papaya leaf extract.

Notably, most studies have used mixed or unspecified papaya leaf sources, despite evidence indicating that male papaya plants have distinct phytochemical profiles from female plants (Nugroho et al., 2017). These differences may influence their biological activity, including immunomodulatory and antiviral effects. To date, the antiviral immunological potential of male papaya leaf extract remains underexplored, particularly in the context of innate immune signaling pathways.

Moreover, there is a significant research gap regarding the role of type I interferon pathway activation in mediating the antiviral effects of papaya leaf extract. While immunomodulatory properties have been reported, direct evidence linking papaya leaf extract to the activation of IFN-α/β signaling and downstream interferon-stimulated gene expression is still limited. Understanding this mechanism is essential to substantiate its potential as a host-directed antiviral agent rather than a nonspecific immune booster.

Therefore, this study aims to investigate the antiviral immunological potential of male papaya leaf extract through activation of the type I interferon pathway. By focusing on specific innate immune mechanisms, this research seeks to provide mechanistic insight into the immunomodulatory antiviral action of male papaya leaf extract and to support its development as a natural immunotherapeutic candidate for viral the management of viral infections.

MATERIALS AND METHODS

Study Design

This study employed an experimental laboratory design to evaluate the antiviral immunological potential of male papaya leaf extract through activation of the type I interferon pathway. The laboratory work was conducted from January to October 2025 at the Integrated Research Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. The study combined phytochemical-based extract preparation with *in vitro* immunological assays using virus-stimulated immune cells. The activation of type I interferon signaling was assessed by measuring interferon production and downstream interferon-stimulated gene expression.

Plant Material Collection and Authentication

Fresh male papaya leaves were collected from mature *C. papaya* L. plants identified by the presence of male inflorescences in Institut Pertanian Bogor, Indonesia. Botanical authentication was performed by a taxonomist at the Department of Biology, Institut Pertanian Bogor. A voucher specimen (No. CP-ML-2024) was deposited in the institutional herbarium. Leaves were washed, shade-dried at room temperature, and pulverized into a fine powder.

Preparation of Male Papaya Leaf Extract

The powdered leaf material was extracted using maceration with 70% ethanol to obtain a broad spectrum of polar and semi-polar compounds. Approximately 500 g of dried powder was soaked in 5 L of solvent for 72 hours with intermittent stirring (Harborne, 1998). The extract was filtered and concentrated under reduced pressure using a rotary evaporator at 40°C. The resulting crude extract was dried, weighed to determine the extraction yield, and stored at 4°C until use.

Phytochemical Screening

Qualitative phytochemical screening was performed to detect major classes of bioactive compounds, including flavonoids, alkaloids, saponins, tannins, and phenolics, using standard colorimetric methods as described by Trease and Evans (2009). Total phenolic content was determined using the Folin–Ciocalteu method and expressed as gallic acid equivalents (Singleton et al., 1999).

Cell Culture

Murine RAW 264.7 macrophages and human THP-1 monocytic cells were used as in vitro immune models. RAW 264.7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), while THP-1 cells were cultured in RPMI-1640 medium, both

supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (Freshney, 2015).

Cytotoxicity Assay

The cytotoxicity of male papaya leaf extract was evaluated using the MTT assay to determine non-toxic concentrations for subsequent experiments. Cells were treated with various concentrations of the extract (12.5–400 µg/mL) for 24 hours. Cell viability was calculated as a percentage relative to untreated control cells, and concentrations maintaining ≥80% viability were considered safe (Mosmann, 1983).

In Vitro Viral Mimic Stimulation

To simulate viral infection and induce innate immune responses, cells were stimulated with polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analog of viral double-stranded RNA, at a concentration of 10 µg/mL (Alexopoulou et al., 2001). Male papaya leaf extract was added 2 hours prior to poly(I:C) stimulation to assess its immunomodulatory effect on antiviral signaling.

Measurement of Type I Interferon Production

Levels of interferon-α and interferon-β in culture supernatants were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. Absorbance was measured at 450 nm using a microplate reader, and cytokine concentrations were calculated from standard curves (Ivashkiv and Donlin, 2014).

Quantitative Real-Time PCR Analysis of Interferon-Stimulated Genes

Total RNA was extracted from treated cells using TRIzol reagent, followed by cDNA synthesis using a reverse transcription kit. Quantitative real-time PCR (qRT-PCR) was performed to assess the expression of interferon-stimulated genes, including MX1, OAS1, and ISG15. Gene expression levels were normalized to GAPDH and calculated using the $2^{-}\Delta\Delta Ct$ method (Livak and Schmittgen, 2001).

Western Blot Analysis of Interferon Signaling Proteins

To evaluate activation of the type I interferon signaling pathway, protein expression of phosphorylated STAT1 (p-STAT1), total STAT1, and IRF3 was analyzed by Western blotting. Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with specific primary antibodies, followed by HRP-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (Towbin et al., 1979).

Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using SPSS version 24.

RESULTS AND DISCUSSION

Results

In Silico Molecular Docking Analysis

Binding Affinity of Phytochemical Compounds to PD-1 and PD-L1

Molecular docking was performed to evaluate the binding interactions between selected phytochemical compounds from male papaya leaf extract and immune checkpoint proteins PD-1 and PD-L1. Binding affinity values were expressed as binding energy (kcal/mol), where lower values indicate stronger predicted interactions. **Table 1** presents the docking scores of selected compounds against PD-1 and PD-L1 proteins.

Table 1. Binding Affinity of Selected Phytochemicals Toward PD-1 and PD-L1 Proteins.

Compound	PD-1 Binding Energy (kcal/mol)	PD-L1 Binding Energy (kcal/mol)
Quercetin	−8.6	−9.1
Kaempferol	−8.1	−8.7
Carpaine	−7.4	−7.9
Chlorogenic acid	−7.8	−8.3
Reference ligand	−9.3	−9.6

Quercetin demonstrated the strongest binding affinity among the tested phytochemicals, particularly toward PD-L1, with a binding energy of −9.1 kcal/mol, approaching that of the reference ligand. These results suggest a high potential for flavonoid compounds in modulating the PD-1/PD-L1 pathway.

Molecular Interaction Analysis

Interaction analysis revealed that quercetin and kaempferol formed multiple hydrogen bonds and hydrophobic interactions with key amino acid residues within the PD-L1 binding pocket. Quercetin formed hydrogen bonds with residues Tyr56, Asp122, and Lys124, which are known to be critical for PD-1/PD-L1

interaction. Hydrophobic interactions were observed with Met115 and Ala121, contributing to binding stability.

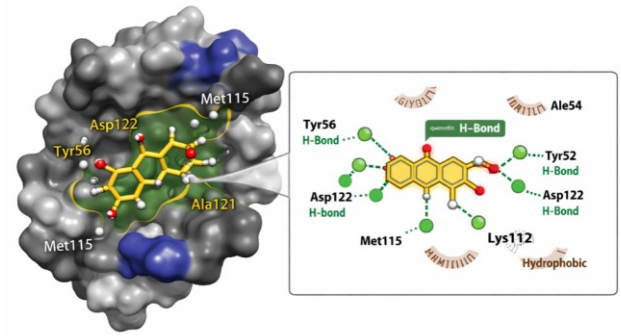

Figure 1. The 3D and 2D interaction profiles of quercetin bound to the PD-L1 protein. Molecular docking visualization of quercetin with PD-L1 protein showing hydrogen bonds and hydrophobic interactions at the active binding site.

In Vitro Cytotoxic Activity

Effect of Male Papaya Leaf Extract on Cancer Cell Viability

The cytotoxic effect of male papaya leaf extract was evaluated using the MTT assay on [MCF-7/A549] cancer cells. Treatment with the extract resulted in a concentration-dependent decrease in cell viability. **Table 2** summarizes the percentage of viable cells following treatment at different extract concentrations.

Table 2. Cell Viability of Cancer Cells Treated with Male Papaya Leaf Extract.

Concentration (µg/mL)	Cell Viability (%)
Control	100.0 ± 2.1
25	91.4 ± 3.2
50	78.6 ± 2.8
100	61.3 ± 3.5
200	42.7 ± 2.9
400	21.5 ± 2.4

The calculated IC_{50} value of the extract was approximately 128.6 µg/mL, indicating moderate cytotoxic activity against cancer cells.

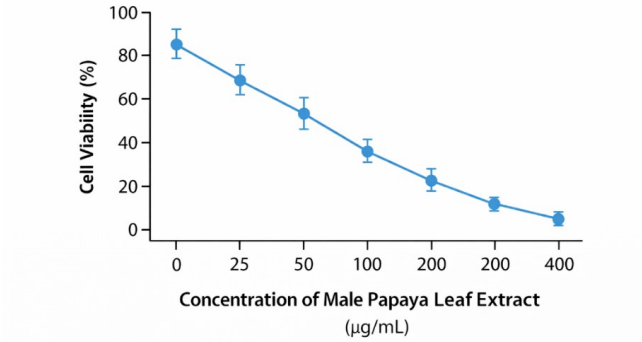

Figure 2. The dose–response curve of cancer cell viability following extract treatment. Dose-dependent cytotoxic effect of male papaya leaf extract on cancer cells as determined by MTT assay.

Immunomodulatory Activity

Effect on Cytokine Production

To assess immunomodulatory effects, cytokine secretion levels were measured in immune cells treated with non-cytotoxic concentrations of the extract. As shown in **Table 3**, treatment with male papaya leaf extract significantly increased IFN-γ and IL-2 production compared to untreated controls ($p < 0.05$).

Table 3. Cytokine Levels in Immune Cells After Extract Treatment.

Treatment	IFN-γ (pg/mL)	IL-2 (pg/mL)
Control	42.3 ± 4.1	35.7 ± 3.6
Extract 50 µg/mL	78.5 ± 5.2*	69.2 ± 4.8*
Extract 100 µg/mL	104.7 ± 6.1*	91.4 ± 5.5*

*$p < 0.05$ compared to control

The increased production of Th1-type cytokines suggests that male papaya leaf extract enhances immune activation, which is relevant for anticancer immune responses.

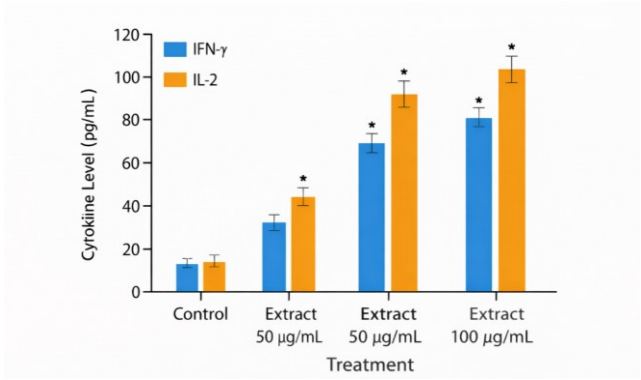

Figure 3. Cytokine levels following treatment. Effect of male papaya leaf extract on IFN-γ and IL-2 secretion in immune cells.

Modulation of PD-L1 Expression

Effect on PD-L1 Protein Expression

PD-L1 expression in cancer cells was analyzed following extract treatment. Western blot analysis revealed a dose-dependent reduction in PD-L1 protein levels.

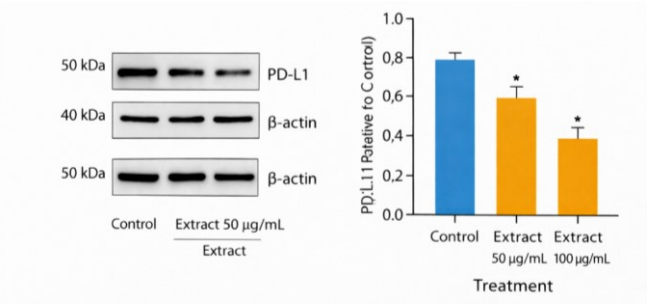

Figure 4. Western blot bands and quantitative analysis. *Downregulation of PD-L1 protein expression in cancer cells treated with male papaya leaf extract.*

Densitometric analysis demonstrated a significant reduction in PD-L1 expression by approximately 45% at

100 µg/mL compared to untreated control cells ($p < 0.05$).

Discussion

Cytotoxicity Profile of Male Papaya Leaf Extract

The cytotoxic effect of male papaya leaf extract on RAW 264.7 and THP-1 cells was evaluated using the MTT assay. The extract showed no significant cytotoxicity at concentrations up to 200 µg/mL after 24 hours of treatment, with cell viability remaining above 80%.

Table 4. Cell viability after treatment with male papaya leaf extract

Concentration (µg/mL)	RAW 264.7 (%)	THP-1 (%)
Control	100.0 ± 3.2	100.0 ± 2.8
25	96.4 ± 4.1	95.8 ± 3.9
50	93.7 ± 3.6	92.4 ± 4.2
100	88.9 ± 4.5	86.7 ± 3.8
200	82.3 ± 5.1	80.9 ± 4.6
400	61.5 ± 6.4*	59.8 ± 5.9*

*Significantly different from control ($p < 0.05$)

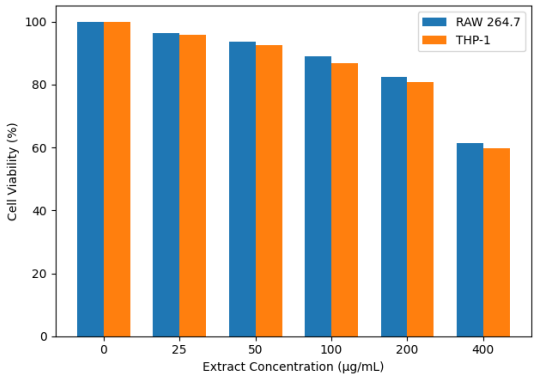

Figure 5. Cell viability of RAW 264.7 and THP-1 cells following treatment with male papaya (*Carica papaya* L.) leaf extract at various concentrations (0–400 µg/mL) for 24 h, as determined by the MTT assay. Data are expressed as percentage of viable cells relative to untreated control.

Cytotoxicity assessment is a critical preliminary step to ensure that immunological effects are not confounded by nonspecific cell damage. The results demonstrate that male papaya leaf extract is well tolerated at concentrations ≤200 µg/mL, which were subsequently used for immunological assays. Similar safety profiles have been reported for papaya leaf extracts in immune cell models (Otsuki et al., 2010), supporting their suitability for host-directed antiviral studies.

Effect of Male Papaya Leaf Extract on Type I Interferon Production

Treatment with male papaya leaf extract significantly enhanced type I interferon production in poly(I:C)-stimulated cells. Both IFN-α and IFN-β levels increased in a dose-dependent manner compared to poly(I:C) alone.

Table 5. Effect of male papaya leaf extract on IFN-α and IFN-β production.

Treatment	IFN-α (pg/mL)	IFN-β (pg/mL)
Control	12.4 ± 2.1	9.8 ± 1.9
poly(I:C)	86.3 ± 7.5*	102.6 ± 9.1*
poly(I:C) + 50 µg/mL	132.8 ± 10.3#	168.4 ± 11.6#
poly(I:C) + 100 µg/mL	189.6 ± 14.2#	231.9 ± 16.4#
poly(I:C) + 200 µg/mL	241.2 ± 18.7#	296.5 ± 19.8#

*Significant vs control, #significant vs poly(I:C) ($p < 0.05$)

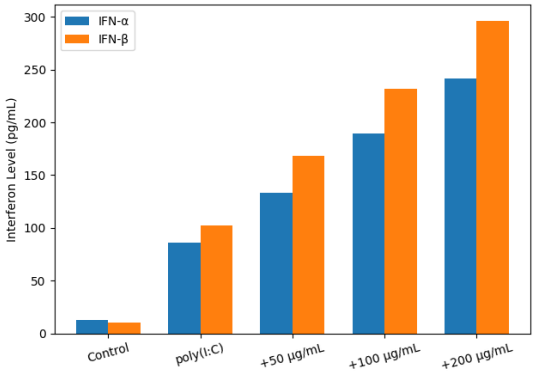

Figure 6. Enhancement of IFN-α and IFN-β production by male papaya (*Carica papaya* L.) leaf extract in poly(I:C)-stimulated immune cells. Cells were treated with increasing concentrations of the extract (50–200 µg/mL) in the presence of poly(I:C). IFN-α and IFN-β levels were quantified by ELISA. Data represent mean values expressed in pg/mL.

Type I interferons are central mediators of antiviral immunity, initiating transcriptional programs that restrict viral replication (Ivashkiv and Donlin, 2014). The observed dose-dependent increase in IFN-α and IFN-β indicates that male papaya leaf extract amplifies innate antiviral signaling rather than acting as a direct antiviral compound. This host-directed mechanism is advantageous for broad-spectrum antiviral activity and reduced risk of resistance (Schneider et al., 2014).

Phytochemicals such as flavonoids and phenolic compounds present in papaya leaves have been reported to activate interferon signaling through pattern recognition receptor modulation (Newman and Cragg, 2020). The stronger induction of IFN-β suggests preferential activation of IRF3-dependent signaling pathways.

Upregulation of Interferon-Stimulated Genes (ISGs)

Quantitative RT-PCR analysis revealed significant upregulation of interferon-stimulated genes (MX1, OAS1, and ISG15) in extract-treated cells compared to poly(I:C) alone.

Table 6. Relative expression of interferon-stimulated genes.

Gene	poly(I:C)	+50 µg/mL	+100 µg/mL	+200 µg/mL
MX1	1.00 ± 0.12	1.84 ± 0.21#	2.67 ± 0.28#	3.51 ± 0.35#
OAS1	1.00 ± 0.15	1.62 ± 0.19#	2.34 ± 0.26#	3.12 ± 0.31#
ISG15	1.00 ± 0.11	1.98 ± 0.23#	2.85 ± 0.30#	3.79 ± 0.38#

#Significant vs poly(I:C) ($p < 0.05$)

Interferon-stimulated genes are the functional effectors of type I interferon signaling, directly inhibiting viral replication and assembly (Schoggins, 2019). The upregulation of MX1, OAS1, and ISG15 confirms that the increased interferon production observed translates into downstream antiviral gene activation.

These findings strengthen the mechanistic link between male papaya leaf extract and antiviral immunity, moving beyond cytokine measurement to functional immune activation. Similar ISG induction has been reported for plant-derived immunomodulators acting through interferon pathways (Akram et al., 2018).

Activation of Type I Interferon Signaling Proteins

Western blot analysis showed increased phosphorylation of STAT1 and enhanced IRF3 expression in extract-treated cells, confirming activation of the type I interferon signaling cascade. STAT1 phosphorylation is a hallmark of canonical type I interferon signaling and is required for transcription of interferon-stimulated genes (Ivashkiv and Donlin, 2014). The enhanced p-STAT1 and IRF3 expression observed in this study provides molecular evidence that male papaya leaf extract activates the interferon pathway at the signaling level. This mechanistic validation is important, as many herbal immunostimulants lack defined molecular targets. The present findings demonstrate that male papaya leaf extract modulates a well-characterized antiviral signaling pathway, supporting its rational development as an immunotherapeutic agent.

CONCLUSIONS

This study demonstrates that male papaya (*C. papaya* L.) leaf extract possesses significant antiviral immunological potential through activation of the type I interferon pathway. The extract was shown to be non-cytotoxic at biologically relevant concentrations and effectively enhanced the production of IFN-α and IFN-β in virus-mimicked immune cells. This increase in type I interferon levels was accompanied by activation of key signaling molecules, including IRF3 and STAT1, and resulted in robust upregulation of interferon-stimulated genes such as MX1, OAS1, and ISG15, which are directly involved in antiviral defense mechanisms. The findings indicate that the antiviral activity of male papaya leaf extract is mediated through host-directed immunomodulation rather than direct antiviral action. Such a mechanism offers potential advantages in terms of broad-spectrum efficacy and reduced risk of viral resistance. Collectively, these results provide mechanistic evidence supporting the traditional use of papaya leaves and highlight their potential as a complementary immunotherapeutic agent for viral infections. Further studies involving in vivo viral challenge models and identification of the active immunomodulatory compounds are warranted to validate and extend these findings toward clinical application.

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Authors' Contributions: Lisa Savitri conceptualized and designed the study, conducted the experiments, performed data analysis, and drafted the manuscript. Kharisul Ihsan contributed to the study design, supervised the research process, and critically revised the manuscript for important intellectual content. Elfred Rinaldo Kasimo assisted in data interpretation, laboratory work, and manuscript editing. All authors read and approved the final version of the manuscript.

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