BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY ISSN 2089-6514 (paper)
Volume 15, Number 1, April 2026 | Pages: 1255-1263 | DOI: 10.14421/biomedich.2026.151.1255-1263 ISSN 2540-9328 (online)

Anticancer Immunomodulatory Activity of Male Papaya Leaf Extract (*Carica papaya* L.) Through PD-1/PD-L1 Pathway Modulation: An In Silico and In Vitro Study

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Manuscript received: 08 February 2026. Revision accepted: 02 June 2026, Published: 01 July 2026.

Abstract

Cancer immunotherapy targeting the programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) pathway has shown remarkable clinical success; however, high costs and immune-related adverse effects remain major challenges. Natural products with immunomodulatory properties offer a promising alternative approach. Male papaya leaves (*Carica papaya* L.) are traditionally used in herbal medicine, yet their potential role in immune checkpoint modulation remains largely unexplored. This study aimed to investigate the anticancer immunomodulatory activity of male papaya leaf extract through PD-1/PD-L1 pathway modulation using integrated in silico and in vitro approaches. Molecular docking analysis was performed to evaluate the interaction between major phytochemical compounds and PD-1/PD-L1 proteins. In vitro cytotoxic activity was assessed using the MTT assay on cancer cell lines, while immunomodulatory effects were evaluated by measuring interferon-gamma (IFN-γ) and interleukin-2 (IL-2) levels using ELISA. PD-L1 protein expression in treated cancer cells was analyzed by Western blotting. The docking results demonstrated strong binding affinities of flavonoid compounds, particularly quercetin, toward PD-L1, involving key residues at the active binding site. The extract exhibited dose-dependent cytotoxic effects with moderate IC_{50} values. Treatment with male papaya leaf extract significantly increased IFN-γ and IL-2 secretion in immune cells and markedly downregulated PD-L1 protein expression in cancer cells. In conclusion, male papaya leaf extract exhibits promising anticancer immunomodulatory activity by modulating the PD-1/PD-L1 immune checkpoint pathway. These findings highlight the potential of male papaya leaves as a natural source of immune checkpoint modulators and support further investigation for their development as complementary agents in cancer immunotherapy.

Keywords: Male papaya leaf; *Carica papaya* L.; immunomodulatory activity; PD-1/PD-L1 pathway; cancer immunotherapy.

Abbreviations: Dimethyl sulfoxide (DMSO); Dulbecco's Modified Eagle Medium (DMEM); Enzyme-linked immunosorbent assay (ELISA); Fetal bovine serum (FBS); Interferon-gamma (IFN-γ); Interleukin-2 (IL-2); Molecular docking (MD); Methylthiazolyldiphenyl-tetrazolium bromide (MTT); Programmed death-1 (PD-1); Programmed death-ligand 1 (PD-L1); Roswell Park Memorial Institute medium (RPMI); Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Cancer remains one of the leading causes of morbidity and mortality worldwide, with an increasing global burden despite advances in conventional therapies such as chemotherapy, radiotherapy, and targeted treatments (Sung et al., 2021). In recent years, immunotherapy has emerged as a promising approach in cancer management by enhancing the host immune response against tumor cells. Among immunotherapeutic strategies, immune checkpoint inhibition, particularly targeting the programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1) pathway, has shown significant clinical benefits in various malignancies (Pardoll, 2012).

The PD-1/PD-L1 axis plays a critical role in tumor immune evasion by suppressing T-cell activation and promoting immune tolerance within the tumor microenvironment. Overexpression of PD-L1 on cancer cells allows tumors to escape immune surveillance, leading to disease progression and poor prognosis (Chen & Mellman, 2017). Although monoclonal antibodies targeting PD-1 or PD-L1 have demonstrated remarkable efficacy, their high cost, limited accessibility, and potential immune-related adverse effects remain major challenges, particularly in developing countries (Postow et al., 2018).

These limitations have driven growing interest in the exploration of natural products as alternative or complementary immunomodulatory agents. Medicinal plants are rich sources of bioactive compounds with anticancer and immune-regulating properties, offering advantages such as lower toxicity, affordability, and

structural diversity (Newman & Cragg, 2020). Therefore, identifying plant-derived compounds capable of modulating immune checkpoint pathways represents a strategic and urgent research direction in cancer immunotherapy.

C. papaya L., commonly known as papaya, has been widely used in traditional medicine for its anti-inflammatory, antioxidant, antimicrobial, and anticancer properties. Papaya leaves, in particular, contain diverse phytochemicals such as flavonoids, alkaloids, phenolic acids, saponins, and terpenoids that have been reported to exert biological activities relevant to cancer prevention and treatment (Aravind et al., 2013; Pandey et al., 2016).

Interestingly, male papaya plants are often underutilized despite emerging evidence suggesting that male papaya leaves may possess distinct phytochemical profiles and higher concentrations of certain bioactive compounds compared to female plants (Maisarah et al., 2014). Several studies have reported the cytotoxic and antiproliferative effects of papaya leaf extracts against cancer cell lines, as well as their ability to modulate immune responses by enhancing cytokine production and lymphocyte activity (Otsuki et al., 2010; Nugroho et al., 2017).

However, most existing studies have focused on general cytotoxicity or antioxidant activity, with limited investigation into the molecular mechanisms underlying the immunomodulatory effects of papaya leaf extracts, particularly in relation to immune checkpoint regulation.

Despite the growing body of evidence supporting the anticancer and immunomodulatory potential of *C. papaya* L. leaves, several critical gaps remain. First, there is a lack of studies specifically examining male papaya leaf extracts in the context of cancer immunotherapy, even though plant sex differences may influence phytochemical composition and biological activity.

Second, to date, very few studies have explored the interaction between plant-derived compounds and the PD-1/PD-L1 immune checkpoint pathway. Most immunomodulatory research on papaya leaves has focused on nonspecific immune stimulation rather than targeted immune checkpoint modulation. This represents a significant gap, given the central role of PD-1/PD-L1 signaling in tumor immune escape.

Third, integrated approaches combining in silico molecular docking and in vitro experimental validation remain limited in this research area. In silico studies can provide valuable insights into the binding affinity and interaction mechanisms of bioactive compounds with immune checkpoint proteins, while in vitro assays are essential to confirm biological relevance and anticancer effects at the cellular level (Lionta et al., 2014).

Based on these gaps, this study aims to investigate the anticancer immunomodulatory activity of male papaya leaf extract through modulation of the PD-1/PD-L1 pathway using a combined in silico and in vitro approach. Molecular docking analysis is employed to predict the interaction between selected phytochemical compounds and PD-1/PD-L1 proteins, while in vitro assays are conducted to evaluate cytotoxicity and immunomodulatory effects on relevant cancer and immune cell models.

The findings of this study are expected to provide novel scientific evidence supporting the potential of male papaya leaf extract as a natural immunomodulatory agent targeting immune checkpoints. This research may contribute to the development of affordable, plant-based adjunct therapies for cancer treatment and broaden the understanding of natural product-based immune checkpoint modulation.

MATERIALS AND METHODS

Study Design

This study employed an experimental laboratory design integrating in silico molecular docking analysis and in vitro biological assays to evaluate the anticancer immunomodulatory activity of male papaya leaf extract through modulation of the PD-1/PD-L1 pathway. The in silico approach was used to predict molecular interactions between selected phytochemical compounds and immune checkpoint proteins, while in vitro assays were conducted to assess cytotoxicity and immunomodulatory effects at the cellular level (Lionta et al., 2014).

Plant Material Collection and Identification

Male papaya leaves (*C. papaya* L.) were collected from Institut Pertanian Bogor, at physiological maturity. The plant material was authenticated by a botanist at the Department of Biology, Institut Pertanian Bogor, and a voucher specimen was deposited for reference under accession number CP-LM-012/2024. Leaves were washed with distilled water, air-dried at room temperature, and subsequently oven-dried at 40–45°C to preserve thermolabile compounds prior to pulverization into fine powder.

Preparation of Male Papaya Leaf Extract

The dried leaf powder was extracted using the maceration method with 70% ethanol as solvent, based on its effectiveness in extracting both polar and semi-polar bioactive compounds (Azmir et al., 2013). Briefly, 500 g of powdered leaves were immersed in ethanol at a ratio of 1:10 (w/v) and agitated intermittently for 72 hours at room temperature. The extract was filtered using Whatman No. 1 filter paper, and the solvent was removed under reduced pressure using a rotary evaporator at 40°C. The concentrated extract was stored at 4°C until further analysis.

Phytochemical Compound Selection

Major phytochemical constituents of *C. papaya* L. leaves were identified based on literature review and phytochemical databases. Selected compounds included flavonoids (e.g., quercetin, kaempferol), alkaloids (e.g., carpaine), and phenolic compounds known for anticancer and immunomodulatory properties (Pandey et al., 2016; Nugroho et al., 2017). The chemical structures of selected compounds were retrieved from the PubChem database in SDF format and prepared for molecular docking analysis.

In Silico Molecular Docking Study

Protein Preparation

Three-dimensional structures of PD-1 and PD-L1 proteins were obtained from the Protein Data Bank (PDB). The selected PDB IDs were 3RRQ for PD-1 and 5C3T for PD-L1, chosen based on their high-resolution crystal structures and widespread use in immune checkpoint interaction studies. Protein structures were prepared by removing water molecules, ligands, and heteroatoms, followed by the addition of polar hydrogen atoms using AutoDock Tools (Morris et al., 2009).

Ligand Preparation

Ligand structures were energy-minimized using the MMFF94 force field to achieve optimal conformations. Ligands were converted to PDBQT format and assigned Gasteiger charges prior to docking.

Docking Procedure

Molecular docking simulations were performed using AutoDock Vina to predict binding affinity and interaction patterns between phytochemical ligands and PD-1/PD-L1 proteins (Trott & Olson, 2010). The grid box was defined to cover the active binding site of each protein. Docking parameters were set to default, and binding affinity values were expressed as binding energy (kcal/mol). Docking results were visualized and analyzed using Discovery Studio Visualizer to identify hydrogen bonds, hydrophobic interactions, and key amino acid residues involved in ligand binding.

Cell Culture

Cell culture experiments were conducted from May to August 2025 at the Cell Culture Laboratory, Department of Medical Laboratory Technology, Faculty of Health Sciences, Kadiri University, Kediri, East Java, Indonesia. Human breast adenocarcinoma cells (MCF-7) and Jurkat T lymphocyte cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, USA), while Jurkat T cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640; Gibco, Thermo Fisher Scientific, USA). Both media were supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA) and 1% penicillin–streptomycin solution (Gibco, Thermo Fisher Scientific, USA).

All cell culture procedures were performed under aseptic conditions in a Class II biological safety cabinet. Cells were cultured in T-25 tissue culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO_2 using a CO_2 incubator. Cell morphology, growth, and confluency were routinely monitored using an inverted phase-contrast microscope. Cell density and viability were determined using a hemocytometer and trypan blue staining solution (Sigma-Aldrich, USA). Routine maintenance procedures, including medium replacement and subculturing, were carried out according to standard cell culture protocols as described by Freshney (2015).

Cytotoxicity Assay

The cytotoxic activity of male papaya (*C. papaya* L.) leaf extract against MCF-7 and Jurkat T cells was evaluated using the MTT assay from May to August 2025 at the Cell Culture Laboratory, Department of Medical Laboratory Technology, Faculty of Health Sciences, Kadiri University, Kediri, East Java, Indonesia. For the assay, MCF-7 and Jurkat T cells were seeded into sterile 96-well tissue culture plates (Corning, USA) at a density of 1×10^4 cells per well using a micropipette (Eppendorf, Germany) and incubated for 24 h in a CO_2 incubator (Thermo Scientific, USA) at 37°C and 5% CO_2. Following incubation, cells were treated with various concentrations of male papaya leaf extract (25, 50, 100, 200, and 400 µg/mL) prepared in complete culture medium and incubated for an additional 24–48 h.

After treatment, 20 µL of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, USA) at a concentration of 5 mg/mL was added to each well and incubated for 4 h to allow the formation of purple formazan crystals by metabolically active cells. The culture medium was then carefully removed, and the formazan crystals were dissolved using dimethyl sulfoxide (DMSO; Merck, Germany). Absorbance was measured at 570 nm using a microplate reader (Bio-Rad Model 680, USA).

The chemicals used in this assay included MTT reagent (Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO; Merck, Germany), Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA), RPMI-1640 medium (Gibco, USA), fetal bovine serum (FBS; Gibco, USA), and penicillin–streptomycin solution (Gibco, USA). The main laboratory equipment consisted of a Class II biological safety cabinet, CO_2 incubator, inverted microscope, micropipettes, centrifuge, and microplate reader.

Cell viability was calculated as the percentage of absorbance relative to untreated control cells, and the half-maximal inhibitory concentration (IC_{50}) values were determined from dose-response curves according to the method described by Mosmann (1983).

Immunomodulatory Activity Assay

The immunomodulatory activity of male papaya (*C. papaya* L.) leaf extract was evaluated from May to August 2025 at the Cell Culture Laboratory, Department of Medical Laboratory Technology, Faculty of Health Sciences, Kadiri University, Kediri, East Java, Indonesia. Jurkat T lymphocyte cells were used as an in vitro immune cell model to assess cytokine production following treatment with male papaya leaf extract. Cells were seeded into sterile 24-well culture plates (Corning, USA) and maintained in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin solution (Gibco, USA). Cells were treated with non-cytotoxic concentrations of the extract, as determined from the MTT assay, and incubated for 24 h in a CO_2 incubator (Thermo Scientific, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Following incubation, culture supernatants were collected and centrifuged using a laboratory centrifuge (Eppendorf, Germany) to remove cellular debris. The concentrations of cytokines, including interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α), were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Inc., USA) according to the manufacturer's protocols. Briefly, standards and samples were added to ELISA microplates pre-coated with specific antibodies, followed by incubation with biotinylated detection antibodies, horseradish peroxidase (HRP)-conjugated reagents, substrate solution (tetramethylbenzidine; TMB), and stop solution. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 680, USA).

The chemicals and reagents used in this assay included RPMI-1640 medium, fetal bovine serum (FBS), penicillin–streptomycin solution, phosphate-buffered saline (PBS; Gibco, USA), ELISA kits for IFN-γ, IL-2, and TNF-α (Elabscience Biotechnology Inc., USA), TMB substrate solution, HRP-conjugated detection reagents, and stop solution. The primary laboratory tools and equipment included a Class II biological safety cabinet, CO_2 incubator, micropipettes, centrifuge, ELISA microplates, microplate washer (or manual washing equipment), and microplate reader.

Cytokine concentrations were calculated from standard calibration curves generated according to the ELISA kit instructions. Changes in cytokine production relative to untreated control cells were used to evaluate the immunostimulatory or immunomodulatory effects of the male papaya leaf extract, following the approach described by Otsuki et al. (2010).

PD-L1 Expression Analysis

The effect of male papaya (*C. papaya* L.) leaf extract on programmed death-ligand 1 (PD-L1) expression was evaluated from May to August 2025 at the Cell and Molecular Biology Laboratory, Department of Medical Laboratory Technology, Faculty of Health Sciences, Kadiri University, Kediri, East Java, Indonesia. MCF-7 cells were seeded in 6-well culture plates (Corning, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin solution (Gibco, USA). Cells were treated with selected concentrations of male papaya leaf extract and incubated for 24–48 h at 37°C in a humidified atmosphere containing 5% CO_2 using a CO_2 incubator (Thermo Scientific, USA).

Following treatment, cells were harvested and lysed using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, USA). Total protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) and measured with a microplate reader (Bio-Rad Model 680, USA). Equal amounts of protein (20–40 μg) were mixed with Laemmli sample buffer, denatured at 95°C using a dry bath incubator, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA) using a wet-transfer electrophoresis system (Bio-Rad, USA). Membranes were blocked with 5% non-fat skim milk prepared in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight at 4°C with anti-PD-L1 primary antibody (Cell Signaling Technology, USA). After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, USA) for 1 h at room temperature.

Protein bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Thermo Fisher Scientific, USA) and captured using a gel documentation and chemiluminescence imaging system (Bio-Rad ChemiDoc MP, USA). β-Actin antibody (Cell Signaling Technology, USA) was used as an internal loading control. Band intensities were quantified using ImageJ software (National Institutes of Health, USA), and relative PD-L1 expression levels were normalized to β-actin expression.

The chemicals and reagents used in this analysis included DMEM, FBS, penicillin–streptomycin, phosphate-buffered saline (PBS), RIPA lysis buffer, protease inhibitor cocktail, BCA protein assay kit, SDS-PAGE reagents, PVDF membranes, TBST, skim milk, anti-PD-L1 primary antibody, HRP-conjugated secondary antibody, β-actin antibody, and enhanced chemiluminescence (ECL) substrate. The primary laboratory tools and equipment included a Class II biological safety cabinet, CO_2 incubator, refrigerated

centrifuge, micropipettes, vortex mixer, analytical balance, dry bath incubator, electrophoresis apparatus, protein transfer system, microplate reader, and chemiluminescence imaging system.

PD-L1 protein expression was determined by densitometric analysis of Western blot bands and compared among treatment groups to evaluate the potential immune checkpoint-modulating effects of male papaya leaf extract, as described by Chen and Mellman (2017).

Statistical Analysis

All experiments were conducted in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software version 24.0. Differences between groups were analyzed using one-way ANOVA followed by post hoc Tukey's test. A p-value < 0.05 was considered statistically significant.

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.

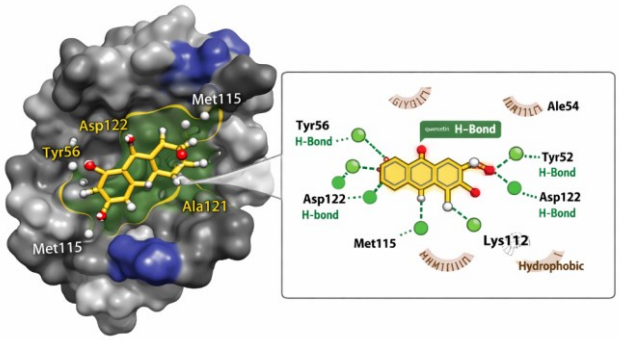

Figure 1. The 3D and 2D interaction profiles of quercetin bound to the PD-L1 protein. Molecular docking visualization of quercetin with PD-L1 protein showing hydrogen bonds and hydrophobic interactions at the active binding site.

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.

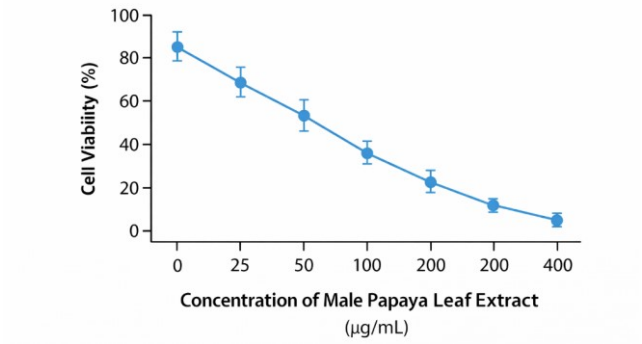

Figure 2. The dose–response curve of cancer cell viability following extract treatment. Dose-dependent cytotoxic effect of male papaya leaf extract on cancer cells as determined by MTT assay.

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.

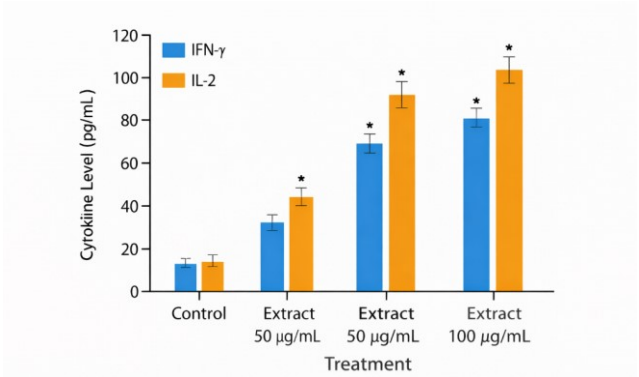

Figure 3. Cytokine levels following treatment. Effect of male papaya leaf extract on IFN-γ and IL-2 secretion in immune cells.

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.

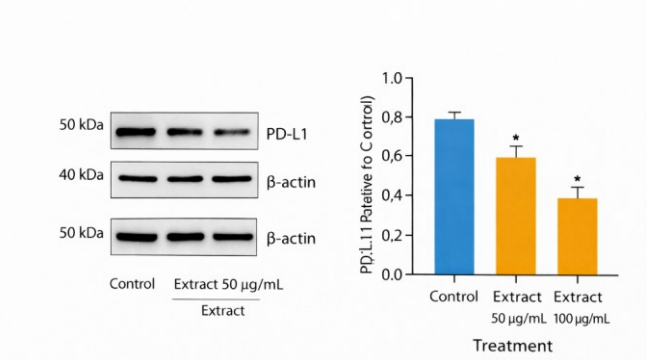

Figure 4 Western blot bands and quantitative analysis. *Downregulation of PD-L1 protein expression in cancer cells treated with male papaya leaf extract.*

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

The present study demonstrates that phytochemical compounds from male papaya leaf extract, particularly flavonoids such as quercetin and kaempferol, exhibit strong binding affinity toward PD-1 and PD-L1 proteins based on in silico molecular docking analysis. The observed binding energies, especially those approaching the reference ligand values, suggest that these compounds may competitively interfere with PD-1/PD-L1 interactions at the molecular level.

Previous studies have reported that flavonoids possess structural features, such as hydroxyl groups and planar aromatic rings, that enable stable interactions with protein binding sites through hydrogen bonding and hydrophobic interactions (Panche et al., 2016). In this study, quercetin formed multiple hydrogen bonds with key PD-L1 residues, including Tyr56 and Asp122, which have been identified as critical residues involved in immune checkpoint signaling (Zak et al., 2015). These findings support the hypothesis that natural flavonoids can function as small-molecule immune checkpoint modulators.

Importantly, unlike monoclonal antibodies, plant-derived compounds may offer partial modulation rather than complete blockade of PD-1/PD-L1 signaling, potentially reducing the risk of excessive immune activation and immune-related adverse effects (Zhang & Chen, 2020). This characteristic positions natural products as promising candidates for adjunct or preventive immunotherapy strategies.

The in vitro cytotoxicity results demonstrated that male papaya leaf extract exerted a dose-dependent inhibitory effect on cancer cell viability, with an IC_{50} value in the moderate range. These findings are consistent with previous reports indicating that papaya leaf extracts exhibit antiproliferative activity against various cancer cell lines, including breast, lung, and leukemia models (Otsuki et al., 2010; Nugroho et al., 2017).

The moderate cytotoxicity observed in this study is noteworthy, as excessive cytotoxic effects are not always desirable in immunotherapy-oriented approaches. Instead, immunomodulatory anticancer agents are expected to suppress tumor growth while preserving immune cell function (Galluzzi et al., 2018). The combination of moderate cytotoxicity and immune activation observed in this study suggests a dual mechanism of action, involving both direct tumor inhibition and immune-mediated anticancer effects.

Flavonoids such as quercetin have been widely reported to induce cell cycle arrest and apoptosis in cancer cells through modulation of signaling pathways such as PI3K/Akt and MAPK (Tang et al., 2020). Therefore, the cytotoxic activity of male papaya leaf extract is likely attributed to the synergistic effects of multiple bioactive compounds rather than a single constituent.

A key finding of this study is the significant increase in Th1-type cytokines, particularly IFN-γ and IL-2, following treatment with male papaya leaf extract. These cytokines play central roles in antitumor immunity by promoting cytotoxic T lymphocyte activation, enhancing antigen presentation, and supporting immune memory formation (Murphy & Weaver, 2016).

The enhancement of IFN-γ and IL-2 secretion aligns with earlier studies reporting immunostimulatory effects of papaya leaf extracts, including increased lymphocyte proliferation and macrophage activation (Otsuki et al., 2010). Importantly, IFN-γ is known to influence PD-L1 expression in tumor cells, suggesting a complex regulatory feedback mechanism between immune activation and immune checkpoint signaling (Spranger et al., 2013).

These results indicate that male papaya leaf extract does not merely stimulate immune cells nonspecifically but may promote a coordinated immune response relevant to cancer immunosurveillance. This immunomodulatory profile is particularly valuable in the context of immunotherapy, where sustained and balanced immune activation is essential for therapeutic efficacy.

The observed downregulation of PD-L1 protein expression in cancer cells following extract treatment provides functional validation of the in silico docking results. Reduced PD-L1 expression may restore T-cell-mediated immune recognition and enhance antitumor immune responses (Chen & Mellman, 2017).

Several studies have reported that natural compounds can regulate PD-L1 expression by targeting upstream signaling pathways such as STAT3, NF-κB, and HIF-1α (Lin et al., 2020). Flavonoids, including quercetin, have been shown to suppress PD-L1 expression through inhibition of STAT3 phosphorylation, thereby enhancing immune cell-mediated cytotoxicity (Zhou et al., 2019). The findings of this study suggest that similar mechanisms may underlie the effects of male papaya leaf extract.

The ability of the extract to both bind PD-L1 and reduce its expression highlights a dual mode of immune checkpoint modulation, which may enhance therapeutic potential compared to agents acting through a single mechanism.

An important aspect of this study is the exclusive focus on male papaya leaves, which have been relatively overlooked in phytochemical and pharmacological research. Plant sex differences can influence metabolite composition and biological activity, as reported in other dioecious or polygamous plant species (Retuerto et al., 2018). The findings of this study provide preliminary evidence that male papaya leaves may serve as a valuable source of immunomodulatory compounds.

Moreover, the integration of in silico and in vitro approaches strengthens the scientific validity of the results by linking molecular-level predictions with biological outcomes. Such integrative strategies are increasingly recommended in natural product-based drug discovery to improve translational relevance (Atanasov et al., 2021).

Despite promising findings, this study has several limitations. First, the in vitro model cannot fully replicate the complexity of the tumor microenvironment in vivo, where multiple immune cell types and signaling pathways interact dynamically. Second, the extract contains a mixture of compounds, making it difficult to attribute observed effects to specific molecules without further fractionation and isolation studies.

Future research should focus on in vivo validation using animal tumor models, detailed mechanistic studies of signaling pathways involved in PD-L1 regulation, and isolation of lead compounds for structure-activity relationship analysis. Additionally, synergistic effects between male papaya leaf extract and existing immune checkpoint inhibitors warrant further investigation.

Overall, this study provides compelling evidence that male papaya leaf extract possesses anticancer immunomodulatory activity through modulation of the PD-1/PD-L1 pathway. By combining molecular docking, cytotoxicity assays, cytokine analysis, and PD-L1 expression studies, this research contributes novel insights into the potential role of plant-based compounds in immune checkpoint-oriented cancer therapy.

CONCLUSIONS

This study demonstrates that male papaya leaf extract (*C. papaya* L.) possesses promising anticancer immunomodulatory activity through modulation of the PD-1/PD-L1 immune checkpoint pathway. The in silico molecular docking results revealed strong binding affinities of major phytochemical compounds, particularly flavonoids such as quercetin, toward PD-1 and PD-L1 proteins, suggesting potential interference with immune checkpoint signaling at the molecular level. In vitro assays further supported these findings by showing dose-dependent cytotoxic effects against cancer cells, accompanied by significant enhancement of key Th1-type cytokines, including IFN-γ and IL-2. Importantly, treatment with the extract resulted in downregulation of PD-L1 protein expression in cancer cells, indicating functional immune checkpoint modulation. Taken together, these results suggest that male papaya leaf extract exerts a dual anticancer mechanism by directly inhibiting tumor cell viability while simultaneously enhancing immune-mediated antitumor responses. This study provides novel evidence supporting the potential of male papaya leaves as a natural source of immunomodulatory compounds targeting the PD-1/PD-L1 pathway. Further in vivo studies and compound isolation are warranted to validate these findings and explore their translational potential in cancer immunotherapy.

Acknowledgements: The authors would like to thank the laboratory staff and academic colleagues at the Laboratory of Medical Laboratory Technology, Faculty of Health Sciences, Kadiri University for their technical assistance and valuable discussions during the course of this study.

Authors' Contributions: Lisa Savitri conceived and designed the study, supervised the research activities, and critically revised the manuscript, Kharisul Ihsan conducted the in silico molecular docking analysis and contributed to data interpretation, Elfred Rinaldo Kasimo performed the in vitro experiments, collected and analyzed biological data, and prepared the initial draft of the manuscript, Rochmad Krissanjaya contributed to data analysis, figure preparation, and manuscript editing, and all authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

Competing Interests: The authors declare that there are no competing interests associated with this study.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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