ORIGINAL ARTICLE

Phytochemical composition and cytotoxic assessment of *Carica papaya* leaf extracts on human squamous carcinoma-3 Cells (in vitro): a laboratory experimental

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ABSTRACT

Introduction: Oral cancer is one of the major diseases that can cause death worldwide. With the advancement of research on new drugs, medicinal plants have been explored as an alternative approach to cancer treatment. Papaya (*Carica papaya*) is a plant that has long been used in traditional medicine to treat various diseases. Ethanolic and aqueous extracts of *C. papaya* leaf have been reported to exhibit anticancer properties through mechanisms such as inhibiting cell proliferation and inducing apoptosis. This study aimed to analyze the cytotoxic effect of *C. papaya* leaf extract obtained with different extraction solvents on HSC-3 cell lines. **Methods**: This laboratory-based experimental study employed a post-test only control group design. The sample size was determined using the Federer formula, yielding four replicates per group. HSC-3 cell lines were treated with ethanolic and aqueous extracts of *C. papaya* leaf at concentrations of 125, 250, and 500 µg/mL for 24 hours, followed by a cytotoxicity test using the CCK-8 assay. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. **Results**: Phytochemical analysis revealed the presence of alkaloids, flavonoids, and tannins in both ethanolic and aqueous extracts, whereas triterpenoids were detected only in the ethanolic extract, and saponins were detected only in aqueous extract. HSC-3 cells did not show any significant cytotoxic response to either extract at any tested concentration. **Conclusion**: Ethanolic and aqueous extracts of *C. papaya* leaf at concentrations of 125, 250, and 500 µg/mL did not exhibit any cytotoxic effects on HSC-3 cell lines.

KEYWORDS

C. papaya leaf, HSC-3 cells, ethanol, aquadest, CCK-8 assay.

INTRODUCTION

Oral cancer is one of the major diseases that can cause death worldwide, including Indonesia.[1] Oral cancer is ranked as the 16th most common cancer globally. The global incidence of oral cancer is expected to reach 389,485 new cases and 188,230 deaths by 2022.[2] The prevalence of oral cancer in Indonesia is around 3-4% of all cancer cases, with a mortality rate of 2-3% of all cancer deaths.[3]

Oral Squamous cell carcinoma (OSCC) is the most common type of oral cancer, accounting for almost 90% of all oral cavity carcinomas.[4] Human Squamous Carcinoma (HSC-3) cell lines are derived from human tongue squamous cell carcinoma with metastatic potential and are widely used in oral cancer drugs research.[5] The etiology of oral cancer is multifactorial and complex. Some of the factors that influence its development include smoking, alcohol consumption and Human Papilloma Virus (HPV) infection.[6] However, multiple factors, including genetics predisposition, contribute to the development of oral cancer.[7, 8]

Due to the high prevalence and mortality rate of oral cancer, effective treatment strategies are required. With the development of research on new drugs, medicinal plants have gained interest as an alternative source for potential cancer treatment. One such medicinal plants with promising potential as an alternative for oral cancer treatment is the papaya plant.

Papaya *(Carica papaya*) is a plant from the Caricaceae family that is widely used in traditional medicine.[9] *C. papaya* leaf extract shows pharmacological activities such as antidiabetic, anti-inflammatory, antimicrobial, antioxidant, and anticancer.[10] The aqueous extract of *C. papaya* leaf has the potential to inhibit breast cancer cell lines (MCF-7) through the suppression of cell proliferation and the induction of apoptosis.[11] Ethanol extract of *C. papaya* leaf is effective in inhibiting the proliferation of pancreatic cancer cell lines (MiaPaCa-2 and ASPC-1).[12]

Because of their distinct polarity, ethanol and water are utilised and this influences the type and concentration of beneficial anticancer phytochemicals obtained from *C. papaya* leaf. Ethanol is expected to yield more cytotoxic phytochemicals given its broader solvation capacity[13, 14]; which aligns with findings indicating that the ethanol extract to have a higher potential for cytotoxicity than water extract.[15, 16] Though more common in water-based extracts, saponins may exert lower overall potency or more cell line-specific anticancer activity.[17, 18]

This study addresses a significant research gap, as previous investigations on *Carica papaya* leaf extracts have primarily focused on breast, pancreatic, and prostate cancer cell lines, with limited evidence regarding their effects on oral squamous cell carcinoma (HSC-3). Moreover, earlier studies rarely compared different extraction solvents, leaving unclear how solvent polarity influences the bioactive composition and cytotoxic potential of *C. papaya*.

The novelty of this research lies in its comparative analysis of ethanol and aqueous extracts of *Carica papaya* leaf on HSC-3 cells, providing new insights into how solvent-dependent phytochemical differences relate to anticancer activity. The aim of this study was to analyze the cytotoxic effects of ethanol and aqueous extracts of *C. papaya* leaf on HSC-3 cell lines.

METHODS

This was a laboratory-based experimental study using a post-test only control group design. *Carica papaya* leaf (a California variety, collected from Bogor, West Java, Indonesia) was dried in indirect sunlight and then ground using a blender until a fine powder was obtained. The powdered sample was extracted using the maceration method for three days by soaking the *C. papaya* leaf powder in 96% ethanol (EMSURE) in a powder to solvent ratio of 1:8 for ethanol extract, and in aquadest in a ratio of 1:8 for the aqueous extract at room temperature.[19] During maceration, agitation was performed several times at 15 minutes intervals for 8 hours. The mixture was filtered using Whatman filter paper (Cytiva, Marlborough, US). The ethanol and aqueous extracts were then concentrated using a rotary evaporator.

Phytochemical tests for alkaloid identification were carried out by adding 2 ml of sample to 5 ml of 2N HCl and then adding Dragendorff reagent. The formation of a reddish-brown precipitate indicates the presence of alkaloid compounds.[20] Identification of steroid and triterpenoid compounds was performed by 0.5 ml of Liebermann–Burchard reagent to the sample. The formation of blue-green color indicates the presence of steroid compounds, while the formation of purple/red color indicates the presence of triterpenoid compounds.[21]

For flavonoid identification 5 ml of ethanol was added to the sample and heated for five minutes. Then a few drops of concentrated 2N HCl and 0.2 g of magnesium powder were added. Then the sample was shaken. The formation of yellow, red or orange color indicates the presence of flavonoid compounds.[22] For saponin identification, the sample was mixed with water and heated for 2-3

minutes, then stirred for 30 seconds, added 2 drops of HCl. If stable foam is formed, then the sample contains saponin compounds.[23] For tannin identification, the sample was dissolved in ethanol and mixed with 4 drops of FeCl3 1%. The appearance of a blue-black color indicated the presence of tannin compounds.[24]

The cell culture process was carried out in a biosafety cabinet. HSC-3 cells were cultured in DMEM (Gibco, Waltham, US) supplemented with FBS (Gibco, Waltham, US) and antibiotics, including 1% amphotericin B and 1% penicillin-streptomycin (Gibco, Waltham, US). HSC-3 cells were incubated at 37°C in an incubator (Esco, Changi, Singapore) humidified with 5% CO_2. The culture medium was regularly replaced, and cells were re-cultured every 3-4 days until the cells reached 80% confluency.[25]

Confluent cells were placed in 96 well plates with a total of 15 x 10^3 cells in each well. Each well was treated according to the designated group, then incubated for 24 hours. 10 µL of CCK-8 reagent (Sigma-Aldrich, Darmstadt, German) was added and incubated for 4 hours at 37°C. After incubation, the medium was discarded, and 0.049% DMSO (Acros Organics, Waltham, US) was added to each well. This stage used a blank, control (-) with cell + medium, control (+) with 3 µM doxorubicin, and solvent control with DMSO. Each treatment group, including controls, was performed in triplicate. Readings were taken at a wavelength of 450 nm using a microplate reader (Tecan, Männedorf, Switzerland).[26]

The number of cells was calculated by subtracting the media absorbance and a correction value (0.1696) from the treatment absorbance, then dividing the result by 0.00005. This calculation provides an estimate of viable cell count based on absorbance readings at 450 nm following treatment and incubation with the CCK-8 reagent. The IC_{50} value, which represents the concentration required to inhibit 50% of cell viability, was calculated using linear regression analysis of the dose–response curve, by plotting the percentage of cell viability against the logarithm of extract concentrations

The collected data were then analyzed using a normality test with the Shapiro-Wilk test. Then, a One-Way ANOVA test is performed, followed by a Tukey Post-Hoc test.

RESULTS

The phytochemical test results are shown in Table 1, and the cell counts from each treatment group are shown in Figure 1. The results showed that the ethanolic extracts of *C. papaya* leaf contained alkaloid, flavonoid, triterpenoid, and tannin compounds, while aqueous extract contained alkaloid, flavonoid, saponin, and tannin compounds. Figure 1 showed that the number of cells obtained from the treatment with ethanol extract of *C. papaya* leaf at concentrations of 125, 250, and 500 µg/mL were 15,300 ± 498.117; 15,805 ± 545.064; and 15,316 ± 873.548, respectively. Meanwhile, treatment with the aqueous extract of *C. papaya* leaf at the same concentrations resulted in cell numbers of 16,038 ± 626.476; 16,107 ± 446.111; and 15,428 ± 793.173, respectively. Figure 1 and table 2 show the number of HSC-3 cells under the tested conditions

A normality test with the Shapiro Wilk method conducted using GraphPad Prism software showed that ethanol and aqueous extracts of C. papaya leaf at concentrations of 125, 250, and 500 µg/mL, as well as the positive and negative controls were normally distributed ($p>0.05$). The One Way ANOVA test showed a significant difference in all test groups with a p value <0.0001. *Post-Hoc* Tukey test showed that ethanol and aqueous extracts of C. *papaya* leaf at concentrations of 125, 250, and 500 µg/mL, the solvent control, and the negative control had significant differences with the positive control.

Table 1. Phytochemical test results

Sample	Compound	Reagent	Result	
Ethanol Extract *C. papaya* leaf	Alkaloid	Dragendorff	Brown sediment	+
	Flavonoid	HCl 2N + Mg	Yellow, red, orange	+
	Saponin	HCl	Stable foam	-
	Steroid	Chloroform + Liebermann-Burchard	Blue-green	-
	Triterpenoid	Chloroform + Liebermann-Burchard	Brownish red	+
	Tannin	FeCl3	Blue-black	+
Aqueous Extract *C. papaya* leaf	Alkaloid	Dragendorff	Brown sediment	+
	Flavonoid	HCl 2N + Mg	Yellow, red, orange	+
	Saponin	HCl	Stable foam	+
	Steroid	Chloroform + Liebermann-Burchard	Blue-green	-
	Triterpenoid	Chloroform + Liebermann-Burchard	Brownish red	-
	Tannin	FeCl3	Blue-black	+

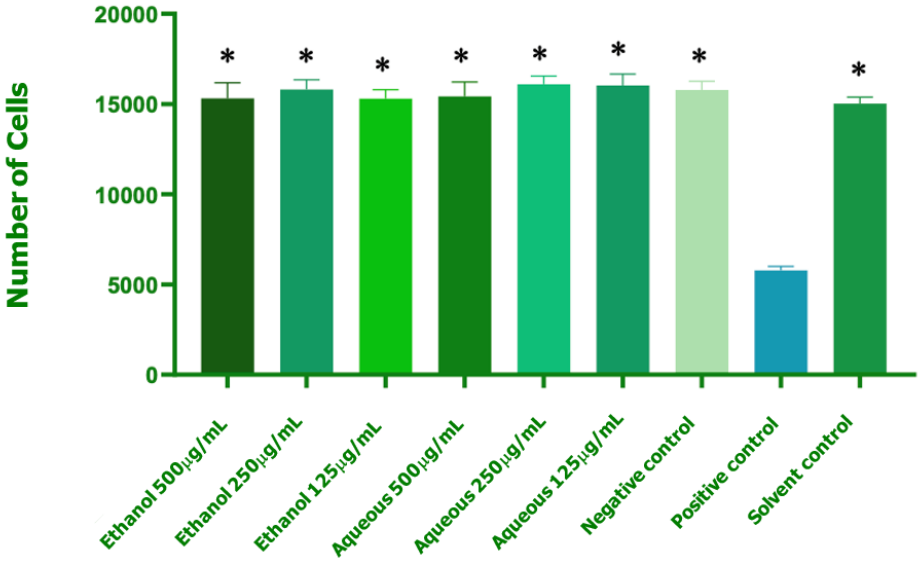

Figure 1. Bar graph showing the number of cells in each treatment. *: indicates a significant difference from the positive control

Table 2. Cytotoxicity test results of ethanolic and aqueous extracts of *C. papaya* leaf on HSC-3 cells.

Treatment Group	Concentration (µg/mL)	Cell Count (Mean ± SD)	Shapiro–Wilk (p-value)	Normality
Ethanol Extract	125	15.300 ± 498.12	0.624	Normal (p > 0.05)
	250	15.805 ± 545.06	0.711	Normal (p > 0.05)
	500	15.316 ± 873.55	0.532	Normal (p > 0.05)
Aqueous Extract	125	16.038 ± 626.48	0.842	Normal (p > 0.05)
	250	16.107 ± 446.11	0.683	Normal (p > 0.05)
	500	15.428 ± 793.17	0.794	Normal (p > 0.05)
Negative Control	—	15.781 ± 480.97	0.658	Normal (p > 0.05)
Solvent Control (DMSO)	—	15.022 ± 366.46	0.719	Normal (p > 0.05)
Positive Control (Doxorubicin)	—	5.781 ± 221.50	0.878	Normal (p > 0.05)

Data are presented as mean ± standard deviation (SD) of triplicate experiments. Normality was assessed using the Shapiro–Wilk test; all groups demonstrated normal distribution ($p > 0.05$).

DISCUSSION

The phytochemical test of ethanolic and aqueous extracts of *C. papaya* leaf showed different compounds. This difference may occur due to the use of different polarity indexes. The use of ethanol and water as solvents for *C. papaya* leaf follows the solvent selectivity principle governed by polarity. Solvents have a tendency to extract compounds with similar polarity, according to the "like dissolves like" principle. Water is better at extracting highly polar substances like saponins and certain glycosides because it has a higher polarity index (9.0).

On the other hand, ethanol can extract a wider variety of moderately polar to slightly nonpolar substances, such as flavonoids, alkaloids, tannins, and

triterpenoids, due to its lower polarity index (5.2).[27] The different biological activities of ethanol and aqueous extracts reflect these differences in phytochemical profiles. For example, the presence of triterpenoids in the ethanolic extract suggests its ability to extract compounds with strong anticancer potential, which were not present in the aqueous extract. Thus, choosing the right solvent is essential for determining the chemical makeup and possible bioactivity of extracts from plants.

Figure 1 shows that there was no discernible cytotoxic effect, as the number of viable HSC-3 cells remained above 15,000 at all concentrations of the ethanol and aqueous extracts of *C. papaya* leaf. While all extract types were significantly different from the positive control group (doxorubicin), none of the tested concentrations (125, 250, or 500 µg/mL) significantly reduced cell viability when compared to the negative control. In addition, the systematic study by Rokis et al. (2025) emphasized that most medicinal plants from Southeast Asia, including *Carica papaya*, exhibit chemopreventive potential through antioxidant mechanisms rather than direct cytotoxic effects. The antiproliferative effect of *C. papaya* tends to appear only at high concentrations (>1000 µg/mL) or after prolonged exposure periods (>72 hours).[28]

These results imply that the tested *C. papaya* extract concentrations are insufficient to cause cytotoxicity in HSC-3 cells during the 24-hour exposure period. Additional information supporting the observed biological activity can be found in Table 1's phytochemical screening results. Alkaloids, flavonoids, triterpenoids, and tannins were present in the ethanol extract, whereas alkaloids, flavonoids, tannins, and saponins were present in the aqueous extract. The study by Barnabas et al. (2022) confirmed a similar pattern, in which alkaloids, flavonoids, saponins, tannins, and triterpenes were detected in the ethanolic extract of *C. papaya*, with higher intensity compared to the aqueous extract.

The authors noted that ethanol was more effective in extracting non-polar compounds such as triterpenoids and flavonoids, which possess stronger antimicrobial and antioxidant potential.[29] In the context of solvent comparison, the study by Ilham et al. (2019) found that the ethanolic extract of *C. papaya* leaf contained higher levels of flavonoids and triterpenoids compared to the aqueous fraction, while the aqueous extract exhibited higher saponin content. This indicates that solvent polarity influences the types of active compounds extracted.[30]

Alkaloids and flavonoids are examples of bioactive substances with known anticancer potential, but their stability or concentrations may not be sufficient to cause cytotoxicity in HSC-3 cells. This is consistent with previous research by Yuliani and Syahdeni (2020), which found that T47D breast cancer cells were not cytotoxically affected by *C. papaya* ethanol extracts. Variations in biological responses across studies may be explained by differences in exposure duration, compound bioavailability, and cancer cell line sensitivity.[31]

Another study by Kong et al. (2021) reported that *C. papaya* leaf extract exhibited no significant acute toxicity at doses up to 2 g/kg, although it did not directly assess cytotoxicity in cancer cell lines. Current research indicates that although *C. papaya* extracts contain potentially anticancer compounds, their efficacy varies widely depending on cancer type and exposure conditions, underscoring the need for more focused oncological research.[32]

Alkaloids have anticancer properties through various mechanisms, such as inhibiting cell proliferation, inducing cell death, and interacting with DNA. As tubulin inhibitors, alkaloids bind to tubulin, a protein that forms microtubules during the cell cycle. The binding of alkaloids to tubulin inhibits the aggregation of microtubule proteins, causing the cell cycle to arrest at metaphase and triggering apoptosis.[33]

The anticancer mechanisms of flavonoid include inhibiting cancer cell growth, triggering apoptosis, and preventing the formation of new blood vessels, which is a process necessary for tumour growth. The anticancer activity of these flavonoids

is highly dependent on several factors such as the flavonoid dosage, the type of cancer cells targeted, and their interaction with the biological environment.[34]

Tannins can inhibit cell proliferation by suppressing protein kinase activity, thereby blocking the signal transduction pathway from the membrane to the cell nucleus.[35] Triterpenoids act as anticancer agents by inhibiting the cell division mechanism and activating the apoptotic pathway in cancer cells.[36] Saponins exhibit anticancer potential by inhibiting the formation of overexpressed Bcl-2, inducing the low-expression caspase-3 protein, increasing p53 expression, and triggering G1 cell cycle arrest.[37]

The anticancer activity of *C. papaya* leaf extract has been tested on several cancer cell lines. Aqueous extract of C*. papaya* leaf has been shown to reduce the viability of breast cancer cell lines (MCF-7).[11] The ethanol extract of *C. papaya* leaf at concentrations of 125, 250, 500 µg/mL exhibited moderate cytotoxic activity on prostate cancer cell lines (DU 145).[38] However, in this study, these concentrations had no cytotoxicity effect on HSC-3.

The use of different cell lines is one of the factors influencing the anticancer effect of *C. papaya* leaf. Each cancer cell has different characteristics, as not all cells have the same growth rate.[34] Therefore, selection of different cell types can yield varying results depending on the experimental conditions. The effectiveness of a compound in inhibiting cell growth is highly dependent on the doubling time of the cells themselves.[39]

To better understand the selectivity and wider applicability of *C. papaya* extracts, future research should include multiple oral cancer cell lines as well as normal oral epithelial cells. It is also advised to look into the molecular processes that underlie cytotoxicity, such as cell cyc0le arrest, reactive oxygen species production, and apoptotic signalling, in order to clarify how the extracts work. Additionally, to identify and measure the active compounds causing the observed effects, phytochemical profiling using HPLC or GC-MS should be carried out. Comparative research using different extraction methods, like ultrasound-assisted extraction or Soxhlet, may also maximise activity and yield. Lastly, in vivo research is necessary to confirm these extracts' safety profile and therapeutic potential in biological systems.

A comprehensive review by Munir et al. (2022) underscored the importance of elucidating *C. papaya*'s mechanisms through studies on cell cycle arrest, apoptosis, and ROS modulation, emphasizing that *in vitro* assays alone are insufficient without subsequent *in vivo* validation. They demonstrated that papaya leaf juice induced S-phase cell cycle arrest and apoptotic signalling, supporting the recommendation to assess molecular mechanisms in future studies.[40] Similarly, another previous study compared supercritical fluid extraction versus conventional extraction for *C. papaya* leaf and revealed significant differences in antioxidant and cytoprotective potentials. Their GC–MS analyses identified unique bioactive compounds (flavonoids, alkaloids, and phenolic acids), reinforcing the need for advanced extraction and profiling methods such as HPLC and GC–MS to optimize yield and activity.[41]

The statistical analysis in this study was performed to evaluate the differences in cytotoxic effects of *C. papaya* leaf extracts on HSC-3 cells across various treatment groups. The normality of the data was first assessed using the Shapiro–Wilk test, which confirmed that all datasets, including ethanol and aqueous extracts, as well as positive, negative, and solvent controls, were normally distributed ($p > 0.05$). Following this, a one-way ANOVA was conducted to compare the mean viable cell counts among groups. The ANOVA results revealed a statistically significant difference ($p < 0.0001$) among all treatments, suggesting that at least one group differed significantly from the others in terms of cell viability. This approach was chosen due to its suitability for analyzing quantitative outcomes derived from independent groups in cytotoxicity assays.

To determine which specific groups differed, a Tukey's post hoc test was subsequently applied. The results indicated that both ethanol and aqueous

extracts of *C. papaya* at concentrations of 125, 250, and 500 µg/mL were significantly different from the positive control (doxorubicin) but not significantly different from the negative or solvent controls. This finding confirms that neither extract produced a measurable cytotoxic effect on HSC-3 cells under the tested conditions. The combination of ANOVA and Tukey's test provided a statistically robust framework for detecting potential differences while minimizing Type I error. Overall, the statistical results support the conclusion that C. papaya leaf extracts, within the concentration range tested, are non-cytotoxic to oral squamous carcinoma (HSC-3) cells, emphasizing the need for further mechanistic studies using molecular assays to validate these findings biologically.

The limitations of this study should also be acknowledged. First, it only used one cell line (HSC-3) for oral squamous carcinoma, which might not accurately reflect the variety of oral cancer.[42, 43] To confirm and expand on the results, more research utilising several cell lines or in vivo models is required. Second, without delving deeper into underlying molecular mechanisms like apoptosis or oxidative stress pathways, the study evaluated cytotoxicity based only on cell viability. Third, only one extraction technique (maceration) and two solvents (ethanol and water) were used, which might not have fully extracted all pertinent bioactive components.

CONCLUSION

The results of this study demonstrate that both ethanolic and aqueous extracts of *C. papaya* leaf at concentrations of 125, 250, and 500 µg/mL did not exhibit cytotoxic effects on HSC-3 oral squamous carcinoma cells. This result suggests that although the extracts contain bioactive substances like alkaloids, flavonoids, triterpenoids, and tannins, the concentrations might not be sufficient to reduce cell viability in this specific cancer cell line. To enhance the possible anticancer effects of *C. papaya* leaf extracts, more research should therefore explore higher concentrations, longer exposure periods, or alternative extraction methods. This conclusion highlights the need for continued investigation within oral cancer therapy research and directly addresses the study goal of comparing the cytotoxicity effects.

The implication of this research lies in supporting the development of plant-based alternatives for oral cancer treatment. The results further emphasise the significance of extraction technique and solvent selection in optimising the medicinal potential of herbal compounds, which may help guide the development of more standardised and potent phytotherapeutic agents in subsequent oncology studies.

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