

Research Article

Cytotoxic and Antioxidant Effects of *Phoenix dactylifera* L. (Ajwa Date Extract) on Oral Squamous Cell Carcinoma Cell Line

Khushboo Shahbaz ¹, Jawaad Ahmed Asif ², Tang Liszen ³, Asma Abdullah Nurul ⁴,
and Mohammad Khursheed Alam ^{5,6,7}

¹Oral Medicine Department, School of Dental Sciences, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia

²Oral and Maxillofacial Surgeon, Prince Mutaib Bin Abdulaziz Hospital, Sakakah, Al-Jouf, Saudi Arabia

³Maxillofacial Surgery Unit, School of Dental Sciences, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia

⁴School of Health Sciences, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia

⁵Orthodontics, Preventive Dentistry Department, College of Dentistry, Jouf University, Saudi Arabia

⁶Center for Transdisciplinary Research (CFTR), Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

⁷Department of Public Health, Faculty of Allied Health Sciences, Daffodil International University, Dhaka, Bangladesh

Correspondence should be addressed to Jawaad Ahmed Asif; doctorjawaad@gmail.com, Tang Liszen; drliszen@usm.my, and Mohammad Khursheed Alam; mkalam@ju.edu.sa

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Aim. The aim of the current study is to investigate the antioxidant and apoptotic potential of Ajwa date flesh (ADF) and Ajwa date pit (ADP) extract on human squamous cell carcinoma cell line (HSC-2). **Method.** ADF and ADP were extracted with a solvent extraction method using hexane, acetone, and ethanol, which were then subjected to antioxidant assay by 2,2-diphenyl-1-picrylhydrazyl (DPPH). HSC-2 cells were then treated with different concentrations of ADF and ADP extract for 24, 48, and 72 hours. MTT assay was performed to assess the antiproliferative effect, and Annexin V-FITC was used for the detection of cellular apoptosis. **Results.** Acetone extracts of ADF and ADP had the highest radical scavenging and antioxidant activities followed by the ethanolic extracts, whereas ADP appeared to have significantly higher antioxidant effects than ADF. MTT assay demonstrated that acetone extracts of ADF and ADP were significantly cytotoxic against HSC-2 cells in a dose- and time-dependent manner. The half inhibitory concentration (IC₅₀) of ADF was found to be 8.69 mg/ml at 24 h, and the maximum cell growth inhibition was observed at 50 mg/ml. The IC₅₀ for the ADP was found to be 0.97 mg/ml at 24 h, and the maximum cell growth inhibition was observed at 5 mg/ml. Statistical analysis of the flow cytometry assay showed that the treatment with ADF and ADP extracts had a significant apoptotic effect which occurred in a dose-dependent manner. HSC-2 cells were seen in the late apoptotic stage with higher doses of ADF and ADP extract. ADP extract demonstrated higher apoptotic activity than ADF extract. In addition, combined treatment of ADF and ADP was also performed on HSC-2 cells which demonstrated higher apoptotic activity when compared to the single extract. **Conclusion.** Ajwa date fruit has a promising cytotoxic effect by inhibiting the growth and proliferation of OSCC cells and inducing cell death by apoptosis.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the 6th most common cancer around the world. It has been documented that 90% of all oral malignancies are squamous cell carcinoma (SCC) [1]. With an increase in the prevalence and mortality

rate, there were an estimated 377,713 new cases and 177,757 deaths from oral cancer in 2020 [2]. The incidence of the disease is influenced by several factors such as use of tobacco, betel quid chewing, alcohol drinking, infection with HPV, genetics, radiation, unhealthy diet, and physical inactivity [3]. The 5-year survival rate is 50%, but if detected in

early stages, it is increased to 60-80% [4]. Despite advancement in surgical approach and radiation therapy, the adverse effects of these treatments include anaemia, loss of appetite, and peripheral neuropathy leading to poor quality of life. Therefore, there is a need to develop an effective treatment with potent anticancer activity and less adverse effects [5].

A new approach in the management of oral cancer treatment is by antioxidant therapy. Reduction in oxidative stress which damages the intracellular structure and DNA of cell can be achieved by antioxidants, which are responsible for the removal of free radicals. Studies have found that date fruit scavenges the free radicals and prevents the occurrence of macromolecular changes in living systems [6]. Studies have also found that Ajwa date flesh and pit have shown gastroprotective [7], hepatoprotective [8], antidiabetic [9], and anti-inflammatory properties [10] due to their strong antioxidant, antimutagenic, and anticancerous activity which can be attributed due to the presence of phenolic compounds and flavonoid glycosides [11] [12]. Ajwa date flesh and pit are also attributed for antimicrobial [13], antifungal [14], and antiviral activities [15]. In the religion of Islam, Ajwa dates have remarkable status as narrated in Hadith by Sahih Al-Bukhari: The Prophet (peace be upon him) said “Whoever eats seven Ajwa dates every morning will not be harmed by poison or witchcraft all that day until night comes” [16].

Ajwa are soft fleshy dates with blackish-brown colour. The edible part is high in sugar content and moisture whereas the pit part is high in crude protein [16], while both parts contain abundant number of dietary fibres and high amount of essential minerals which are necessary for the skeletal growth and maintenance of cellular functions in human body in contrast with other date varieties [12, 16–18]. Provitamin A, C, D, E, and K; riboflavin (B2); pyridoxine (B6); and niacin (B3) were also found in Ajwa date flesh [19–21]. Date fruits also contain sulphated-flavanol glycosides. This form of flavanols was not detected before in any fruit as well as vegetables [22, 23].

Ajwa date fruit reported to have high amount of carotenoids like beta-carotene and lutein and polyphenols like quercetin, isoquercetin, luteolin, apigenin, rutin, and anthocyanins when compared to other varieties [24]. These phytochemicals are known for their antioxidant property which help in the prevention and treatment of cancer through inhibition of cell cycle progression and induction of apoptosis by modulating signalling pathways which can regulate intracellular reactive oxygen species (ROS), Bcl-2/Bax, and p53/p21 pathways [25, 26].

The anticancerous mechanism was investigated in a study on human breast adenocarcinoma (MCF7) cell line with methanolic extract of Ajwa date. The result showed significant inhibition of cell proliferation in a dose- and time-dependent manner. Flow cytometric analysis showed that the cell death was due to apoptosis [27]. In another study, date seed oil also has shown chemoprotective effects and can be used in the prevention of skin cancer caused by UV radiation [28, 29]. Few other *in vitro* studies showed similar results of cytotoxic properties of Ajwa date extract with aqueous acetone on cervical (HeLa) cell line [30], Ajwa date

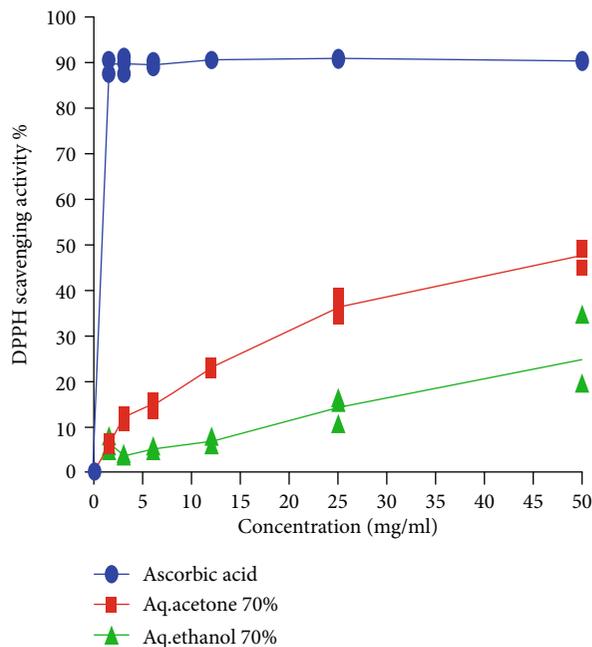


FIGURE 1: DPPH scavenging activity of different solvents of ADF extract at different concentrations. The curve for ascorbic acid has been used as a standard. Each value represents the mean of three independent experiments ($n = 3$).

extract with ethanol on hepatocellular carcinoma (HepG2) cell line [31], and lung cancer (NCI-H460) cell line [32].

The extraction of different phytochemical compounds is influenced by the polarity of the solvent. Anthocyanins are best extracted by ethanol [33], whereas acetone was the best solvent for the extraction of phenols and flavonoids [34]. Since polyphenols, flavonoids, and flavones attributed to the Ajwa date antioxidants and tissue protective properties, it is important to select a single solvent which can yield the maximum antioxidant from the fruit [35]. In the previous literature, different solvents have been used to extract different bioactive components according to their research needs; therefore, three solvents were selected in the present study to extract the maximum amount of polyphenols and flavonoids. To this date and to our best knowledge, no study has been conducted to oversee the cytotoxic and apoptotic effects of Ajwa date flesh and pit on human oral squamous cell carcinoma cell line (HSC-2).

2. Materials and Methods

2.1. Preparation of Ajwa Date. Ajwa dates are only cultivated in the outskirts of city of Madinah Al Munawwarah. Four kg of Ajwa dates was purchased from the “Tamar Market” of Al-Madinah Al Munawwarah, Kingdom of Saudi Arabia. Medium-sized Ajwa dates without any visible physical damage were selected and washed, first with tap water and then with distilled water three times under sterile environment to remove all the dust and soil particles; then dried with sterile cotton cloth; and air-dried under shade for one night. Ajwa dates were then manually pitted, and the seeds were washed again to remove any remaining date flesh and air-

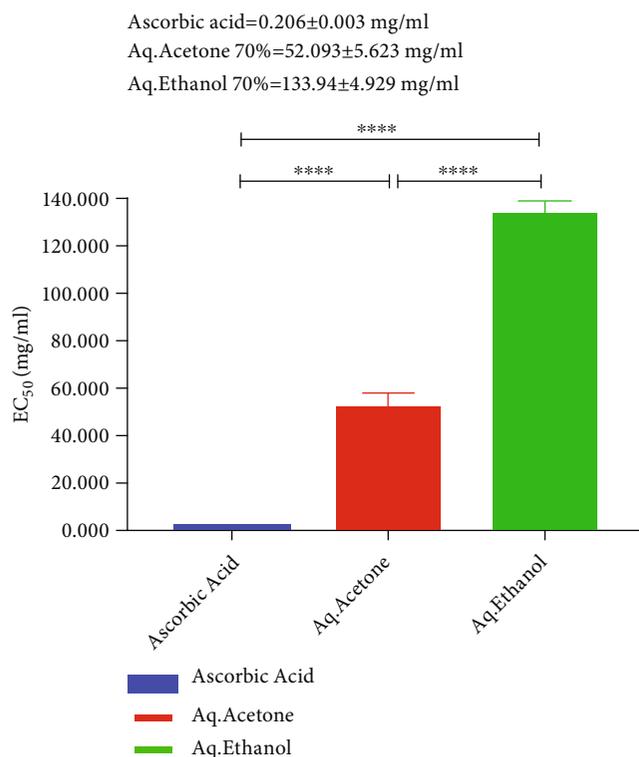


FIGURE 2: Comparison of EC₅₀ value of aq. acetone and aq. ethanol extract of ADF with the standard EC50 value of ascorbic acid. ****Significant difference from control at $p < 0.05$.

dried for another night. Ajwa date flesh was chopped to small pieces approximately 1 cm each using a surgical blade no. 10. Ajwa pits and the cut date flesh were then further dried completely using a freeze-drying method for a week separately in order to stabilize the samples and prevent microbial spoilage and hydrolytic rancidity [36]. Date pits were milled using a laboratory milling grinder with 30~300-micron finesse. The flesh part was crushed coarsely using a pestle and mortar. The powdered pits and flesh were weighed and made into aliquots of 50 g and sealed into airtight plastic bags stored at -40°C in dark until required for extraction.

2.2. Extraction of Date Flesh and Seed. In this study, different solvents such as n-hexane, acetone/H₂O (70:30, v/v), and ethanol (70:30, v/v) were used successively in order to isolate a wide range of antioxidant compounds present in Ajwa date's flesh and pits. In this study, the order in which extraction was performed was n-hexane, acetone 70%, and ethanol 70%. Ajwa date's flesh and pit extractions were performed separately following the protocol described previously by [27, 37] with slight modifications.

A portion of freeze-dried contents (15 g) was extracted in n-hexane (150 ml) with a ratio of 1:10 (weight to volume) for 48 hours at room temperature in a flat bottom flask on a shaking incubator. Following extraction, the resultant extract was filtered using Whatman No. 1 filter paper. The remaining residue was further extracted in acetone/H₂O (70:30) for 48 hours at room temperature with continuous agitation. The solution was filtered and evaporated under

vacuum using a concentrator to give a dark brownish extract. Remaining insoluble residue was subjected to ethanol/H₂O (70:30) extraction for 48 h at room temperature. The solution was filtered, and the filtrate was evaporated under vacuum to give dark brown concentrate. All the crude extracts were frozen, and the H₂O removed by freeze-drying to yield a brown solid. All the extracts of Ajwa date's flesh and pits were weighed, sealed, labelled, and stored at -20°C in 50 ml tubes for analytical purposes.

2.3. Antioxidant Properties of Date Extract

2.3.1. DPPH Assay. To evaluate the antioxidant capacity of Ajwa date's flesh and seed extracts from three different solvents, diphenyl picrylhydrazyl (DPPH) assay was carried out to obtain the highest biologically active extract according to the previously described protocol [38]. The working solution of DPPH in methanol was prepared daily for the measurement of antioxidants in the extracts using a UV spectrophotometer. To prepare the 0.1 mM of DPPH (molecular weight 394.32 g/mol) solution, 3.94 mg of DPPH was dissolved in 100 ml of methanol in a flask which was covered by aluminium foil. Three ml of this solution was then mixed with 100 µl of various concentrations of sample extract solution in disposable microcuvettes. The samples were kept in a dark place for 30 min at room temperature before being measured for absorption at 517 nm using the spectrophotometer. A blank sample containing 3 ml of DPPH solution was measured daily to obtain an absorbance of 0.0 ± 0.02 units at 517 nm. Ascorbic acid was used as a

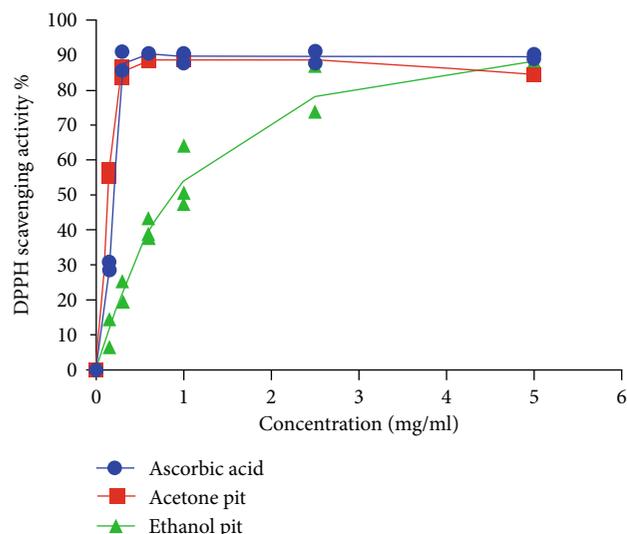


FIGURE 3: DPPH scavenging activity of various fractions of Ajwa date pits at different concentrations. The curve for ascorbic acid is used as a standard. Each value represents the mean of three independent experiments ($n = 3$).

standard reference for the comparison of results. A standard curve was prepared for calibration using six concentrations of ascorbic acid ranging from 1.5 to 50 mg/ml. The total antioxidants are expressed as milligrams per milliliter of ascorbic acid. The experiment was run in triplicate, and average was taken to calculate the radical scavenging activity using the following formula:

$$\% \text{inhibition} = \left[\frac{(A_b - A_s)}{A_b} \right] \times 100, \quad (1)$$

where A_b is absorbance of control and A_s is absorbance of the sample.

2.4. Cytotoxic Properties of the Date Extract

2.4.1. HSC-2 Cell Culture. HSC-2, RCB-1945, is a human oral squamous carcinoma cell line, which was purchased from Riken Cell Bank, Japan. HSC-2 cells were cultured in minimum essential medium (MEM) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cells were incubated in 5% CO_2 at 37°C incubation in a humidified CO_2 incubator. Cells were maintained as a monolayer, and cell passage was performed every 3rd or 4th day.

2.5. Evaluation of Morphological Changes of HSC-2 Cells. Cells were cultured in 6-wells (10 mm) cell culture petri dishes at a seeding density of 2×10^5 cells/well. After 24-hour attachment, the cells were treated with different concentrations of Ajwa date's flesh and pit extracts separately and in combination, whereas untreated cells served as control. The morphological changes were observed under an inverted microscope, and images were taken for the comparison with the untreated cell images at 48-hour treatment.

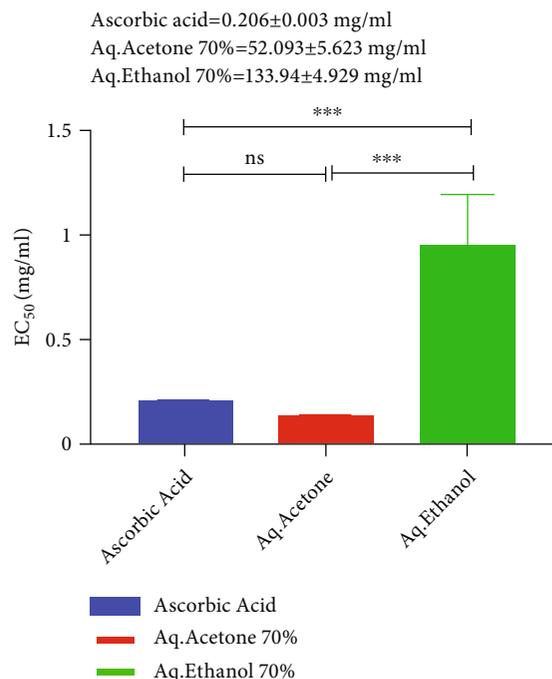


FIGURE 4: Comparison of EC₅₀ value of aq. acetone and aq. ethanol extract of ADP with the standard EC₅₀ value of ascorbic acid. ***Significant difference from control at $p < 0.05$; NS: no significant difference from control.

2.6. Assessing Cell Viability by MTT Assay. In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxic effect of Ajwa date's flesh and pit extracts on oral squamous cell carcinoma. A range of concentrations (0.8, 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml) of ADF extract [27, 31, 39] and (0.08, 0.31, 0.63, 1.15, 1.25, 2.5, and 5 mg/ml) of ADP extract [40, 41] were selected with 70% aq. acetone.

The HSC-2 cells were harvested and seeded in a 96-well plate at a cell density of 5×10^3 cells/well. Cell's attachment was confirmed under a microscope after 24 hours; the cells were then treated with different concentrations of ADF and ADP extracts separately. Control wells were treated with the same amount of complete growth media only. For every treatment and untreated control group, complete growth media without cells were added as a blank to reduce the background absorbance values. For every concentration of ADF and ADP extracts, 6 wells were used, three with treatment and three as blanks. All the experiments were carried out three times independently with 3 replicates in each experiment.

The cells were grown with sample extracts up to three different time points (24 h, 48 h, and 72 h) after which MTT assay was performed by removing the medium gently and adding 10 μl of MTT solution with a final concentration of 5 mg/ml per well, and incubated at 37°C for about 4 hours until the purple crystals were formed. After that, the MTT solution was discarded from every well and 100 μl of DMSO was added to dissolve the crystals. The 96-well plate was mounted on a microplate shaker and shaken for 15 minutes until the crystals completely dissolved. The absorbance value

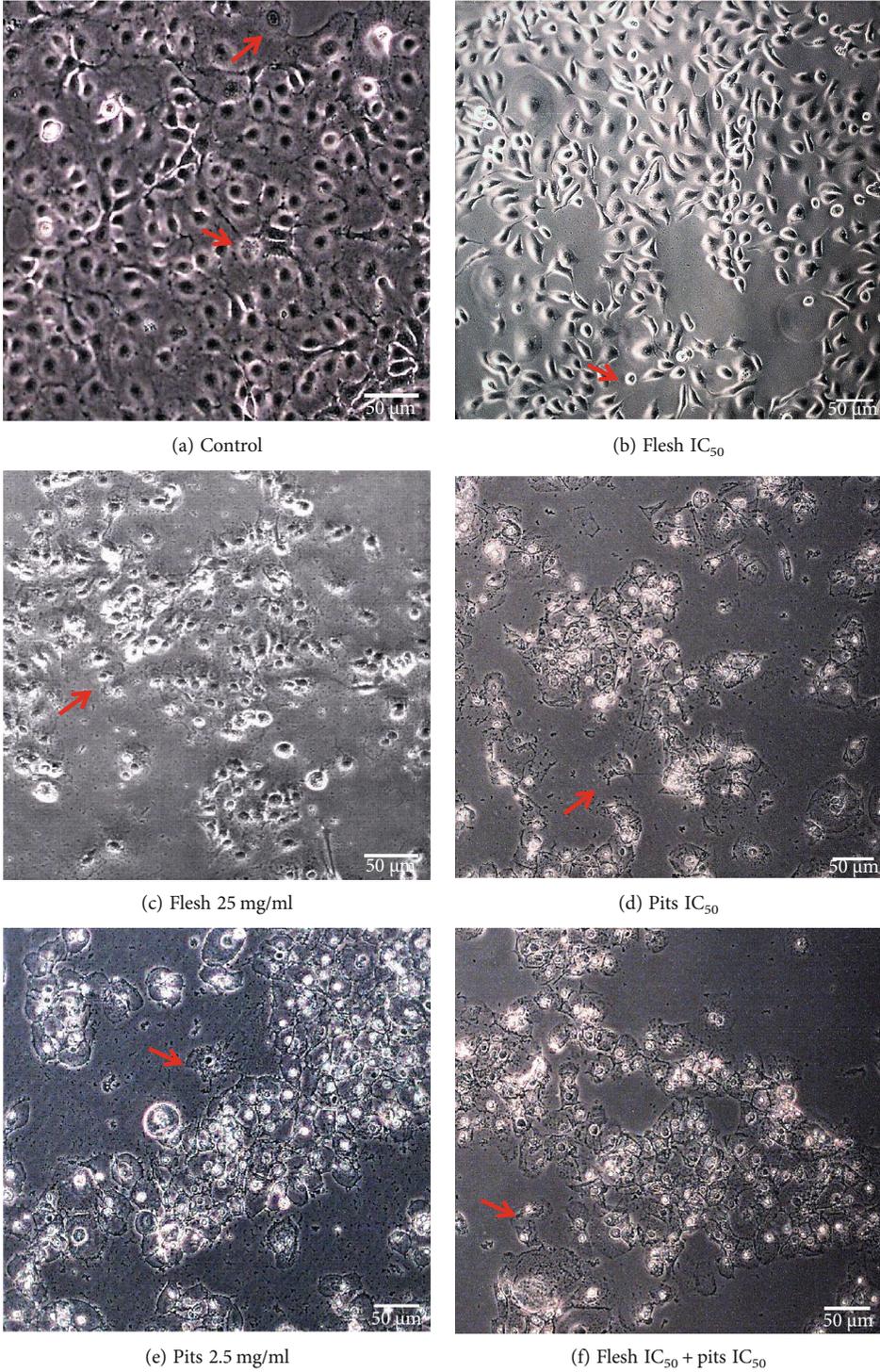


FIGURE 5: Continued.



(g) Flesh 25 mg/ml + pits 2.5 mg/ml

FIGURE 5: Morphological appearance under an inverted light microscope after 48 hours. Mitotic figures can be seen in (a), cell shrinkage and cell detachment can be seen in (b) and (c), and membrane disruptions, cytoplasmic condensation, and cell death can be seen in (d), (e), (f), and (g); magnification 100x.

for each well was determined at an optical density at 570 nm wavelength using an ELISA microplate reader [42]. The 50% inhibitory concentration (IC_{50}) of the ADF and ADP extract was also calculated at 24 h.

The cell viability (CV) percentage after treatment with ADF and ADP extract was calculated with the formula below:

$$CV(\%) = \frac{\text{absorbance of treatment cells} - \text{absorbance of blanks}}{\text{absorbance of control cell} - \text{absorbance of blanks}} \times 100. \quad (2)$$

2.7. Detection of Apoptosis by Flow Cytometry Assay.

Annexin V-FITC (fluorescein isothiocyanate) Apoptosis Detection Kit I was used to quantify apoptotic cells by flow cytometry following the manufacturer's instructions. For negative control, untreated cells were used. Briefly, HSC-2 cells at 2×10^5 cells/ml density were incubated for 24 h to allow adherence of cells to the 6-well culture plate. After 24 hours, the cells were incubated with 8.69 mg/ml (IC_{50}) and 25 mg/ml of ADF extract and 0.97 mg/ml (IC_{50}) and 2.5 mg/ml of ADP extracts for 24, 48, and 72 hours. After the given time point, the cells were harvested by washing with PBS and trypsinizing with 400-450 μ l of trypsin. Cells were then centrifuged with 1 ml of cold PBS twice and once with 100 μ l of 1X binding buffer solution for 5 minutes each time at $157 \times g$. The cells were stained with 5 μ l Annexin V-FITC and 5 μ l Propidium Iodide (PI) for 15 min at 25°C in the dark and resuspended in 400 μ l of 1X binding buffer solution and immediately analysed by BD Accuri™ C6 flow cytometry in 5 ml round-bottom polystyrene FACS tubes. Wavelengths of 533 nm and 585 nm were used for Annexin V-FITC and PI, respectively. 10,000 events per sample were recorded on forward scatter versus side scatter plot using BD Accuri™ C6 software. Control samples were prepared separately for the purpose of recording the HSC-2 cells according to their granularity and size. Cells with PI-only stain represent necrosis, PI and Annexin-V represent late apoptosis,

cells stained with Annexin-V only represent early apoptosis, and unstained cells were evaluated as viable healthy cells.

2.8. Combination Treatment. A combination treatment of both ADF and ADP extracts was performed together to analyse if there was any synergistic effect in apoptosis of the cells.

IC_{50} concentrations from the MTT assay of ADF and ADP were combined together (8.69 mg/ml + 0.97 mg/ml) as a single treatment, and the results were recorded.

Likewise, next higher concentrations were combined for both the extracts (25 mg/ml + 2.5 mg/ml) and used as a single treatment to observe if it had significantly higher apoptotic effect than if used separately.

2.9. Statistical Analysis. The statistical analysis was carried out using SPSS 24 version. To compare the difference between the control and the treated groups for DPPH, cell proliferation and apoptosis assay. One-way ANOVA was conducted followed by either Tukey's post hoc or Dunnett's multiple comparison test. The IC_{50} values for DPPH and MTT assay were calculated using nonlinear regression analysis function with Prism GraphPad software (version 7). All experimental data was represented as the mean \pm standard deviation (SD) of three independent experiments which were performed in triplicate. A p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. DPPH Assay. Assessment of the total antioxidant contents of the Ajwa date flesh's extract was performed. The assay could not be performed with n-hexane solvent due to the insufficient amount of extracted residue present to make a working solution to perform the assay. Therefore, antioxidant activity from the acetone/ H_2O (70:30, v/v) and ethanol/ H_2O (70:30, v/v) was studied.

The results of ADF sample of aq. acetone 70% and aq. ethanol 70% of 1 to 50 mg/ml concentrations are shown in Figure 1. Data shows that the percentage of inhibition of

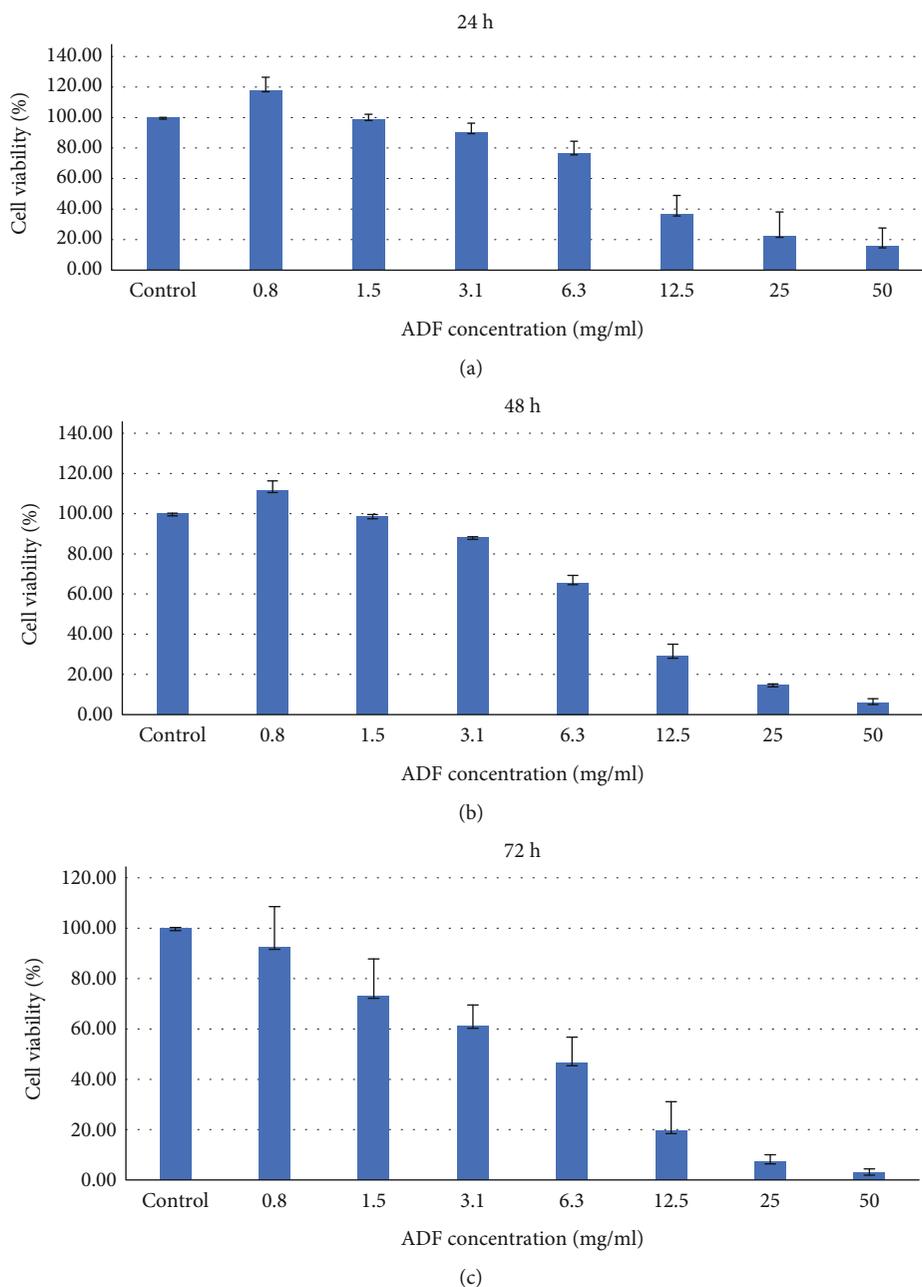


FIGURE 6: Inhibition of proliferation of HSC-2 cell. MTT assay of HSC-2 cells treated with ADF extract at different concentrations at (a) 24 h, (b) 48 h, and (c) 72 h. These decreases in percentage of cell viability were statistically significant. The values are expressed as mean SD from triplicate samples of three independent experiments. * indicates $p < 0.05$.

aq. acetone 70% ADF extract was higher than that of the aq. ethanol 70% ADF extract. The curve for ascorbic acid also showed the same inhibition pattern, but at a very low concentration of 1.5 mg/ml, it reached the highest inhibition percentage of 89.8%.

The graph (Figure 1) presented showed that the extract concentrations of both solvents are proportional to the percentage of inhibition, which means the greater the concentration of extracts, the greater the DPPH scavenging activity. The inhibition percentage was significantly greater for the aq. acetone extract of ADF (median = 15.1%) than the aq. ethanol extract of ADF (median = 5.6%) ($p = 0.011$).

3.2. Determination of EC_{50} Value for Ajwa Date Flesh Extract with Various Solvents. EC_{50} is the dose concentration of the sample required to reduce 50% of the free radicals of DPPH. The half-maximal effective concentration (EC_{50}) of 70% aq. acetone extract of ADF was 52.09 mg/ml, and EC_{50} of 70% aq. ethanol extract of ADF was 133.94 mg/ml. Ascorbic acid $EC_{50} = 0.206$ mg/ml. The graphical representation is shown in Figure 2. These results suggest that 70% acetone extract has more antioxidant activity than the 70% ethanol extract of flesh sample. The high EC_{50} value of 70% ethanol extract suggests the minimal amount of antioxidant present in this extract.

3.3. Assessment of the Total Antioxidant Contents of the ADP. For ADP sample, concentrations ranging from 0 to 5 mg/ml were selected for both solvents. 70% aqueous acetone extract has greater DPPH free radical scavenging activity at a lower concentration than 70% aqueous ethanol extract of ADP, but the highest average yield of the total antioxidants for both the solvent samples was reached at the concentration of 5 mg/ml as shown in Figure 3. At the $p < 0.05$ level of significance, the results showed that there is significant difference in the inhibition percentage ($p = 0.04$) between the 70% acetone (median = 86.2%) and 70% ethanol (median = 39.1%) solvent extract.

3.4. Determination of EC_{50} Value of Ajwa Date Pits with Various Solvents. The EC_{50} of 70% aq. acetone extract of ADP was 0.153 mg/ml, and that of 70% aq. ethanol extract of ADP was 0.954 mg/ml. The EC_{50} value of ascorbic acid was 0.206 mg/ml, as shown in Figure 4.

3.5. ADF and ADP Induced Changes in Cell Morphology. Images taken from the light microscope of HSC-2 cells of control sample demonstrated characteristic of epithelial nature and growth proliferation as a monolayer. The cells appeared to be attached together in an ovoid shape with a large central nucleus; dividing cell can also be seen with two or more nucleoli in Figure 5(a). In contrast, the ADF and ADP extract-treated cells showed mild to severe decrease in cell numbers (Figures 5(b)–5(g)) which was dose dependent leaving behind very few live cells. At a concentration of ADF extract IC_{50} , cell shrinkage and partial cell-to-cell detachment can be seen; cells have also started losing their shape. At a concentration of ADF extract 25 mg/ml, more drastic changes can be seen; cells have become rounded in shape with complete cell-to-cell detachment, with decrease in number of viable cells. For ADP extract at a concentration of IC_{50} , cytoplasmic vacuolization, nuclear condensation, and cluster shrinkage can be seen. At a concentration of 2.5 mg/ml, a lot of cellular fragmentation can be seen with very few viable cells. For the combination treatment with ADF and ADP extract, drastic morphological changes can be seen in Figures 5(f) and 5(g)); nuclear condensation, cell membrane blebbing, and fragmentation are vastly present with very few viable cells.

3.6. ADF Inhibited HCS-2 Cell Proliferation. The MTT assay results from the present study demonstrated HSC-2 cell growth inhibition following the treatment with ADF extract. At 24 h treatment period, ADF extract reduced cell viability to 99, 90.3, 76.5, 36.5, 22.4, and 15.6% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml, respectively. Negative correlation can be seen between the concentration of ADF extract and viable cells of OSCC. As the concentration of the ADF extract increases from 1.5 to 50 mg/ml, the percentage of viable cells decreased from 99% to 15.6% indicating a dose-dependent manner. At 48 h treatment period, ADF extract exerted a more pronounced effect, drastically reducing the viability of treated cells to 98.5, 88.6, 65.6, 29, 14.8, and 5.9% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml of extract, respectively. Moreover, a further decrease in cell viability percentage

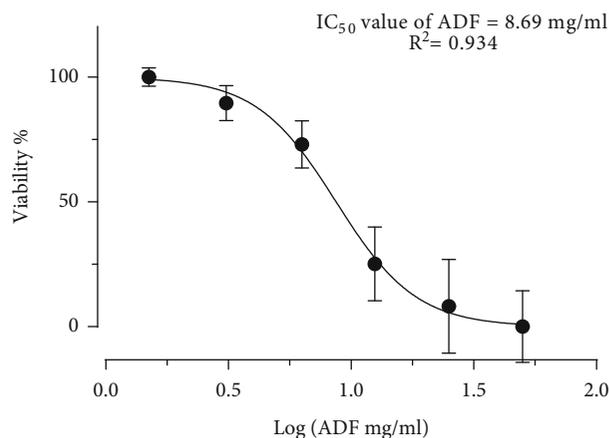


FIGURE 7: Determination of IC_{50} value of (ADF) on HSC-2 cells.

was seen at 72 h with a percentage of 66.5, 61.1, 46.3, 19.4, 7.3, and 2.9% at 1.5, 3.1, 6.3, 12.5, 25, and 50 mg/ml, respectively, stipulating that ADF extract is more toxic at higher concentrations, as presented in Figure 6. From the above results at 50 mg/ml concentration, the cell viability decreased to 15.6% at 24 h, 5.9% at 48 h, and 2.9% at 72 h. Therefore, the cell viability data proposed that treatment with ADF extract significantly reduced HSC-2 cell growth in both dose- and time-dependent manners, signifying its ability to impair proliferation potential. IC_{50} value is estimated to comprehend the basic pharmacological and biological characteristics; the lower the IC_{50} value, the more potent the drug is [43]. The IC_{50} value was determined to be 8.69 mg/ml at 24 h duration, in Figure 7.

3.7. ADP Inhibited HSC-2 Cell Proliferation. MTT assay demonstrated HSC-2 cell growth inhibition following the treatment with ADP extract in a dose-dependent manner (Figure 8). Ajwa date pit extract showed similar but enhanced results compared to the flesh part with significant cytotoxic effect on the viability on HSC-2 cells. At 24 h treatment incubation time, ADP extract reduced cell viability to 82.2, 73.7, 54.9, 40.7, 22.3, 15.1, and 6.6% at 0.08, 0.3, 10.6, 31.1, 51.2, 2.5, and 5.00 mg/ml concentration of ADP extract, respectively. At 48 h treatment period, ADP extract reduced cell viability 79.3, 70.1, 46.5, 25.3, 14.2, 10.6, and 3.7% at 0.08, 0.3, 10.6, 31.1, 51.2, 2.5, and 5.00 mg/ml concentration of ADP extract, respectively, as shown in Figure 8. Thus, the data demonstrated cell growth inhibition in a dose- and time-dependent manner. The significant decrease in percentage of cell viability started from 0.63 mg/ml concentration at 24 h and at 48 hours and 1.25 mg/ml at 72 h. The IC_{50} value was determined to be 0.97 mg/ml at 24 h duration from Figure 9.

3.8. ADF and ADP Induced Apoptosis in HSC-2 Cells. The percentage of early apoptotic and late apoptotic cells increased in the treated group when compared to that of the early and late apoptotic cells of control cells. Among the various extracts, ADP 2.5 mg/ml showed the highest percentage of late apoptotic cells and combination of ADF and ADP IC_{50} concentration showed the highest percentage of early apoptotic cells at 24 h. At 48 h, ADP IC_{50} showed the

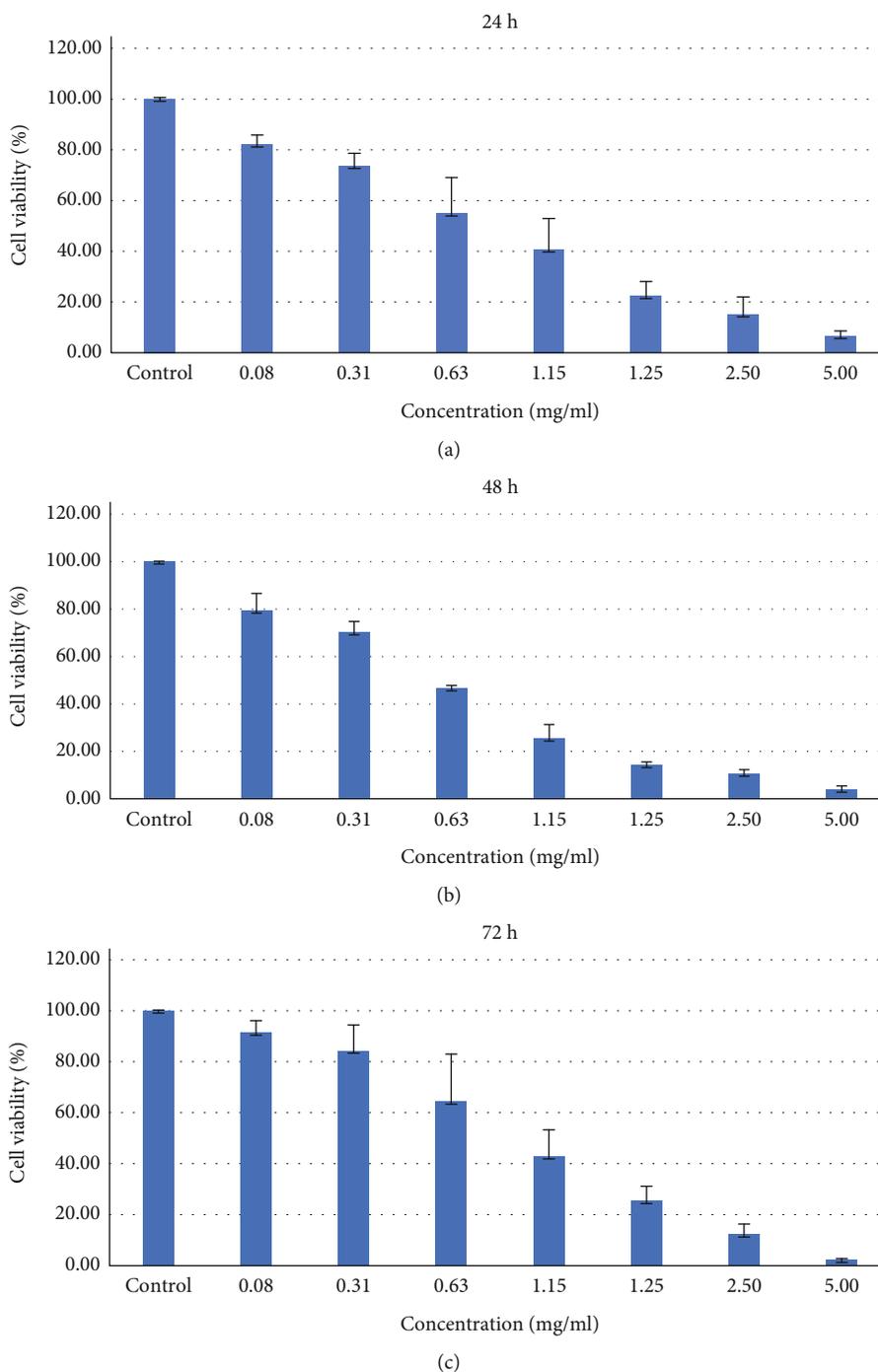


FIGURE 8: MTT assay of HSC-2 cells treated with ADP extract at different concentrations at (a) 24 h, (b) 48 h, and (c) 72 h. The values are expressed as mean SD from triplicate samples of three independent experiments. * indicates $p < 0.05$.

highest percentage of late apoptotic cells and combination of ADF 25 mg/ml+ADP 2.5 mg/ml showed the highest early apoptotic cells. At 72 h, the increase in total apoptotic cells was $40 \pm 47.09\%$ and $62.37 \pm 50.09\%$ for IC_{50} (8.69) mg/ml and 25 mg/ml of ADF, respectively; for ADP, total apoptotic cells were $82.13 \pm 50.7\%$ and $69.77 \pm 37.03\%$ for IC_{50} (0.97) mg/ml and 2.5 mg/ml, respectively, compared to control $10.83 \pm 3.87\%$. (Figures 10 and 11).

When the combination of both ADF and ADP extracts as a single-treatment ADF and ADP IC_{50} (8.69 + 0.97) mg/ml

and ADF and ADP (25 + 2.5) mg/ml is given, the result of total apoptotic cells was $76.57 \pm 50.21\%$ and $75.47 \pm 21.64\%$ (Figures 10 and 11(a)–11(d)).

4. Discussion

The DPPH results of ADF and ADP extracts demonstrated that when acetone was used as a solvent, higher antioxidant activity was obtained in both flesh and pits compared to the values obtained with ethanol at the same solvent

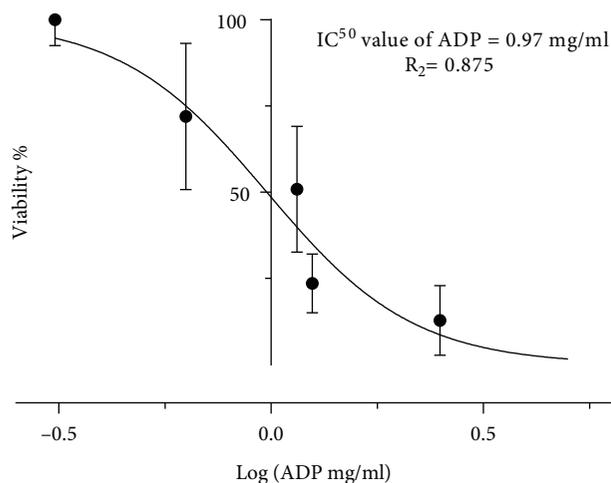


FIGURE 9: The graph was constructed to determine the IC_{50} value of ADP extract on HSC-2 cells.

concentrations (70%). These results are in line with a previous study conducted by Nematallah *et al.* in 2018 where 50% aq. acetone yielded the highest antioxidant activity in Ajwa date, followed by ethanolic extract. The variations in the values can be due to the difference in extraction procedure [44]. Another important finding observed from the DPPH assay was that the pit extract exhibited higher antioxidant activity with both the solvents when compared with the flesh part. These results are in support with a study reported by Maqsood *et al.* where the concentration of acetone and ethanol between 60 and 80% yielded the highest DPPH free radical scavenging activity from a date pit [45]. Therefore, it can be concluded that pit appeared to be a richer source for phenols and flavonoids; these results can be supported by previous studies, where date pits are the highest source of total polyphenols among tea, flaxseed, nut seeds, grapes, and even date flesh [46, 47]. Date fruit flesh also contains polyphenols like quercetin and kaempferol [48], which possess anticancer activity against oral squamous cell carcinoma [49, 50]. Therefore, it can be said that the anticancer effect of Ajwa date extract against HSC-2 cells might be due to the integrated or collective effect of the potential bioactive components of Ajwa dates. The anticancer activity of these bioactive compounds can be mediated by several molecular mechanisms including free radical scavenging activity, deactivation of carcinogenic metabolites, antiproliferation, induction of apoptosis, and cell cycle arrest [51].

One of the hallmarks of cancers is its ability to replicate and invade through escaping apoptosis, being insensitive to antigrowth signals, and self-sufficient in growth signals to proliferate uncontrollably. Hence, finding new drugs and treatments, targeting various pathways in the induction of apoptosis and inhibition in the proliferation, plays an important role in the treatment of cancers. Interestingly, the morphological data of the present study revealed ADF- and APD-mediated changes which are indicative of apoptosis. Treated cells appeared shrunken, nonadherent, partially detached, and rounded in shape, with also a decrease in cell number. As the concentration of ADF and ADP extracts

increased, more drastic changes were observed like cluster shrinkage, membrane blebbing, and cellular fragmentations leading to cell death, exhibiting a dose-dependent severity. These are typically initial characteristic features of apoptotic cell death [52]. In addition, both MTT and annexin V-FITC costaining with PI assays demonstrated inhibition in cell growth following treatment with ADF and ADP extract, supporting the morphological observations in the HSC-2 cells. In previous studies, apoptosis or initiation of apoptotic pathways has been induced by bioactive components and secondary metabolites of natural products [53].

Interestingly, in the present study, the data from MTT assay showed that, at a concentration of 0.8 mg/ml of ADF extract, there was an increase in cell viability percentage of 117.9 and 111.4% at 24 and 48 h of incubation period, respectively, when compared to the untreated control group. This could be explained by a phenomenon known as hormesis, where some cells might try to adjust to the toxic environment at very low dose resulting in a higher MTT signal compared to the control [54]. ADP extract showed increased cytotoxic effect on the viability on HSC-2 cells, which can mainly be attributed to the presence of high amount of phenolics, flavonoids, and vitamin C in the seeds compared to the flesh part [46]. The date seeds contain high amount of total polyphenols, close to 3942 mg/100 g, whereas date flesh contains 239.5 mg/100 g wet weight [34].

In a study, the IC_{50} value of ADF extract against human hepatocellular carcinoma (HepG2) cells was 20.03 mg/ml after 24-hour exposure [31]. In comparison to the present study, the IC_{50} value of 8.96 mg/ml after 24-hour treatment period indicates that ADF extract was more effective against HSC-2 cells. The variation in the IC_{50} value could be due to many reasons. It could be the difference in proliferation rate of the cells or the cell density during the assay period [55].

On the contrary, the 50% inhibition of HSC-2 cells by ADP extract with an extremely low IC_{50} in the present study confirmed the anticancer property of ADP extract. In a similar study conducted by Thouri and colleagues in 2018, it was shown that date pit extract of two different varieties induced significant growth inhibition and apoptosis in a human cervical cell line (HeLa) and human liver cell line (HepG2) with an IC_{50} value of 0.028 mg/ml and 0.034 mg/ml, respectively. They also reported that the seed extract had no cytotoxic effects on normal fibroblast cell line [41]. Interestingly, polyphenols were distinguished by their low cytotoxic effects towards normal cell line and increased cytotoxicity towards cancer cell line [56].

The exact mode of action of ADF and ADP extract on HSC-2 cells is not fully understood. Nonetheless, a likely route of action for the extract could be via modulating oxidative stress and scavenging free radicals within the cells. Various stimuli including reactive oxygen species are known to activate HSC-2 cells [57]. In the present study, flow cytometry analysis indicated that the cytotoxic effects in HSC-2 cells were due to apoptosis induction. Furthermore, the results also displayed that with a lower concentration of both ADF and ADP extracts, HSC-2 cells were observed more in early apoptosis stage while late apoptosis stage was identified at a higher concentration of extracts. The data also

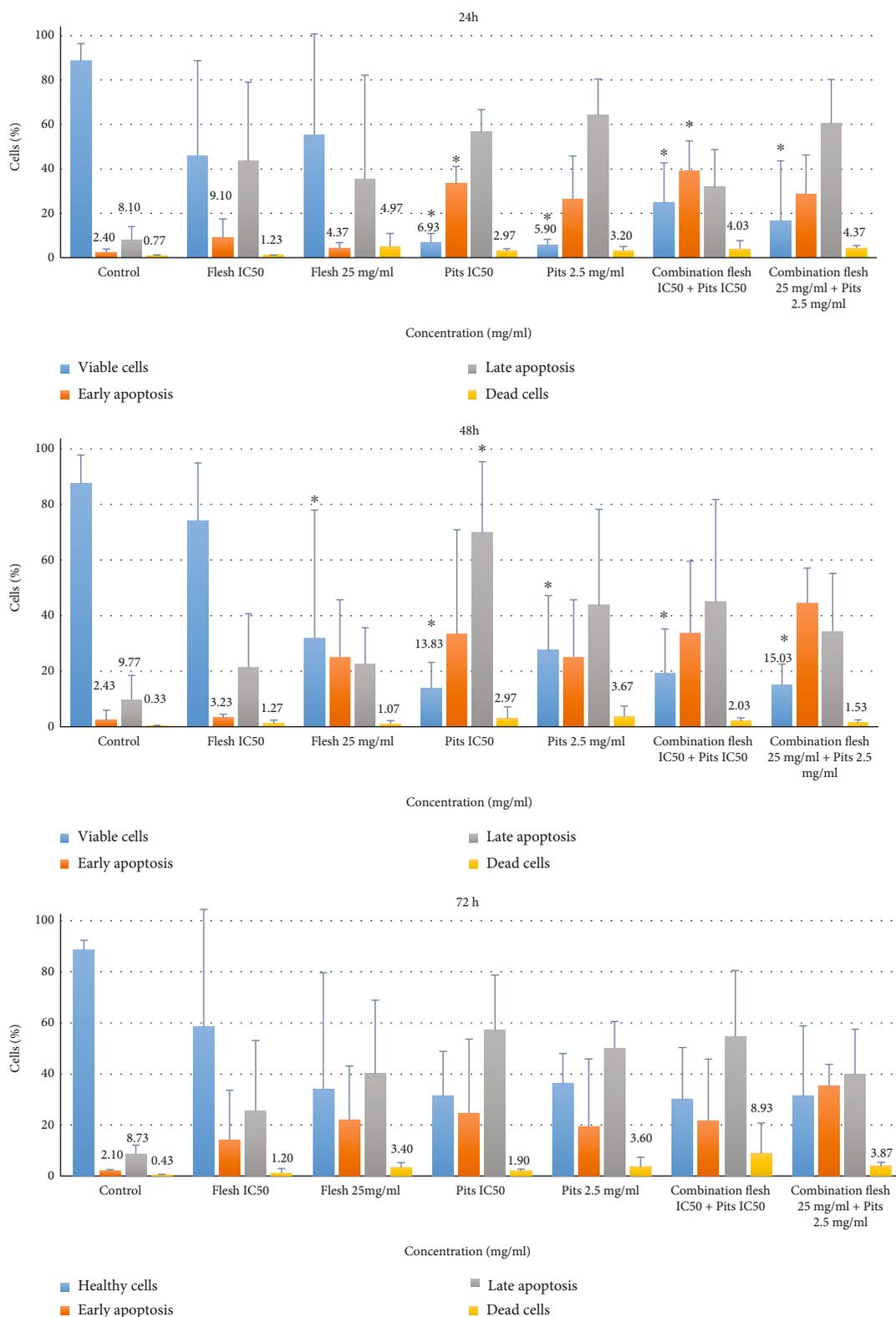
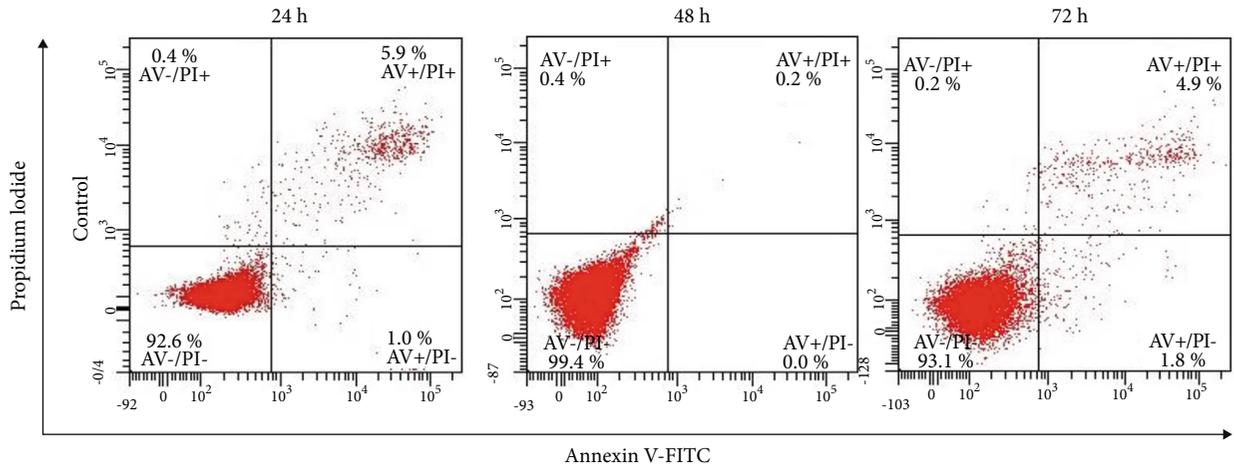
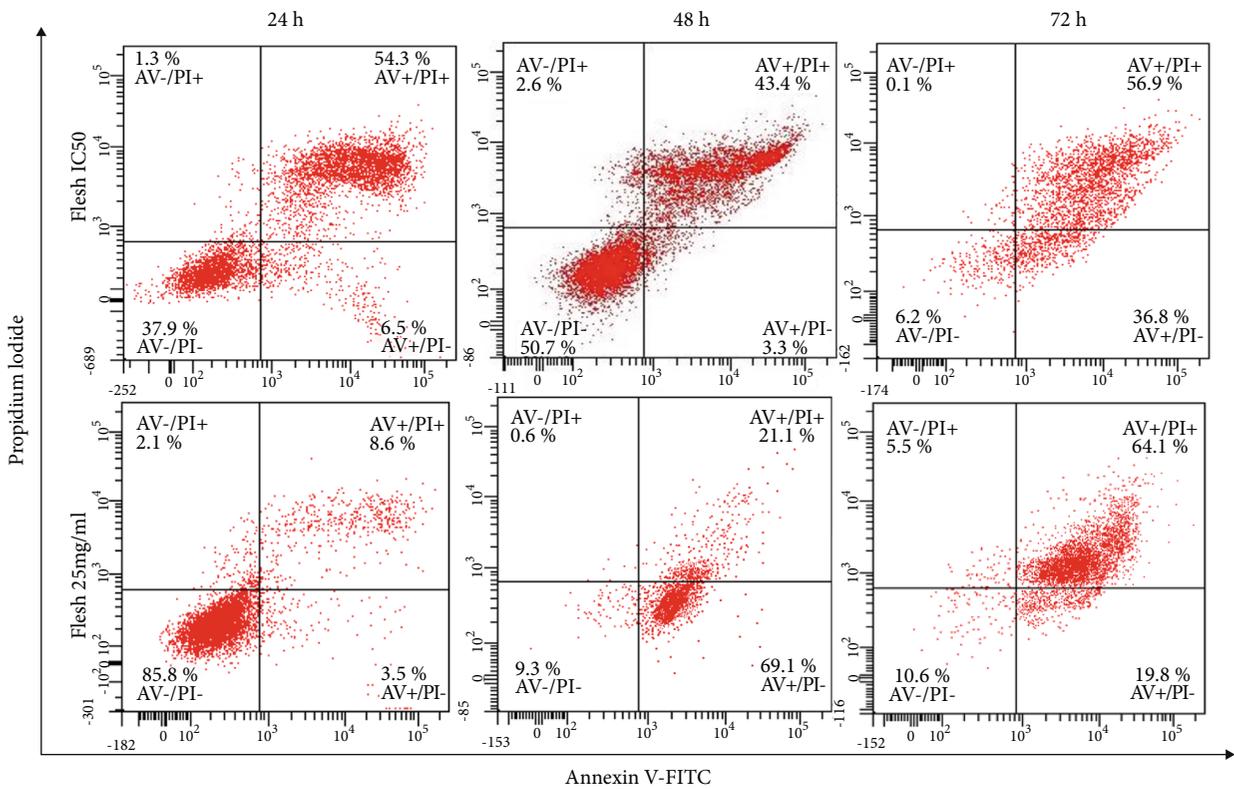


FIGURE 10: The effect of adding various concentrations of Ajwa date flesh and pit extracts on the apoptotic activity of HSC-2 cell line at 24 h, 48 h, and 72 h. The percentage of cells is shown in four stages, healthy cells, cells in early apoptotic stage, cells in late apoptotic stage, and dead or necrotic cells. The flesh IC50 value is 8.69 mg/ml, and pits IC50 value is 0.97 mg/ml. * indicates that the treatment is significantly different from the control group at $p < 0.05$.

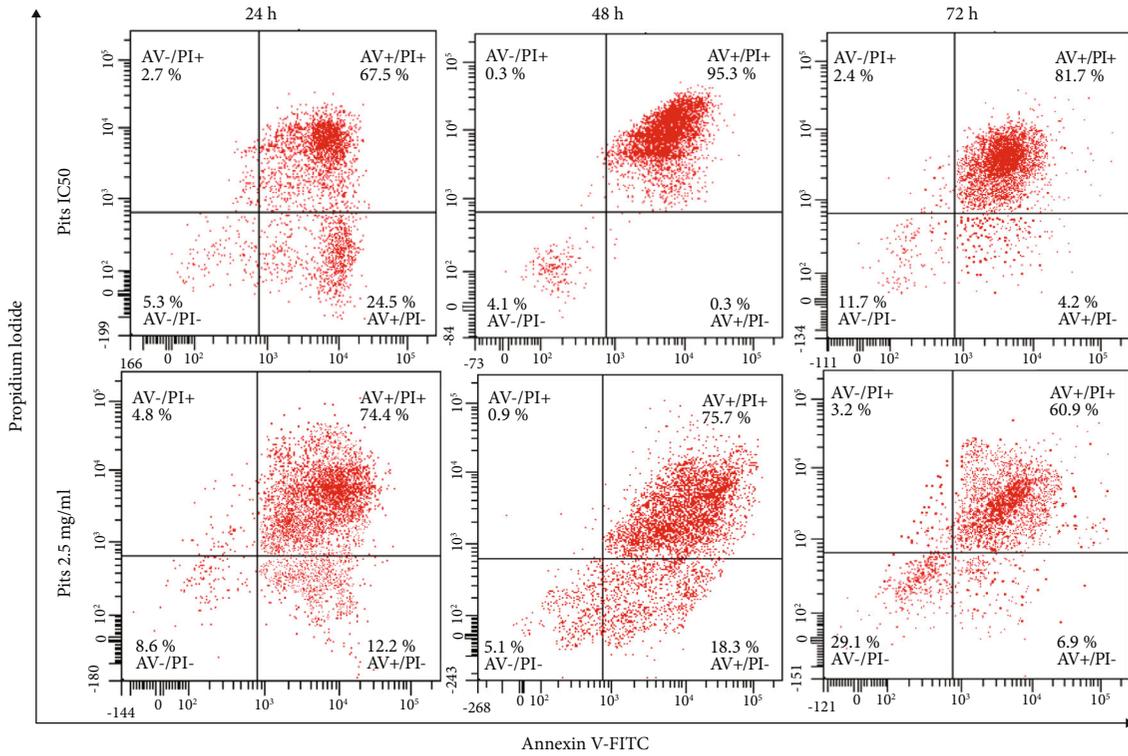


(a)

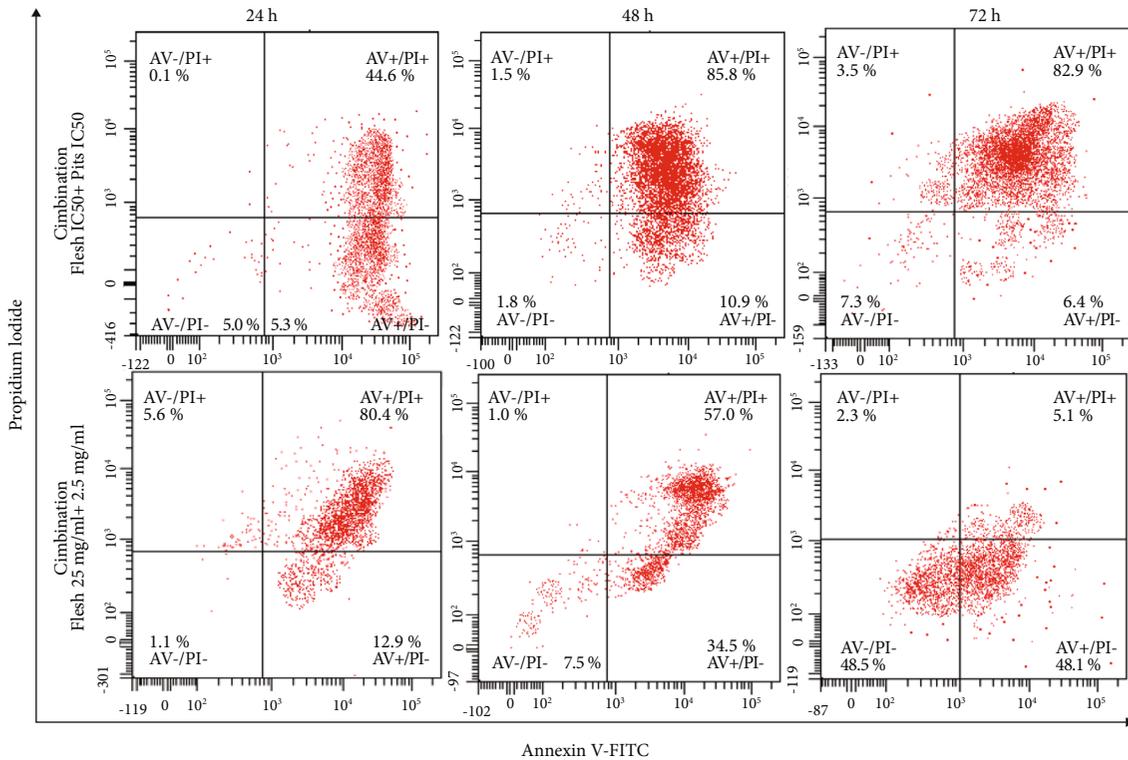


(b)

FIGURE 11: Continued.



(c)



(d)

FIGURE 11: Annexin V-FITC and PI assays on HSC-2 cells treated with various concentrations of ADF and ADP for 24, 48, and 72 hours are represented in a dot-plot graph, where the AV-/PI- quadrant represents viable cells, AV+/PI- quadrant represents cells in early apoptosis, AV+/PI+ quadrant represents cells in late apoptosis, and AV-/PI+ quadrant represents dead or necrotic cells. (a) Represent the control group, (b) represent flesh with IC₅₀ value and flesh with 25 mg/ml, (c) shows pits with IC₅₀ and 2.5 mg/ml value, and (d) shows the combination of flesh IC₅₀+pits IC₅₀ and flesh 25 mg/ml+pits 2.5 mg/ml.

suggests a time-dependent manner. The apoptotic activity exhibited by the ADF and ADP extracts may be attributed to the presence of phenols and flavonoid content like rutin, catechin, caffeic acid, apigenin, and quercetin which are present in Ajwa date flesh and pit [46, 58].

A previous study has reported the induction of apoptosis in breast cancer MCF-7 cells by the methanolic extract of Ajwa date flesh [27], in which it was reported that the percentage of total apoptotic cells was 68.1% at 25 mg/ml at 48 h of treatment duration, which correlates with the findings of present study where the total apoptotic cells at 48 h were 67.1% at 25 mg/ml of ADF extract. With the loss of cell membrane asymmetry, the phosphatidylserine (PS) flips towards the outside, which is considered to be a hallmark of cell in later stages of apoptosis [59]. Furthermore, ADP extract had increased apoptosis, compared to ADF. This result supports the results of MTT assay, in which there was significant cell death after the treatment of Ajwa date extract; apoptosis assay elaborates that the cell went through early and late stages of apoptosis before dying, thus confirming that Ajwa date induced apoptotic cell death in oral cancer cells.

The present study also investigated the combined effect of ADF and ADP extracts for the induction of apoptosis in HSC-2 cells. At 24 h, the percentage of early apoptotic cells with the combination treatment was 39.2% whereas when treated separately, it was 9.10% and 33.4% for ADF and ADP extract, respectively. Although it was not significantly higher than the single treatment, this may suggest that Ajwa date can induce higher level of apoptotic effect when used as a whole. Previously, many studies have evaluated the bioactive phenolic compounds of fruit seed more than the fruit flesh. Similar to the present study, grape seed extract had induced apoptotic cell death in OSCC [60]. Not only the seed but the peel (skin) and the edible part of grape have demonstrated apoptotic activity [61, 62]. Many other fruits have demonstrated higher polyphenol content in their seed than the edible flesh [47]. Therefore, it can be suggested that the fruit as a whole can be more beneficial in providing protection against carcinogenic effects and the seed part can be utilized in many different forms; like recently, date pit powder was used to make noncaffeinated coffee with coffee flavour [63].

5. Conclusion

In conclusion, the results from the present study suggest that Ajwa date (flesh and pit) demonstrated significant cytotoxic and antiproliferative activity against HSC-2 cells. The IC_{50} value demonstrated that Ajwa pit had a stronger antiproliferative effect than the flesh extract signifying its higher anticancerous potential, which was further confirmed in morphological alteration such as nuclear shrinkage, blebbing of cell membrane, and reduction in cell number, which are characteristic features of apoptotic cells. Furthermore, ADF and ADP extract was found to cause cell death via apoptosis in OSCC cells by flow cytometry assay in a dose- and time-dependent manner. The present study also demonstrates that Ajwa date as a whole can induce apoptosis more effec-

tively than as separately. As research is still ongoing, further studies can be conducted to purify and identify individual components of the Ajwa date flesh and seed that are responsible for the anticancer properties and to better understand the in-depth molecular mechanism of action of apoptosis so that a novel chemotherapeutic drug can be made with less/no conventional side effects.

Data Availability

All data are available within the manuscript.

Conflicts of Interest

There are no conflicts of interest between the authors.

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