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Original Article

Determination of Anti-Cancer and Photo-Aging Potential of Ripened *Carica papaya* Fruit Extract in *in vitro* Cell Line Models

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ABSTRACT

Aim and Background: *Carica papaya* has been widely used in traditional medicine. However, the ripened papaya fruit remains underexplored regarding its potential as an anti-cancer and photo-aging aspect. Thus, our aim is to investigate the potential therapeutic opportunities of ripened *Carica papaya* fruit against colorectal cancer and photo-aging in *in vitro* cell models. **Materials and Methods:** 80% methanolic extracts were prepared using Ultrasound-Assisted Extraction (UAE) and maceration techniques. The cytotoxicity and therapeutic efficacy of the extracts were evaluated through DPPH, MTT, DCFH-DA staining, TMRM staining, Hoechst staining, scratch assay, and clonogenic assay in colorectal cancer (HT-29) cells. The photoprotective potential was also assessed by measuring ROS levels and cell viability in UVC-irradiated HaCaT and 3T3-L1 cells. **Results:** The UAE extract exhibited an IC_{50} of 0.1 mg/mL against HT-29 cells and significantly increased ROS levels, promoting ROS-induced apoptosis. The extract also inhibited cancer cell migration and reduced tumor-initiating potential. Furthermore, it demonstrated antioxidant and photoprotective effects by reducing ROS levels in UVC-irradiated HaCaT and 3T3-L1 cells, improving cell viability. **Conclusion:** In summary, the results demonstrated the specific efficacy of ripened papaya fruit extract against colorectal cancer progression in *in vitro* conditions. Also, these findings supported its role against skin photoaging, further highlighting the value of natural products in promoting good health and skincare.

Keywords: *Carica papaya*, Colorectal cancer, Photoaging, Anti-aging, Ripened papaya fruit.

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INTRODUCTION

As reported by the World Health Organization (WHO) in 2023, Colorectal Cancer (CRC) is the third most common cancer accounting for approximately 10% of all cancer cases, and globally, the second leading cause of cancer-associated deaths.[1] Currently, the treatment of CRC involves surgery, targeted therapy, radiotherapy, and chemotherapy. Although chemotherapy has reduced the tumor burden and prolonged survival, however, most CRC patients eventually develop adverse reactions or resistance to chemotherapeutic drugs.[2] Thus, there is a need to investigate natural products with lower side effects that have displayed selective anti-cancer activities against malignant cells.[3] Tropical fruit, *Carica papaya* has been used in traditional medicine to treat various illnesses. Papaya has shown chemoprotective properties such as activating tumor-suppressor genes, deactivating oncogenes, and minimizing oxidative damage by scavenging free radicals.[4] The myrosinase enzyme produced by human gut microflora breaks down the benzyl glucosinolate present in papaya pulp into an anti-cancer compound benzyl isothiocyanate.[5] Furthermore, a strong relationship between intake of fibrous foods and reduction in the rate of CRC is evidenced from literature, as these dietary fibers are rich in galactosides which can competitively inhibit the interaction between galectin-3, a lectin biomarker in CRC, and glycans on the cell surface and papaya is one of such fruits rich in these dietary fibers.[6]

Skin undergoes intrinsic or chronological aging as well as extrinsic aging due to exposure to factors like UV. However, only 3% of skin aging is contributed by intrinsic factors while extrinsic factors are the main cause.[7] Both intrinsic and extrinsic aging factors involve the production of Reactive Oxygen Species (ROS), the accumulation of which can cause cellular dysfunction.[8] Photoaging-induced ROS leads to the activation of Mitogen-Activated Protein Kinases (MAPKs) and upregulation of the Activator Protein-1 (AP-1) complex. A cytokine, Transforming Growth Factor β (TGF-β1) stimulates

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the proliferation of fibroblasts, promotes collagen synthesis, and suppresses Matrix Metalloproteinase (MMP) expression. However, in the case of UV-exposed cells, the activated AP-1 leads to downregulation of TGF-β1 which in turn leads to reduced type I procollagen production, a precursor of collagen. There is also upregulation of Nuclear Factor kappa B (NF-κB) seen which leads to increased expression of genes associated with a pro-inflammatory response such as IL-1β, TNF-α, IL-6, and IL-8.[9] Although, mammalian cells do have an endogenous defense system against oxidative stress, impairment in the redox capacity of the system with age can result in the accumulation of ROS.[8] Intrinsic aging of the skin is inevitable but premature aging due to extrinsic factors is preventable.[7] *Carica papaya* is a potential candidate for reducing extrinsic skin aging owing to its anti-oxidant and anti-inflammatory properties. Papaya contains flavonoids mainly kaempferol, myricetin, quercetin, and phenols like caffeic acid, and ferulic acids which act as ROS suppressors.[10] Caffeic acid and rutin were found to be the main anti-aging components in papaya as they downregulated MMP expression.[9]

Although papaya seeds, leaves, and unripe fruit have been investigated for their anti-cancer and anti- aging potential, to our knowledge, no study has been done on the ripened papaya fruit against colorectal cancer progression and photoaging of the skin in *in vitro* models. In this study, we demonstrated the same.

MATERIALS AND METHODS

Materials

Analytical-grade Methanol, Gallic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and Hydrochloric acid (HCl) were obtained from SRL Pvt. Ltd., India. Dulbecco's Modified Eagle Medium (DMEM) with glutamine and sodium pyruvate and without glucose and sodium bicarbonate, Sodium pyruvate, Fetal Bovine Serum (FBS), Antibiotic solution with 10000 U Penicillin/mL and 10 mg Streptomycin/mL in 0.9% normal saline, Phosphate-Buffered Saline (PBS) and 0.25% Trypsin (1X) were procured from HiMedia Laboratories Pvt. Ltd., India. 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) reagent was obtained from TRC, Canada. Hoechst 33342 and 2,7- Dichlorodihydrofluorescein Diacetate (DCFH-DA) were obtained from Sigma-Aldrich Pvt. Ltd., India. 100% isopropanol and 96% Crystal violet were obtained from Loba Chemie Pvt. Ltd., India. Ethanol was procured from Changshu Hongsheng Fine Chemicals Co. Ltd., China. Acetic acid was obtained from Research Lab Fine Chem Industries, India.

Cell culture

For the determination of anti-cancer potential Human colorectal adenocarcinoma cell line HT-29 was used while for photo-aging potential human keratinocyte cell line HaCaT and murine fibroblast cell line 3T3-L1 were considered, and were obtained from the National Centre for Cell Science (NCCS), India. These cells were maintained in DMEM high glucose (25 mM) supplemented with 10% FBS, 1% sodium pyruvate, and 1% pen-step antibiotic solution in T-25 flasks and incubated at 37°C and 5% CO_2.

Extract preparation from ripened *Carica papaya* fruit

Ripened papaya fruit was obtained from a local market (Bhiwandi, Maharashtra, India). The Ultrasound-Assisted Extraction (UAE) and maceration method were used to prepare the extract. 150 g of fruit without skin and seeds was blended with 500 mL of 80% analytical-grade methanol for 2 min. For UAE, the blended mixture was sonicated using a bath-sonicator (PCI Analytics Pvt. Ltd., India) at 20 kHz for 1 hr whereas, for preparation of macerated extract, the blended mixture was agitated at slow speed for 150 min. Both the mixtures were double filtered, first with muslin cloth and then re- filtered with Whatman No. 1 filter paper to get clear liquid. After that filtered solvent was subjected to rotary vacuum evaporation (Biobase Pvt. Ltd., China) and further dried in a hot air oven.

Free Radical-Scavenging Activity

Free-radical scavenging activity of the extracts equivalent to the standard gallic acid was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.[11] Two-fold serial dilutions of gallic acid (2.5-20 µg/mL) and ten-fold dilutions of the extracts (0.1-10 g/mL) were used. To a 96-well plate, 90 µL DPPH working stock (0.097 mM) was added to which 10 µL of different concentrations of extract or standard was added; the control was without the extract. 100 µL methanol was used as blank. After incubation for 30 min at RT (Room Temperature) in the dark, absorbance was measured at 517 nm using BioTek Cytation 5 (Agilent Technologies, USA). The DPPH radical-scavenging capacity (%) was calculated as

$$\% \text{ Radical scavenging (GAE)} = \frac{Corrected\ OD\ of\ control - Corrected\ OD\ of\ sample}{Corrected\ OD\ of\ control} X\ 100.$$

Determination of Total Phenolic Content (TPC)

The TPC of the extracts equivalent to the standard gallic acid was determined by Folin-Ciocalteu assay.[12] Two-fold serial dilutions of gallic acid (10-1000 µg/mL) and ten-fold dilution (0.1-10 g/mL) of extracts were used. To a 96-well plate, 50 µL of 2N Folin-Ciocalteu reagent was added followed by 10 µL of extract or standard, and incubated for 3-5 min in the dark. Control was without the extract. Later 40 µL 7.5% sodium carbonate was added and again incubated at RT in the dark for 30 min. Absorbance was measured at 765 nm using BioTek Cytation 5. 100 µL methanol was used as blank.

$$\text{TPC (mg GAE)} = \frac{Corrected\ OD\ of\ sample}{Corrected\ OD\ of\ control}\ x\ 100$$

Determination of Total Flavonoid Content (TFC)

The TFC of the extracts equivalent to the standard quercetin was determined by the aluminium chloride colorimetric method.[11] Dilutions of Quercetin (QE) and extracts were used as mentioned previously. 25 µL extract or standard was added to an equal 2% aluminium chloride volume in a 96- well plate and gently mixed. The control was without the extract. 150 µL of 5% potassium acetate was added to all the wells and incubated for 30-40 min. Later, absorbance was measured at 415 nm using BioTek Cytation 5.

$$\text{TFC (mg QE)} = \frac{Corrected\ OD\ of\ sample}{Corrected\ OD\ of\ control}\ x\ 100$$

Evaluation of cell viability

For the evaluation of efficacy and toxicity of the extracts, HT-29 cells and HaCaT cells were used and to evaluate the photo-aging effect, cell viability of UVC-irradiated HaCaT and 3T3-L1 along with their non-irradiated counterparts was done by MTT.[13] 5×10^3 cells/100 µL were seeded in a 96-well plate and incubated at 37°C in 5% CO_2 for 48 hr. Treatment of the cells with the extracts serially diluted using DMEM supplemented with 1% FBS and incubated for 72 hr. 10 µL of 5 mg/mL MTT reagent was added and incubated for 4 hr in the dark under culture conditions. Previous media was removed from the wells and 100 µL of MTT solvent (4mM HCl in 100% isopropanol) was added to the wells, mixing was done by retro-pipetting, and incubation was done for 5-10 min in the dark. Later, absorbance was measured at 570 nm using BioTek Cytation 5.

$$\%\ \text{Viability} = \frac{Corrected\ OD\ of\ sample}{Corrected\ OD\ of\ control}\ x\ 100$$

Evaluation of intracellular ROS by DCFH-DA staining method

Intracellular ROS level was evaluated by DCFH-DA staining with some modifications.[14] UVC- irradiated HaCaT and 3T3-L1 along with their non-irradiated counterparts and HT-29 cells (8×10^3 cells/100 µL) were seeded in DMEM with 10% FBS and incubated at 37°C in 5% CO_2 for 48 hr. The cells were treated with 0.1 and 1 mg/mL concentrations of the UAE extract for 4 hr. Washing was done with serum-free media twice. DCFH-DA stock was diluted 1:1000 using serum-free media and 100 µL of this was added to the wells. After incubation for 30 min under culture conditions, washing was done with chilled 1X PBS twice. Fluorescence was measured at excitation and emission wavelengths 485 nm and 535 nm respectively; GFP images were also taken using BioTek Cytation 5.

$$\%\ \text{ROS production} = \frac{(Fluorescence\ of\ sample - Fluorescence\ of\ blank)}{(Fluorescence\ of\ control - Fluorescence\ of\ blank)} \times 100$$

Evaluation of apoptosis by Hoechst staining method

Evaluation of apoptosis was done by using Hoechst 33342 stain with some modification in the protocol.[15] 3×10^3 HT-29 cells/100 µL of DMEM with 10% FBS were seeded in a 96-well plate and incubated at 37°C in 5% CO_2 for 48 hr. Treatment with 0.1 and 1 mg/mL concentration of the UAE extract was done and incubation was done for 48 hr. Washing with 1X PBS was done and cells were fixed with 100 µL 100% chilled ethanol followed by incubation at room temperature for 15 min. After the removal of ethanol, cells were stained with 40 µL Hoechst stain (2.5 µg/mL) and incubated for 15 min in the dark. Washing with 1X PBS was done thrice. DAPI images of random three sections of each well were taken using BioTek Cytation 5. Cells showing changed morphology or fragmented DNA were counted as apoptotic.

$$\%\ \text{Apoptotic cells} = \frac{No.of\ apoptotic\ cells}{Total\ cell\ count}\ X\ 100$$

Evaluation of Mitochondrial Membrane Potential (ΨΔM) by TMRM Assay

To determine the difference in electric potential in interior and exterior of the mitochondrial membrane after the treatment of papaya extract was determined by Cayman's Tetramethylrhodamine, Methyl Ester (TMRM) kit.[16] Briefly, 1×10^4 were seeded in 96 well plated and incubated for 24 hr. Selected concentration of papaya extract 0.1 mg/mL and 1 mg/mL was exposed for 24 hr followed by 1X PBS twice. For Carbonyl cyanide p-trifluoromethoxyphenylhydrazone control wells, dilute the 100 nM working stock prepared using serum free media and incubated for 30 min. Add equal volume of TMRE and incubated for 30 min at 37°C. Aspirate the media and wash the cells gently with 200 µL of 1X assay buffer and followed by addition 100 µL of 1X assay buffer to each well. Equilibrate the plate at room temperature for 15 to 30 min. Read the plate in a fluorescence plate reader (excitation/emission=555/575 nm). Representative image was captured at Texas Red Mode BioTek Cytation 5, Gen 3.1 Software.

Evaluation of migratory potential by Scratch assay

The HT-29 cells, 2×10^5 cells/500µL DMEM with 10% FBS were seeded in a 24-well plate and incubated at 37°C in 5% CO_2. After the cells reached confluence and a monolayer was obtained, they were treated with/out the UAE extract. Scratch was made using a micropipette tip. After giving 1X PBS wash, DMEM media supplemented with 1% FBS was added. Bright field images were taken on 10X at 0 hr and scratch width was measured. At the same X and Y axes co-ordinates this scratch was measured at different time points.

$$\%\ \text{Scratch width remaining} = \frac{Scratch\ width\ at\ any\ time\ point}{Scratch\ width\ at\ 0\ hr}\ X\ 100$$

Evaluation of tumour-forming potential by Clonogenic assay

The HT-29, 1×10^4 cells/500µL DMEM with 10% FBS were seeded in a 24-well plate and incubated at 37°C in 5% CO_2 for 48 hr. The cells were then treated with/out the UAE extract and again

incubated under culture conditions for another 48 hr. Later, washing was done with 1X PBS twice and the cells were fixed with chilled 100% ethanol. After incubation for 15 min, the cells were stained with 300 µL 0.1% crystal violet (dissolved in ethanol), and the plate was incubated for 20 min. Washing was done gently with water to remove the excess stain and then, the plate was dried completely. 300 µL 20% acetic acid was added and scrapping of the colonies was done to get a homogenous mixture. Contents of the well were transferred to a 96-well plate and absorbance was measured at 595 nm using BioTek Cytation 5.

$$\% \text{ Relative Colony Forming Efficiency (\% RCFE)} = \frac{Corrected\ OD\ of\ sample}{Corrected\ OD\ of\ control} \times 100$$

UVC irradiation of the HaCaT and 3T3-L1 cells

Protocol for UVC irradiation of cells was adapted from the method previously reported by Hwang *et al.*, (2014).[17] Cells were seeded in 96 well Plate of the assays and incubated at 37°C in 5% CO_2 for 48 hr. Later, cells were rinsed with 1X PBS twice, and a thin layer of PBS was maintained in the wells. The cells were exposed to UV-C (253.7nm) inside the biosafety cabinet for 5 min. After irradiation, cells were washed with PBS again and immediately treated with UAE 80% methanolic extract at 0.1 and 1 mg/mL concentrations prepared in DMEM with 1% FBS. Non-irradiated controls were also maintained without UV-C exposure.

Statistical analysis

All the assays were performed in triplicates or pentaplicates. All the data were shown as Mean±Standard Error Mean (SEM). The significant value was analyzed using one- and two-way ANOVA using Prism software. *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$, were consider significant.

RESULTS

Comparative evaluation of DPPH Scavenging Activity, TPC, and TFC compositions between UAE and macerated 80% methanolic extracts

The free radical scavenging activity by DPPH, total phenolic and flavonoid content of UAE, and macerated 80% MeoH extracts were performed. As evident from Table 1, UAE and macerated 80% MeoH extract at 1 mg/mL concentration shows good DPPH scavenging potential. The macerated extract exhibited a significantly higher activity (6.01±1.06 mg GAE/g) compared to the UAE extract (1.2±0.2 mg GAE/g). Further, the total phenolic and flavonoid content of the extracts was determined by Folin-Ciocalteu assay and aluminum-chloride colorimetric assay respectively. Phenolic and flavonoid content varied between the two extraction methods. The UAE extract showed a TPC of 11.19±0.41 mg GAE/g, comparable to the macerated extract (11.67±0.48 mg GAE/g). In terms of flavonoid content, the macerated extract had a higher TFC (38.33±0.24 mg QUE/g) compared to the UAE extract (35.71±1.49 mg QUE/g).

Comparative MTT-based cytotoxicity evaluation between UAE and macerated 80% methanolic extracts

The anticancer activity of the UAE and macerated 80% methanol extracts were evaluated using the MTT assay on HT-29 colorectal cancer cells (Figure 1(i)). Treatment with the UAE extract significantly decreased the percentage viability of HT-29 cells at both 0.1 mg/mL ($p \leq 0.05$) and 1 mg/mL ($p \leq 0.01$) concentrations compared to the control. However, the macerated extract did not exhibit any significant cytotoxic effect at either concentration, suggesting that the UAE extract possesses a stronger anticancer activity. The specificity of the extracts was evaluated by testing their effects on HaCaT normal keratinocyte cells (Figure 1(ii)). The UAE extract did not show any significant reduction in HaCaT cell viability, indicating its selective cytotoxicity towards cancer cells. Conversely, the macerated extract significantly reduced HaCaT cell viability at 1 mg/mL ($p<0.001$), demonstrating potential toxicity to normal cells. These findings suggest that the UAE extract may be a more suitable candidate for selective anticancer applications.

The ROS and apoptosis evaluation in UAE 80% methanolic extracts

The intracellular ROS production in HT-29 cells treated with UAE 80% methanol extracts was evaluated using DCFH-DA staining (Figures 2(i) and 2(ii)). Treatment with UAE extract at concentrations of 0.1 mg/mL and 1 mg/mL resulted in a significant increase in green fluorescence intensity, suggesting elevated levels of Reactive Oxygen Species (ROS) when compared to the untreated control group. Quantitative analysis revealed that ROS production increased in a concentration-dependent manner, with the highest level observed at 1 mg/mL, suggesting oxidative stress as a possible mechanism of cytotoxicity. The apoptotic potential of the UAE extract was assessed using Hoechst 33342 staining to examine nuclear morphology (Figures 2(iii) and 2(iv)). Cells treated with 1 mg/mL UAE extract exhibited a significant

Table 1: Evaluation of the radical scavenging activity (DPPH), TPC, and TFC. The TPC, TFC, and DPPH activity of 1 mg/mL of UAE and macerated 80% MeoH extract correlated with respective standard gallic acid and quercetin. Experiments were performed in triplicate and data are expressed as Mean±SD.

Sl. No.	Extraction Type	TPC (mg GAE/g)	TFC (mg QUE/g)	DPPH (mg GAE/g)
1	UAE- 80% MeOH	11.19±0.41	35.71±1.49	1.2±0.2
2	Maceration-80% MeOH	11.67±0.48	38.33±0.24	6.01±1.06

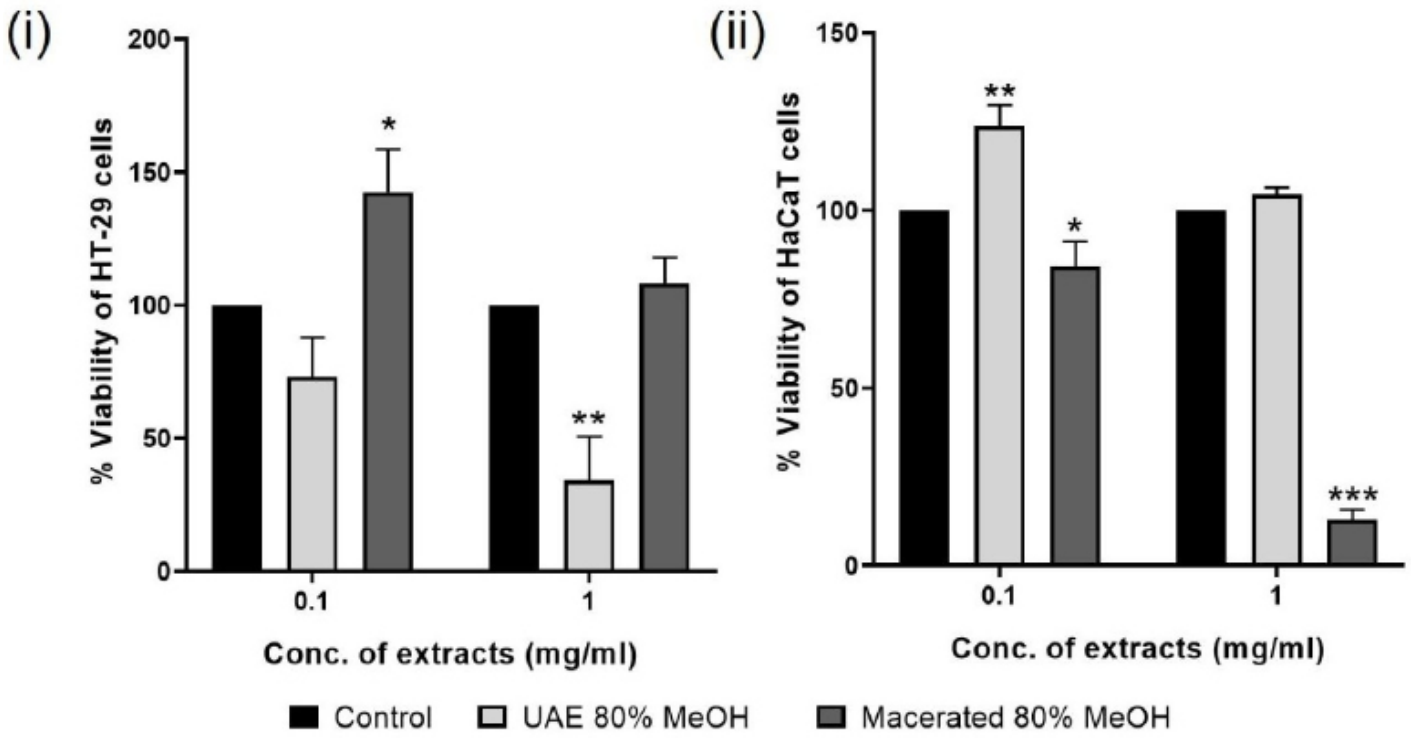

Figure 1: MTT-based evaluation of cytotoxicity of the extracts. (i) Effect of extracts on HT-29 cells. (ii) Effect of extracts on HaCaT cells. All experiments were conducted in triplicate. Data are presented as Mean±SD (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

increase ($p<0.01$) in the percentage of apoptotic cells compared to the control, as evidenced by truncated and blebbed nuclei. At 0.1 mg/mL, a moderate increase in apoptotic cells was observed, indicating a dose-dependent effect. These results highlight that the UAE extract induces apoptosis, possibly mediated through ROS generation.

Mitochondria Membrane Potential (MMPs)

Decrease in MMPs has been considered a characteristic feature in the early stage during apoptosis. As shown in Figure (Figure 2(v) and 2(vi)), HT-29 cells treated with the UAE extract of 0.1 mg/mL and 1 mg/mL respectively, resulted in moderately decreased MMPs (10% and 20% respectively) *(*$p<0.01$ for 1mg/mL group*)*, at 24 hr treatment. These data suggests that *Carica papaya* extracts leads to degenerate and depolarization of MMPs in HT-29 cells, which may potentially leads to increased ROS production. These data also corroborate with apoptosis data shown as nucleus staining by Hoechst staining.

The anti-migratory and anti-tumorigenic potential of the UAE 80% methanolic extracts

The scratch assay evaluated the anti-migratory effect of UAE extract, as evident from Figure 3 (i). UAE extracts reduced the rate of migration of HT-29 cells as the treated group exhibited a marginal reduction in the scratch gap unlike observed in the control group after 24 h. Quantitative analysis confirmed a dose-dependent reduction in migration, with both concentrations demonstrating statistical significance compared to the control ($p<0.05$, $p<0.01$). These findings suggest that the UAE extracts impair the migratory potential of HT-29 cells. The tumor-initiating potential was evaluated by clonogenic assay. As evident from Figure 3(iii), the untreated control cells had formed macroscopic colonies resembling the tumor-forming potential. In contrast, cells treated with 0.1 mg/mL and 1 mg/mL UAE extract displayed a dose-dependent reduction in colony formation, with significant inhibition observed at both concentrations ($p<0.001$) indicating a reduction in the tumor- initiating potential.

The photo-aging effect of the UAE 80% methanolic extracts

The protective effects of UAE 80% methanol extracts against photo-aging were evaluated in HaCaT and 3T3-L1 cells. Cells were exposed to UV-C radiation (253.7 nm) for 5 min, followed by treatment with UAE extract, and cell viability was assessed after 48 hr. As shown in Figure 4(i), the UAE extract significantly enhanced HaCaT cell viability, with notable increases observed both in non-irradiated and UV-C-irradiated conditions at 0.1 mg/mL ($p<0.05$) and 1 mg/mL ($p<0.001$). Similarly, the extract improved 3T3-L1 cell viability under UV-C exposure in a dose-dependent manner, with a statistically significant effect at 1 mg/mL ($p<0.01$) (Figure 4(ii)). These results suggest that the UAE extract promotes cell recovery and demonstrates protective effects against UV-C- induced damage, supporting its anti-photo-aging potential.

The antioxidant effect of UAE 80% methanolic extracts in UVC-irradiated cells

ROS generation because of external or internal factors indirectly plays an important role in cell aging and senescence. Therefore, to evaluate the anti-photo-aging effect, the antioxidant potential (ROS) of UAE extract was determined. As shown in Figure 5(i) and 5(ii), UV-C exposure significantly increased green fluorescence, indicative of elevated ROS levels. Treatment with UAE extract at 0.1 mg/mL and 1 mg/mL reduced the ROS levels in both HaCaT and 3T3-L1 cells, as evidenced by a decrease in fluorescence intensity. Quantitative analysis confirmed a dose-dependent reduction in ROS production in both non-irradiated and UV-C-irradiated groups (Figure 5(iii) and 5(iv)). Significant reductions were observed, particularly at 1 mg/mL ($p<0.01$, $p<0.001$), correlating with the extract's protective effects against photo-aging. The ROS results also correlate with the previous MTT results as additional supportive data about extracts' mediated photo-aging functions especially under UVC exposures.

DISCUSSION

For the present study, the 80% methanolic extracts of ripened papaya fruit were prepared by the green extraction method, Ultrasound-Assisted Extraction (UAE), and traditional maceration method. However, the UAE extract was the only one that manifested potent activity specifically against the HT-29 with the IC_{50} of 0.1 mg/mL without showing any toxicity towards the normal cells (HaCaT cells) (Figure 1). Previous studies with papaya seed extract showed the protective effect in human skin fibroblast as oxidative stress-induced apoptosis was reduced by 30%.[18] Our study with the ripened papaya fruit extract showed the papaya extract is specific in inducing apoptosis in malignant cells but protective in the case of normal cells. The activity of papaya leaf on HT-29 was reported by past studies with the IC_{50} of 0.3-0.4 mg/mL.[19] This IC_{50} value was comparatively higher than that was observed in the present study; thus, proving more efficacy of the ripened papaya fruit against HT-29 than that of the papaya leaf.

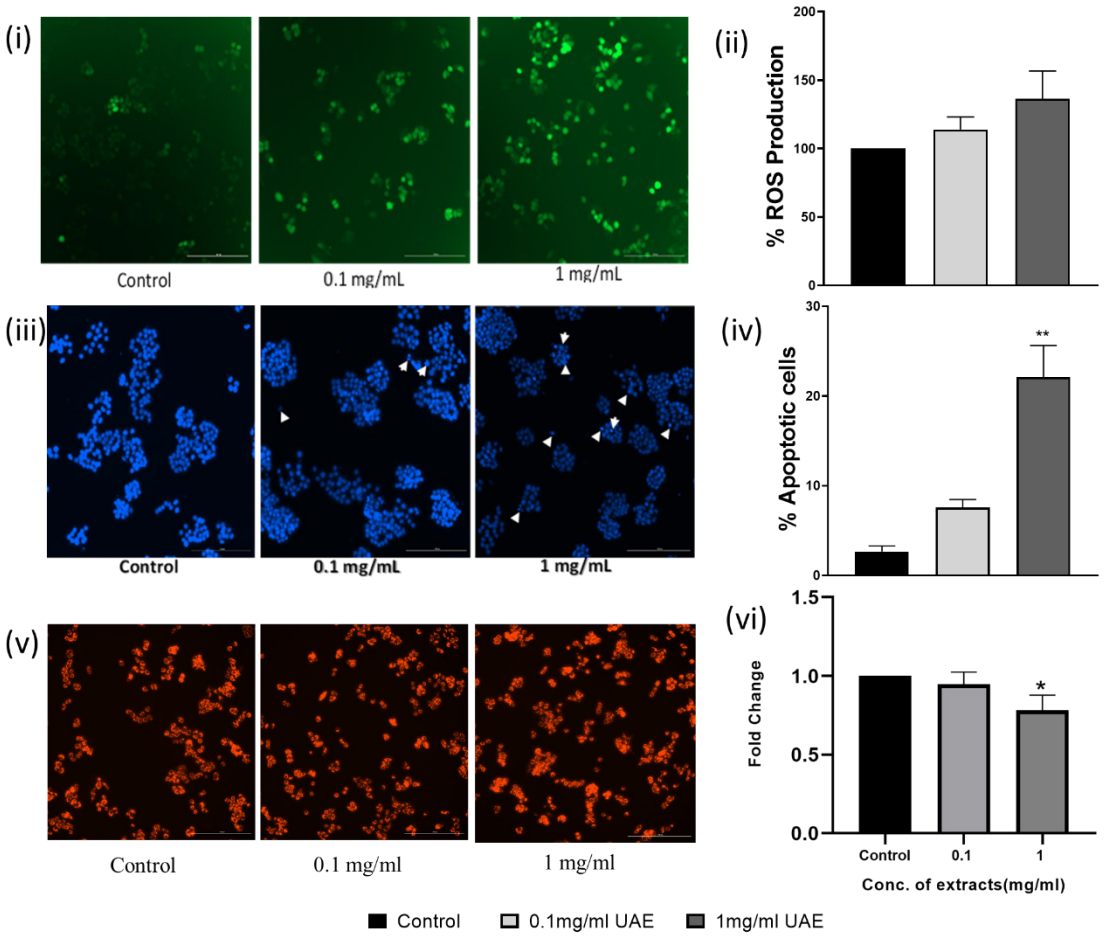

Figure 2: Evaluation of intracellular ROS and apoptosis in HT-29 after treatment with the UAE extract by DCFH-DA, Hoechst staining and Mitochondrial Membrane potential respectively. (i) images capture in green fluorescence channel at 20X magnification, (ii) Graph of % ROS production. (iii) DAPI images at 20X magnification of HT-29 cells. The white pointers indicate the truncated nucleus of the apoptotic cells, (iv) Graph of % apoptotic cells (v) cells were stained with TMRM images captured in red change (vi) Fold change difference in ΨmΔ. All experiments were conducted in triplicate. Data are presented as Mean±SD (*, $p\leq0.05$; **, $p\leq0.001$).

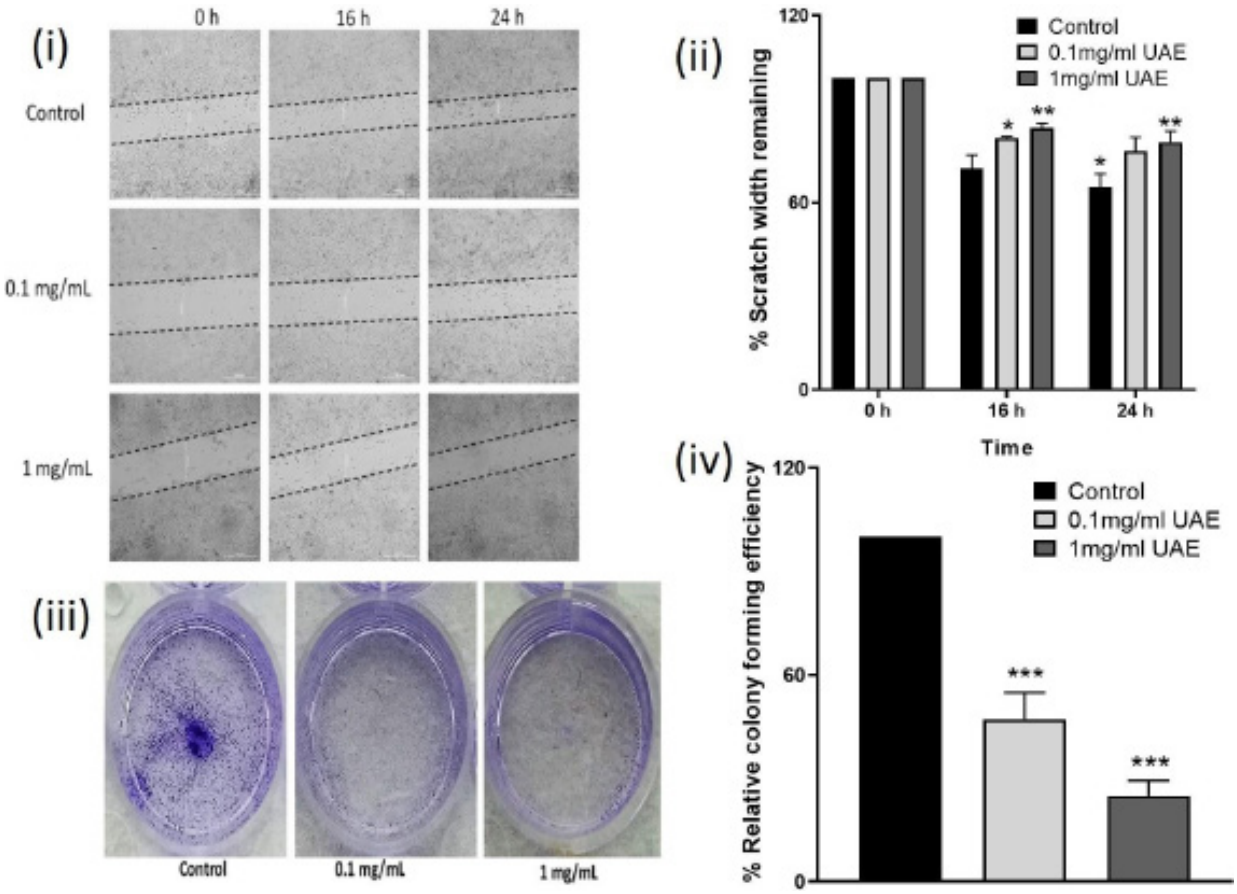

Figure 3: Evaluation of the anti-migratory and anti-tumor-initiating potential of the UAE extract by scratch assay and clonogenic assay, respectively. (i) Bright-field images at 10X magnification of scratch at 0, 16 and 24 h. (ii) Graph of % scratch width remaining at those time points. (iii) Images of untreated and treated cells after staining with crystal violet (iv) Graph of % relative colony forming efficiency (% RCFE). All experiments were conducted in triplicate. Data are presented as Mean±SD (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

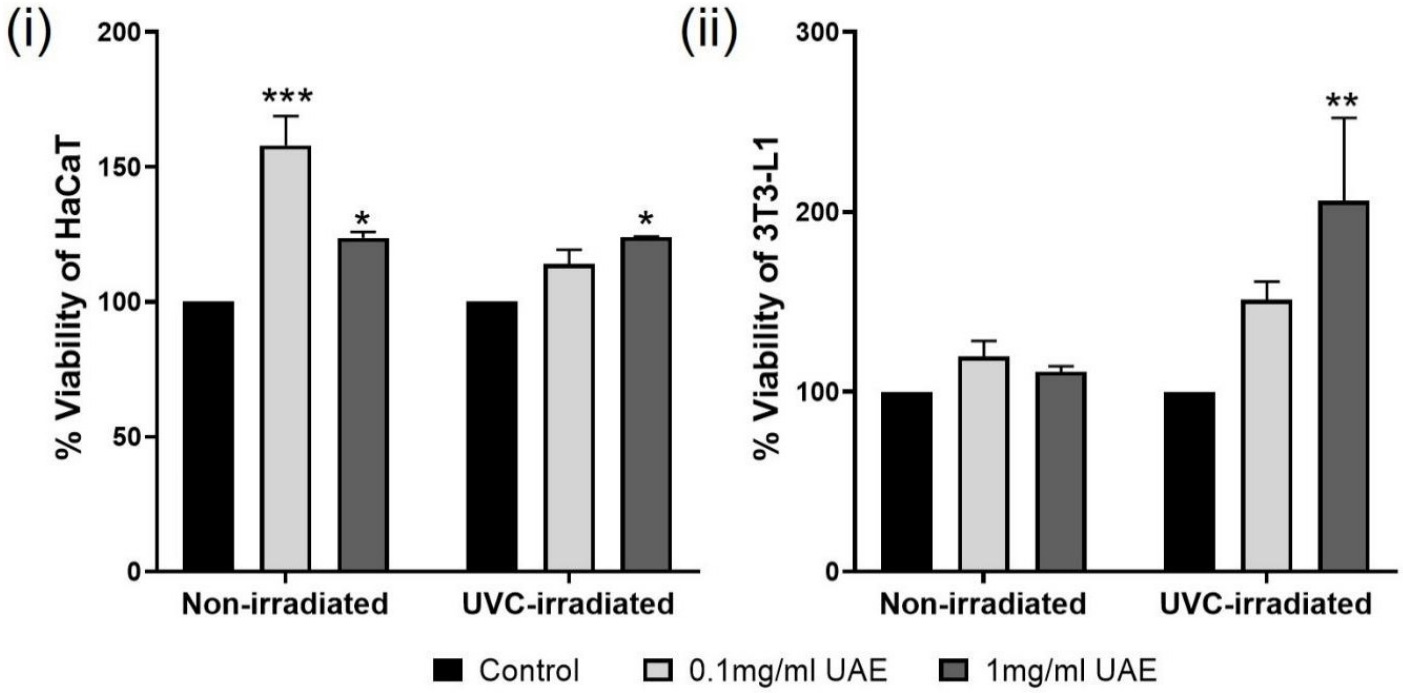

Figure 4: Evaluation of the photo-aging effect of the extract on UVC-irradiated HaCaT and 3T3- L1 cells along with their non-irradiated counterparts by MTT assay. Cell viability of (i) HaCaT (ii) 3T3-L1. All experiments were conducted in triplicate. Data are presented as Mean±SD (*, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$).

Cancer cells have increased levels of ROS than normal cells owing to their high metabolic rate and bioenergetic dysfunctions which makes them much more susceptible to oxidative stress.[20] Any further increase in their ROS levels causes these malignant cells to reach their oxidative stress threshold earlier than the normal cells leading to oxidative stress-induced death.[21] Most chemotherapeutics work by increasing the ROS in the cancer cells.[22] Our study also interlinked increase in ROS productions and apoptosis associated with decrease in MMPs, when treated with different concentrations of UAE extracts. A similar effect was observed in *Ficus carica* fruit extract, where ROS generation, driven by mitochondrial membrane disruption, led to cell death in PANC-1 and QGP-1 cell lines.[23] Additionally, another study reported that methanolic extracts of D. kaki at concentrations of 200 µg/mL and 400 µg/mL triggered mitochondrial dysfunction in SW80 and E705 cells.[24] The given evidence potentially indicating that UAE extracts could exhibit cytotoxic effects on the same way by increasing the ROS production in a dose-dependent manner in HT-29 cells (Figure 2). Moreover, as reported earlier, the phytochemicals such as polyphenols and flavonoids could alter the mitochondrial functions thereby leading to ROS-induced apoptosis (Figure 2).[25]

Scratch assay helps to assess the migratory potential of the cells. In the present study, the UAE extract reduced the rate of migration of HT-29 indicating its potential ability to reduce the metastatic capabilities of the cancer cells (Figure 3). Significant reduction in the rate of migration of colon cancer cell line CT-26 after treatment with papaya black seeds extract was reported before.[26] Similar results were obtained in the case of liver cancer cell line HepG2 after treatment with papaya blackseed extract.[27] Although papaya reduced the migration of malignant cells, it showed a wound-healing effect in the case of skin fibroblast cell line HSF1184, again proving the specificity of papaya.[28] The clonogenic assay provides information regarding the

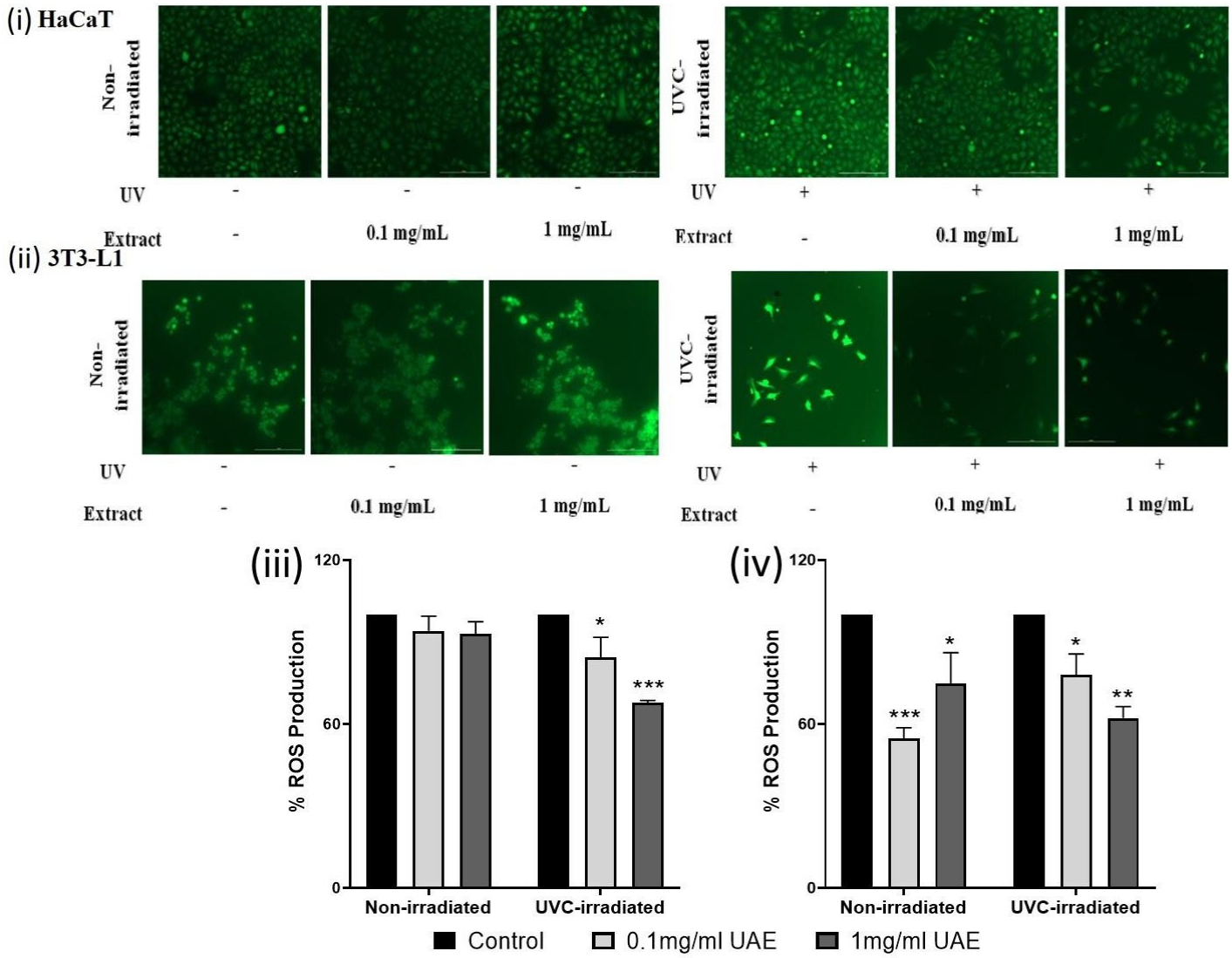

Figure 5: Evaluation of the intracellular ROS levels in UVC-irradiated cells with their non- irradiated counterparts. GFP images of non-irradiated and UVC-irradiated (i) HaCaT and (ii) 3T3-L1 cells. Graph of % ROS production in (iii) HaCaT and (iv) 3T3-L1 cells. All experiments were conducted in triplicate. Data are presented as Mean±SD (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

tumor-initiating potential of the cancer cells and their ability to survive and proliferate under the influence of stress induced by therapeutic agents. The UAE extract significantly declined the number of colonies formed indicating the ability of the extract to reduce the chances of relapse (tumor-initiating properties) of this cancer (Figure 3).

This study provides *in vitro* functional evaluation of anti-cancer potential of ripened papaya fruit extracts. However, considering the limitations of 2D monolayer derived cell models, further explorative investigation of this extract at the molecular level using 3D cell models (spheroids) could provide more meaningful data closure to *in vivo* physiology. Nonetheless, molecular pathway/signaling analysis such as EMT markers or Receptor Tyrosine Kinase (RTKs) within this complex 3D models could also pave the way to better understanding the molecular mechanism of action.[29,30]

In the present study, for evaluation of the photo-aging potential of ripened papaya fruit, murine fibroblast cell line 3T3-L1 and human keratinocyte cell line HaCaT were used as fibroblasts are responsible for the secretion of collagen; while keratinocytes are essential for re-epithelization process involved in skin repair necessary for restoration of the epidermal barrier.[31] DNA absorbs maximally at 245-290 nm and thus, it is one of the main cellular targets of UV radiations. UV-C can damage DNA significantly by inducing strand breaks, bipyrimidine photoproducts and oxidatively damaging the bases by ROS production.[32] In the present study, cells were exposed to UVC (253.7 nm) for 5 min. This duration was chosen because 10 min of exposure of HaCaT cells to UVC induced apoptosis and membrane blebbing was seen after 30 min of exposure; whereas, in the case of 5 min exposure a temporary mutation was created in the cells.[33] The UAE extract did not induce significant cytotoxicity in the irradiated cells instead it increased the cell viability (Figure 4). This protective effect of the extract may be due to its antioxidant potential as there was a decline in ROS levels seen in a dose-dependent manner in UVC-irradiated cells (Figure 5). The antioxidant potential of cells may delay the UV-induced inflammation and aging of the skin owing to the synergistic relationship between oxidative stress and inflammation.[34]

However, the activity of this extract is yet to be studied at the molecular level such as its effect on EMT or anti-inflammatory aspects for a better understanding of its true potential. Detection and quantification of phytochemicals of this extract could also be explored to better understand the molecular signaling part.

CONCLUSION

In the present study, the potential of ripened *Carica papaya* fruit against colorectal cancer and photoaging of the skin was successfully evaluated. The extract prepared by ultrasound-assisted extraction showed specificity in its action against a colorectal cancer cell line. The anti-cancer nature was attributed to increased ROS levels leading to ROS-induced apoptosis following treatment with this extract. This extract was found to be capable of reducing the metastatic potential of colorectal cancer. Furthermore, this extract was proven to have a photoprotective nature as it significantly reduced the ROS level in UVC-irradiated cells owing to its antioxidant potential. Consequently, all the compelling evidence obtained throughout the study strongly advocates for the utilization of ripened papaya fruit for further investigation and development of a formulation for the treatment of colorectal cancer and photoaging of the skin.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

CRC: Colorectal Cancer; **ROS:** Reactive Oxygen Species; **UAE:** Ultrasound-Assisted Extraction; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoid Content; **MTT**: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; **DCFH-DA:** 2′,7′-Dichlorodihydrofluorescein diacetate; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **PBS:** Phosphate-Buffered Saline; **IC50:** Half Maximal Inhibitory Concentration; **AP-1:** Activator Protein-1; **MAPK:** Mitogen-Activated Protein Kinase, **TGF-β1:** Transforming Growth Factor Beta 1; **NF-κB:** Nuclear Factor Kappa B; **IL-1β, IL-6, IL-8:** Interleukins; **TNF-α:**Tumor Necrosis Factor-alpha; **UVC:** Ultraviolet-C radiation; **HT-29:** Human Colorectal Adenocarcinoma Cell Line; **HaCaT:** Human Keratinocyte Cell Line; **3T3-L1:** Murine Fibroblast Cell Line; **RCFE:** Relative Colony-Forming Efficiency; **MeOH:** Methanol.

SUMMARY

The study investigates the anti-cancer and photo-aging potential of ripened *Carica papaya* fruit extract. Using Ultrasound-Assisted Extraction (UAE), the extract showed selective efficacy against colorectal cancer (HT-29 cells) by inducing ROS-mediated apoptosis, reducing cell migration, and inhibiting tumor-initiating potential. It also demonstrated antioxidant and photoprotective effects in UVC-irradiated HaCaT and 3T3-L1 cells, improving

cell viability and reducing ROS levels. These findings highlight the potential of ripened papaya as a natural candidate for cancer treatment and skincare applications.

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