Microwave assisted extraction of polyphenols from Pithecellobium dulce benth fruit peels and evaluation of its anticancer and antioxidant activity

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Abstract

Polyphenols are great interest in recent decades due to the potential health benefits such as protection against development of carcinoma, diabetes, obesity, osteoporosis neurodegenerative and cardiovascular diseases etc. Therefore, researchers and scientists have been more interested in the extraction of polyphenols from plant resources. The present study investigates the microwave-assisted extraction of polyphenols from the *Pithecellobium dulce* fruit peels. ANOVA pareto analysis and Response surface methodology was employed to analyse the effect of process variables on delignification. Four independent process variables such as microwave irradiation power, microwave irradiation time, pH and Liquid to solid ratio (LSR) were analysed. Microwave-assisted aqueous extraction facilitated in maximum yield of polyphenols from the fruit peels (79.18 mg GAE/g dw). The polyphenol extract exhibited potential antioxidant (IC_{50} of 63.18 µg/ml) and anticancer (LD_{50} of 61.3 µg/ml) activity using radical scavenging DPPH and MTT assay respectively. Therefore, our study indicates that the polyphenolics rich, biologically potent *Pithecellobium dulce* fruit peel extracts can be a good therapeutic and nutraceutical supplement to treat cancer and related complications.

Statement Of Novelty

Pithecellobium dulce fruit peels have potential biological properties due to it high polyphenolic content. Hence recycling of *P. dulce* fruit peels are attracting topic in food and agriculture for extraction of high value-added products especially polyphenols. Several research articles have been reported on the effective extraction of polyphenols using conventional extraction (solvent based) methods such as solid-liquid extraction, liquid-liquid extraction, boiling, maceration etc. However, these final extracts (solvent based) contain solvent traces which can affect the health. The non-conventional methods are green based extraction techniques which lead to absence of precarious chemicals in final products (extracts) for human health and environmental safety. The present study describes the effective utilization of *P. dulce* fruit peels for extraction of polyphenols using microwave-assisted extraction method. In addition, this study showed that the aqueous based polyphenols have potential antioxidant and anticancer activities which can be useful for food, nutraceutical, pharmaceutical and cosmetic applications.

1. Introduction

Plants have been used for treatment of various diseases since prehistoric times. As per the world health organization report, around 21,000 plant species are used for medicinal purpose (Miransari et al., 2021). Medicinal plants contain a significant amount of natural products (phytochemicals) such as flavonoids, terpenoids and steroids (Giannenas et al., 2020). In recent days food and biopharmaceutical industries have focused their interest in some medicinal plants due to the presence of polyphenolic profile (Yu et al., 2021). Polyphenolic compounds are major secondary metabolites/phytochemicals mainly found in plant tissues. In recent years, extensive attention has been paid to polyphenols due to their diverse pharmacological properties such as antioxidant, anti-inflammatory, anti-carcinogenic, antiviral and anti-allergic activities (Bhat et al., 2020). These phenolics compounds are mainly involved in the defence

mechanism against oxidative stress, radiation and aggression by pathogens in human body (Ekalu and Habila, 2020).

Pithecellobium dulce belonging to the family of Leguminosae (subfamily: Mimosoideae) locally known by its English name as Manila Tamarind. It is a small to medium sized, evergreen, spiny woody legume tree up to 18m height and its nativity belongs to the tropical America and also found throughout India and Pakistan (Murugesan et al., 2019). *P. dulce* parts such as fruits, leaves, seeds, bark and etc have a phenomenal source of biological active compounds which relates both high nutritional and wide range of pharmaceutical applications due to the presence of higher amount of polyphenols (Dhanisha et al., 2020; Selvakumar et al., 2021). However, the *P. dulce* fruit and seed contains rich nutrition like vitamins (ascorbic acid, thiamine, riboflavin) and essential amino acids (lysine, phenylalanine, tryptophan, and valine). The bark and pulp are used as a traditional remedy against gum ailments, toothache, haemorrhage, dysentery, and diarrhoea. The leaves are also used as a feed for goat because of its higher nutritional content (Dhanisha et al., 2021). Each part of *P. dulce* contains dignified medicinal values, like anti-inflammatory activity (using fruits saponin fraction), estrogenic activity (root extracts), antimicrobial (leaves and seed extracts), Adultical activities (leaves and seeds extract), anti-venomous activity (bark polyphenols) (Dhanisha et al., 2021; Murugesan et al., 2019; Roselin and Parameshwari, 2022). The fruit and fruit peel extract also have potential hypolipidemic, antidiabetic, antiulcer and nephroprotective effect. Megala et al and Gabriela López-Angulo et al reported that the acute and sub-acute toxicity profile of *P. dulce* extract and concluded that it can be used safely for experimental and clinical trials (López-Angulo et al., 2021).

Polyphenolic compounds from natural sources are known to possess various significant biological activities. In recent years, extensive attention has been paid for isolation of polyphenols due to their diverse pharmacological properties (Venkateswaran et al., 2021). Therefore, a lot of interest is now focused towards the extraction of polyphenols from plants and evaluation of their bioactive potential. Numerous articles have been reported for the effective extraction of phenolic content using conventional methods (Solid-Liquid extraction/Soxhlet extraction, liquid-liquid extraction, boiling, maceration and soaking) (Alara et al., 2021). However, these methods have several disadvantages such as utilizing the huge amount of solvents, thermal degradation of desired molecules, cost, lesser yield, high energy, longer extraction time etc (Chemat et al., 2020). Microwave assisted extraction (MAE) is a suitable method for extraction of bioactive compounds from natural resources. It is a greener extraction method, water is used as a solvent and it is very safe, fast, eco-friendly and inexpensive (Vernès et al., 2020). However, excessive heat generation involved during the extraction process and this excess heat destroy the desired bioactive molecules, thus the extraction conditions such as power, irradiation time, type of solvents, solvent ratio, and composition etc needs to be optimized for to achieve the maximum yield of the phenolic compounds (Jha and Sit, 2021). The present study aims to optimize the process parameters for maximising the phenolic yield from the *P. dulce* fruit peel and evaluating the antioxidant and antiproliferative activity of the *P. dulce* fruit peel phenolics.

2. Materials And Methods

2.1 Preparation of the fruit peels

P. dulce fruits were collected from Virudhunagar district, Tamil Nadu, India. The fruit peels were removed manually, washed thoroughly and dried in a hot air oven at a temperature of 60 ^{0}C. The dried peels were further powdered, sieved and stored in dark bags at dried environment in prior to the experimental analysis.

2.2 Chemicals and Reagent

All the chemicals and the solvents used in the study were analytical grade and purchased from Merck Chemicals, Chennai, India

2.3 Microwave assisted Extraction

The MAE experiments were conducted using domestic microwave reactor (IFB-25SC2). The waves were generated from microwave signal generator in a perpendicular to the experimental setup and the signal generator kept in the center of the oven in order to obtain uniform distribution of heat. The continuous and precise microwaves were produced by using magnetron (2450MHz). The microwave extraction device has an adjustable knob to control the Microwave power and Irradiation time operational parameters (Alexander et al., 2020).

2.4 Experimental work - Extraction procedure

2.4.1 Microwave Assisted Extraction of Polyphenols from *Pithecellobium dulce*

Initially 1g of powdered sample was used for extraction process and the samples were treated with water under the process conditions of the solid-liquid ratio (LSR) 30:1 mL/g, irradiation time 60s and microwave power 810W. After extraction, the samples were allowed to room temperature, filtered using Whatmann no.1 filter paper, centrifuged at 5000 rpm for 10 min. The supernatant was collected and subjected to analysis of total phenolic content (Li et al., 2017).

2.4.2 Optimization Using OVAT

One variable at a time (OVAT) approach is a univariate, conventional technique used for optimization of process variables. A holistic view of the entire process parameters can be obtained using this approach. In the present study, the effect of Microwave power 90-630W, irradiation time 30-330s, LSR 10–60 mL/g and pH (4 to 11) were optimized in a sequential manner. One parameter was varied and other parameters were maintained at a fixed level. After OVAT, the factors were optimized using RSM studies.

The principle of heating by microwave energy is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. Polar molecules (such as polyphenols) and ionic solutions

absorb microwave energy strongly because they have a permanent dipole moment (Soria et al., 2015). This results in rapid rise of the temperature and fast completion of the reaction. In the second stage, after analysing the free phenolic acids and the optimal conditions of pre-treatment, it was used to carry out further steps. The time of exposure to microwaves plays a critical role. Higher power level shall require lesser time and vice versa.

2.5 Statistical Optimization Using CCRD

Statistical optimization of the process parameters was performed using Central Composite Rotatable Design (CCRD). Optimization of process parameters for the extraction process is considered essential in order to reduce the time and cost of the experiment. The statistical analysis of the parameters was performed by Design Expert statistical software package 7.0.0. Total of 30 runs was performed for four factors. The parameters affecting the microwave-assisted extraction process are microwave power (X_1), time (X_2), liquid-solid ratio (X_3) and pH (X_4) respectively. The levels were represented as, 1 for high level, 0 for central point and − 1 for the low level, which was selected based on preliminary single factor experiment. The total phenolic content (TPC) present in the sample is represented as Y value. The levels and experimental range of the parameters are mentioned in Table 2.1.

Pareto analysis of variance (ANOVA) gives the regression coefficients of quadratic, interaction and quadratic terms and their effects. Statistical testing was executed by F-test and the probability levels ($p \leq 0.05$) were used to analyze the significance of the F-values. Adjusted determination coefficient (R^2adj), determination coefficient (R^2), the coefficient of variance (CV %), adequate precision and predicted determination coefficient (R^2 pre) of the model were analysed to identify the fitness of the polynomial model. The quadratic equation is represented as contour plots to establish the relation between the parameters and responses. After optimization, triplicates of experiments were performed at optimal process conditions and the average value was compared with the predicted values of developed model equation.

Table 1
Factors and their levels in RSM Studies

Factor	Variables	Levels of factors		
		-1	0	1
X_1	Microwave power (W)	450	540	630
X_2	Time (s)	100	150	200
X_3	Liquid-solid ratio (mL/g)	20	30	40
X_4	pH	8	9	10

2.6 Estimation of Total Phenolic Content

The concentration of Total polyphenol content (TPC) of the *P. dulce* fruit peel obtained from the MAE extraction techniques was determined by Folin-Ciocalteu procedure (Ravichandran et al., 2021). Briefly, 10µL of the sample solution (polyphenol extract) was added to the 100µL of Folin reagent. The reaction setup was kept in room temperature for 2 minutes and later neutralized using sodium carbonate solution (7.5% w/v). The reaction mixture was incubated at dark for 10 minutes at room temperature. The solution was diluted with distilled H_2O and absorbance was read at 750 nm using UV-Vis spectroscopy. Gallic acid was used as standard for to estimate the phenolic content (Zeković et al., 2017).

2.7 DPPH radial scavenging assay

The scavenging potential of the phenolics was estimated by DPPH radicals using customized protocol of (Murugesan et al., 2020). Ascorbic acid was used as a reference standard. The stock DPPH solution (60 µM) was prepared using methanol. A total volume of 4 mL solution was prepared which contains 3.9 mL of DPPH solution and 100 µL of sample solution in a different concentration manner. In the control, the exact amount of sample extract was replaced with methanol. The test tubes were allowed for 15 minutes at ambient temperature in dark and the absorbance was read at 517nm (Senthilkumar et al., 2022). Radical-scavenging activity was expressed as the % inhibition of free radicals by the sample and calculated as,

% Inhibition = (Absorbance of Control - Absorbance of Test) / Absorbance of Control at 15 minutes x 100

Where; C = absorption of control sample (t = 0 min), C = absorption of control (t = 15 min), T = absorption of test solution.

2.8 *In vitro* Antiproliferative effect

2.8.1 Cell culture

The human cervical carcinoma cell line (HeLa) was procured from National Centre for Cell Sciences (NCCS), Pune, India and grown up in culture medium consisting of DMEM (Dulbecos modified Eagles medium) with 10% FBS, antibiotics (penicillin G: 100 µg/mL and streptomycin sulfate: 50 µg/mL) and the cells maintained using the standard protocol (37°C, 5% CO2, 95% air and 100% humidity). The sub-culturing of HeLa cells was performed by trypsination.

2.8.2 Determination of cell viability by MTT assay

The MTT assay was a colorimetric assay for the non-radioactive quantification of cellular proliferation, viability and cytotoxicity. The HeLa cells were preincubated with or without extract in the 96-well plates. The untreated cells were used as a control. After 24 h of incubation, MTT (15 µl; 5 mg/mL) in phosphate buffered saline (PBS) was added to respective wells and allowed to incubate at 37°C for 4 h. After incubation, the supernatant was collected, and the formed formazan crystals were then dissolved in DMSO (100 µl). The absorbances at 570 nm were then measured using a microplate reader (Venkateswaran et al., 2021). The cell viability fraction was calculated by the following formula with respect to absorbance of control:

Cell viability fraction = mean experimental absorbance / mean control absorbance ×100%.

2.8.3 Apoptosis detection by Acridine Orange/Ethidium Bromide Staining

Initially the HeLa cells (1×10^5 cells/mL) were grown in 96-well plates and incubated with different concentration of phenolic extracts. After 24 h incubation, PBS solution (1X) was added to the cells with 0.25% trypsin for washing purpose. Thereafter cells were resuspended with binding buffer (200 µl). The treated and untreated cells were incubated with acridine orange (AO) and FITC-annexin V (both 100µg/ml) in the dark condition for 15 min. The stained apoptotic cells were washed twicely with PBS (1X) and subsequently the cells were examined under fluorescent microscope (Zhu et al., 2018). The cells are divided into four categories as living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragment chromatin) and late apoptotic (orange-stained nucleus).

3. Results And Discussion

3.1 Effect of different process parameters on MAE Of *P. dulce*

The *P. dulce* bioactive compounds have tremendous health benefits such as antioxidant, anti-proliferative, anti-inflammatory and anti-microbial activities. The present study deals with extraction of high value-added product (polyphenols) from the plant solid matrix by using a significant low-cost solvent. In this study, we investigated effect of microwave irradiation on aqueous extraction of fruit peels for maximal yield of polyphenols. As water have the highest dielectric constant among the common solvents, the rate at which water absorbs microwave energy is higher than the rate at which the system can dissipate the heat. These phenomena account for the “superheating” effect which occurs when water is used as a solvent (Đurović et al., 2018).

3.2 Single-Factor Experiments

A univariate, conventional One Variable at A Time (OVAT) approach was employed to optimize the parameters influencing the extraction procedure. It gives the correlation between the various process variable by varying one parameter against the other fixed parameters. The work extends to optimize the significant condition with the use of Response Surface Methodology (RSM).

3.2.1 Effect of Microwave Power

The extraction of polyphenols increased linearly with increase in microwave power from 90W to 630W Fig. 1 (a). It has been experimentally valuated that while the microwave power was increased from 300-700W the release of phenols has been gradually increased. The microwave assisted treatment improves reactivity of biomass as the heat transformation is from the surface to core of the material. The dipole interactions inside the reaction system also facilitates the disruption of the plant matrix (Li et al., 2017).

The penetration power of the solvent in to the cell matrix increases up to 540W, the point where the extraction of phenols are more effective. Further increase in power beyond 540 W did not exhibit a significant effect on the phenol extraction. The high irradiation power offered superfluous energy to the solvent and matrix and drastically disturbed molecular interactions. This could be attributed to the fact that free radicals generated due to the breakage of oxygen bridge between the monomeric units (Prakash Maran and Priya, 2015). Hence, based on the observations, moderate microwave power levels of 450, 540 and 630W were selected as the lower, middle and upper levels, respectively, to apply in RSM optimization.

3.2.2 Effect of Time

Time is one of the key factor which influences any process in terms of cost economics as well as process scale up. Hence, in this current work, different irradiation time (30–330 sec) were analyzed and the response was depicted in Fig. 1 (b). As the reaction time was increased linearly from 30sec to 150 sec, the extraction of polyphenol also improved linearly from 15 to 29mg GAE/g dw. This could be due to the increased collision between the molecules and disruption of intermolecular forces due to the electromagnetic irradiation. Increasing trend of irradiation time facilitate the rapid disintegration of cell wall up to a certain level. Prolonged exposure beyond 180 s of irradiation time had a negligible effect on the extraction of phenols and it causes the reduction in TPC. Similar results were reported in studies on the extraction of anthocyanins from grape juice waste and polysaccharides from *Moringa oleifera* Lam. Leaves (Chen et al., 2017; Varadharajan et al., 2017). Thus, an extraction time of 158 seconds was selected in the subsequent experiments.

3.2.3 Effect of Liquid-Solid Ratio

LSR is yet another significant operating parameter to be optimized for effective extraction. For example, in an industrial extraction process, it is important to maximize extraction yield, while minimizing consumption of solvent. Figure 1 (c) shows the effect of L/S ratio on extraction efficacy of polyphenols at diverse ratios ranging from 10–60 mL/g. Increasing the LSR from 10-31mL/g enhanced the extraction yield and improved the contact area between the fruit peels and solvent. Hence the release of polyphenols the material was increased. But further increase in the LS ratio decreases the supply of microwave power and negatively affects the extraction yield (Moorthy et al., 2017). The reduction in the polyphenol yield with the increase of solvent amount is not in accordance with mass transfer principles, since the concentration gradient which is the driving force is thought to be higher when a lesser solid to solvent ratio is used. When LSR reach 31 mL/g, the mass transfer process reached its maximum. Similar results were reported in the studies on the extraction of polysaccharide from *Genetiana Scabra* and extraction of solvents from flavonoids from *Crotalaria sessiliflora*.

3.2.4 Effect of pH

It is common to treat plant-extracts with alkali. In this study, the stability of the natural polyphenols extracted from the fruit peels were tested for the effect of pH in the range 4–11 with the aid of ultraviolet spectroscopy. Figure 1 (d) represents the results illustrated that when the samples were treated at high pH (8.5–9.3) condition, high poly phenolic contents were obtained without any degradation of the sample.

Because plants contain a large number of structurally different antioxidant, anti-carcinogenic, and antimicrobial polyphenolic compounds. Further increase in pH higher than 9, the concentration of phenols as well as the yield was decreased sharply due to its aggregation and interactions of monomeric and polymeric subunits of the extracts. The analyses of TPC at low pH also seems to be reduced due to hydroxylation of phenolic groups in water solvent medium. Therefore, the optimum pH for the maximum recovery of polyphenols from *Pithecellobium dulce* was found to be 8.6 pH.

Maximum phenol recovery was obtained at microwave power of 540 W, irradiation time of 200 sec, LSR of 40 ml/g and pH of 8.6. Under these conditions the phenol yield was 79.18 mg GAE/g dw.

3.3 Statistical Optimization of Microwave Assisted Polyphenol Extraction

3.3.1 Modelling and fitting the model using response surface methodology (RSM)

The empirical relation expressed by second-order polynomial equation was fitted to the obtained results to develop a mathematical model, which will help to predict the extraction of polyphenols.

Final equation derived in terms of un-coded factors is given below.

TPC = 78.27 + 2.65 X1 + 1.75*X2 + 0.68*X3 + 0.36 *X4 -0.85*X1*X2–0.11 * X1 * X3 + 0.49 * X1 * X4 + 2.59 * X2 * X3 -1.91 * X2 * X4 -0.17 * X3 * X4–5.36 * X12–5.46* X22–3.31 * X32–3.68 * X42

Where the variables are coded as X_1 – Power ; X_2 – Time ; X_3-LSR and X_4- pH

Experimental data were analyzed by Pareto analysis of variance (ANOVA) and the results were shown in Table 3.1. From the data it is evident that the developed quadratic model was highly significant. The higher F value of 95.34956 associated with a low p-value of < 0.0001 clearly indicated the significance of the developed model (Maran and Priya 2015). The goodness of fit of model was evaluated by determination coefficient (R2), adjusted determination coefficient (R2adj), predicted determination coefficient (R predicted) and coefficient of variance (CV%). In the present study the value of determination coefficient (R2) is 0.988888 and it ensures a satisfactory correlation between the developed model and the experimental data (Maran, Sivakumar, Thirugnanasambandham, et al. 2013). The calculated adjusted determination coefficient (Radj2) for present extraction was 0.9984 and predicted determination coefficient (Rpred) was 0.942774. The high values indicated a good correlation between the observed and predicted values. In addition, the low CV values (1.94) expressed a low deviation between predicted and experimental values. Adequate precision measures the ratio of response to deviation and a value greater than four is desirable (Maran, Sivakumar, Sridhar, et al. 2013). The ratio was found to be higher, indicating an adequate signal. The results derived from the ANOVA were further evaluated by study of three-dimensional response surface plot constructed from the regression model.

3.3.2 Diagnostics of model adequacy

The Adequacy of the developed model was evaluated from the diagnostic plots such as actual phenol recovery versus actual phenol recovery, normal % probability. The diagnostic plots are displayed in Fig. 2. It is mandatory to affirm that the fitted values provide an ample approximation to actual values. This will facilitate in ignoring poor and misleading results on optimization. The predicted extraction values derived from the developed model was in line with the experimental responses. The values are found to lie close in straight line and indicated the reliability and significance of the developed model (Fig. 2 (i)). Similarly, the normal % probability plot of the residuals was normally distributed and the values are close in position to the straight line which indicates and acceptable deviation of variance (Fig. 2 (ii)).

3.3.3 3-Dimensional Response Plot

In the present study, four factors at five level CCRD design is used to investigate the effect of microwave power, reaction time, pH and liquid solid ratio on extraction of polyphenols from *Pithecellobium dulce* fruit peels. To study the main and interacting effects of independent variables three dimensional response surface and contour plots were constructed (Fig. 3). The graphs are constructed by maintaining two factors at constant and the other two factors so as to understand the main and interacting effects of variables. It was clear from the response plot between microwave power and irradiation time that the phenol extraction increases with increase in microwave power and irradiation time up to a certain level and afterwards there is a slight decrease in the phenol recovery which might be due to the fact that thermal accumulation would be facilitated at higher conditions of microwave power and irradiation time.

It was evident from the response plot between microwave power and LSR with an increase in microwave power and LSR, the percent phenol recovery increases and thereafter reduces markedly, this might be due to the improper mass transfer reactions at higher temperature. At a microwave power of 560 W and LSR of 31 ml/g the maximum phenol recovery of 78.16 mg GAE / gm. dw was obtained. Similarly, from the response plot between microwave power and pH, with an increase in microwave power and pH the phenol recovery improved significantly up to a certain extend and then decreased. Maximum extraction was observed at a microwave power of 540 W and pH of 8.6. The response surface plot between irradiation time and liquid solid ratio, it is evident that the there is a increase in phenol recovery with increase in between irradiation time and liquid solid ratio, thereafter no further increase in phenol recovery was observed. Maximum phenol recovery was observed at irradiation time of 158 sec and a liquid solid ratio of 31ml/g.

3.3.4 Optimization and Validation of Process Variables:

The main objective of the current study is to derive at an optimal solution for extraction of polyphenols from *Pithecellobium dulce* fruit peels. Derringer's desired function methodology was applied in the present study to analyse the suitability of the developed model equation so as to predict the optimum response values. From the three-dimensional response plots and the contour plots it is evident that maximum phenol recovery was obtained at microwave power of 540 W, irradiation time of 158.94 sec, LSR of 31.67 ml/g and pH of 8.6. The experiments were conducted in triplicates to identify the suitability

of optimized conditions. The mean values were correlated well with the predicted values at optimized condition.

Table 2
Analysis Of Variance (ANOVA) for the Central Composite Rotatable Design.

Source	Sum of Squares	df	Mean Square	F-Value	P-Value	
Model	2079.468	14	148.5334	95.34956	< 0.0001	significant
X_1	168.54	1	168.54	108.1926	< 0.0001	
X_2	73.5	1	73.5	47.1826	< 0.0001	
X_3	10.935	1	10.935	7.019615	0.0182	
X_4	3.081667	1	3.081667	1.978245	0.1800	
X_1X_2	11.56	1	11.56	7.420827	0.0157	
X_1X_3	0.2025	1	0.2025	0.129993	0.7235	
X_1X_4	3.8025	1	3.8025	2.440977	0.1391	
X_2X_3	107.1225	1	107.1225	68.76623	< 0.0001	
X_2X_4	58.5225	1	58.5225	37.56794	< 0.0001	
X_3X_4	0.49	1	0.49	0.314551	0.5832	
X_12	787.5219	1	787.5219	505.5419	< 0.0001	
X_2^2	817.1905	1	817.1905	524.5873	< 0.0001	
X_3^2	300.2076	1	300.2076	192.7153	< 0.0001	
X_4^2	372.1219	1	372.1219	238.88	< 0.0001	
Residual	23.36667	15	1.557778			
Lack of Fit	20.06667	10	2.006667	3.040404	0.1157	not significant
Pure Error	3.3	5	0.66			
Cor Total	2102.835	29				

Table. 3 The optimized condition obtained from the reduced model.

Number	Microwave Power (W)	Time (s)	LSR (ml/g)	pH	TPC (mg GAE /gm.dw)
1	540	158	31	8.6	79.16

3.4 DPPH radial scavenging assay

The radical scavenging effect of polyphenols from *P. dulce* fruit peel was analysed using DPPH method. The free radical which is capable of producing purple/violet colour. It reduces in presence of an antioxidants giving purple/violet to colourless. In the present study, the polyphenols from *P. dulce* fruit peel extract exhibited significant antioxidant activity in the dose dependent manner (20, 40, 60, and 80 µg/mL). The extracts exhibited a significant result with an IC50 value of 63.18 µg/ml (Fig. 4). The experimental results were very stable and produced reliable results in repeated tests, as the DPPH possess an odd electron giving away a strong absorption at 515 nm. As the electron gets paired off, in presence of free radical scavengers the absorption fades and the resulting decolorization is in line with the number of electrons taken up (Choi et al., 2002).

3.5 *In vitro* anti proliferative activity

3.5.1 Determination by MTT assay

The HeLa cells were treated with polyphenol extract (200µg, 100µg, 50µg, 25µg, 12.5µg in 100µl of 5% DMEM) and showed the significant decrease in cell viability respectively. The viability of the cells at different concentration of polyphenol extract were displayed in Figs. 5 & 6. The morphology of cultured cancer cell significantly changed with treatment of polyphenol extract. The effect of inhibition of cell growth showed significant cytotoxic against HeLa cell lines with the usage of an LD50 of 61.3 µg/ml. The overall results indicate the promising baseline information for the potential uses of the aqueous extract of *P. dulce* fruit peels as a significant chemo-preventive agent.

3.5.2 Determination of Apoptosis

Damaged and apoptotic morphology of cell lines treated with polyphenols were analysed using AO and EB staining. The method discriminates between viable and non-viable cells. The viable cells were witnessed with green nuclei whereas the dead cells were visualized with orange to red nuclei. The morphological changes at the cellular level were studied using AO/EB staining under fluorescence microscopy (Fig. 7). HeLa cells treated with the polyphenolic extract of *P. dulce* exhibited condensed nucleus and apoptotic bodies. An intact nucleus was observed in control (Abutaha et al., 2022).

Conclusion

Plant based medication is one of the most important research areas in recent years for treatment of cancer and autoimmune diseases. The increased economic burden along with unintentional side effects limits, the researchers focus on development of pure and novel plant-based compounds such as

polyphenols, flavonoids, alkaloids etc as a therapeutic drug against cancer. The present work established an improved and optimised procedure for extracting polyphenols from *Pithecellobium dulce* fruit peels using Microwave Assisted Extraction method. Pareto analysis showed that all four parameters Microwave power, Time, LSR and pH were significant and quadratic models were obtained for predicting the response using CCRD. The Response Surface Methodology- Central Composite Rotatable Design was successfully employed to optimize total phenolic yield extracted from dried fruit peels which are rich in polyphenol content with high antioxidant activity. Maximum yield of Phenol was obtained at a Power of 540 W, pH 8.6, Time of 158 Sec, LSR of 31 ml/gm, The extracted phenols exhibited a significant antioxidant, anti-proliferative and apoptotic activity (IC 50: 63.18 µg/ml: LD 50: 61.3 µg/ml). These results demonstrated that *Pithecellobium dulce* fruit peels might be a novel and attractive therapeutic candidate for cancer treatment.

Declarations

Conflict of interest

The authors declared that they have no conflict of interest.

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Figures

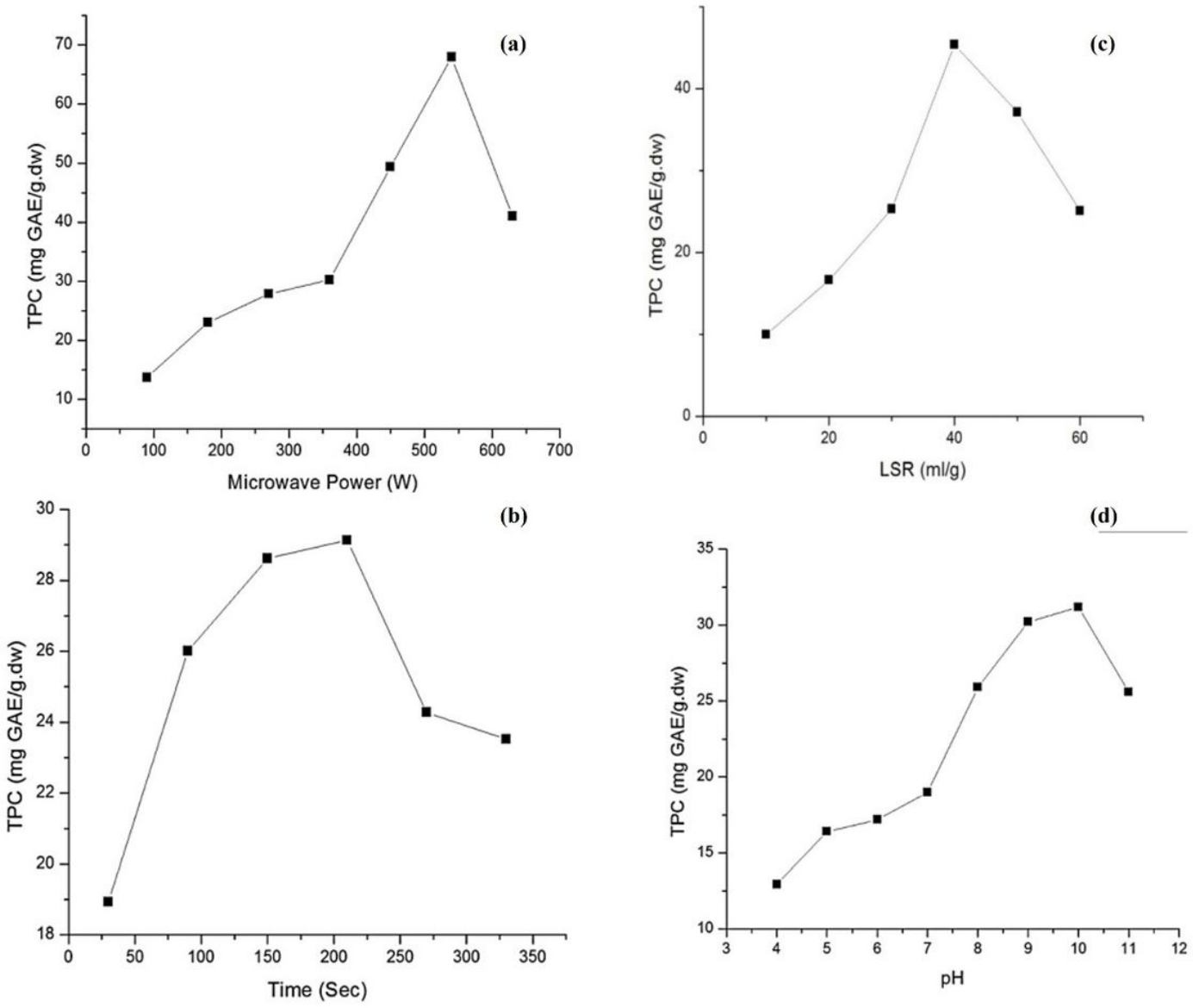

Figure 1

Effect of the process parameters (a) Microwave power, (b) Irradiation time, (c) Liquid-solid ratio and (d) pH variation on the yield of the Total Phenolic Content.

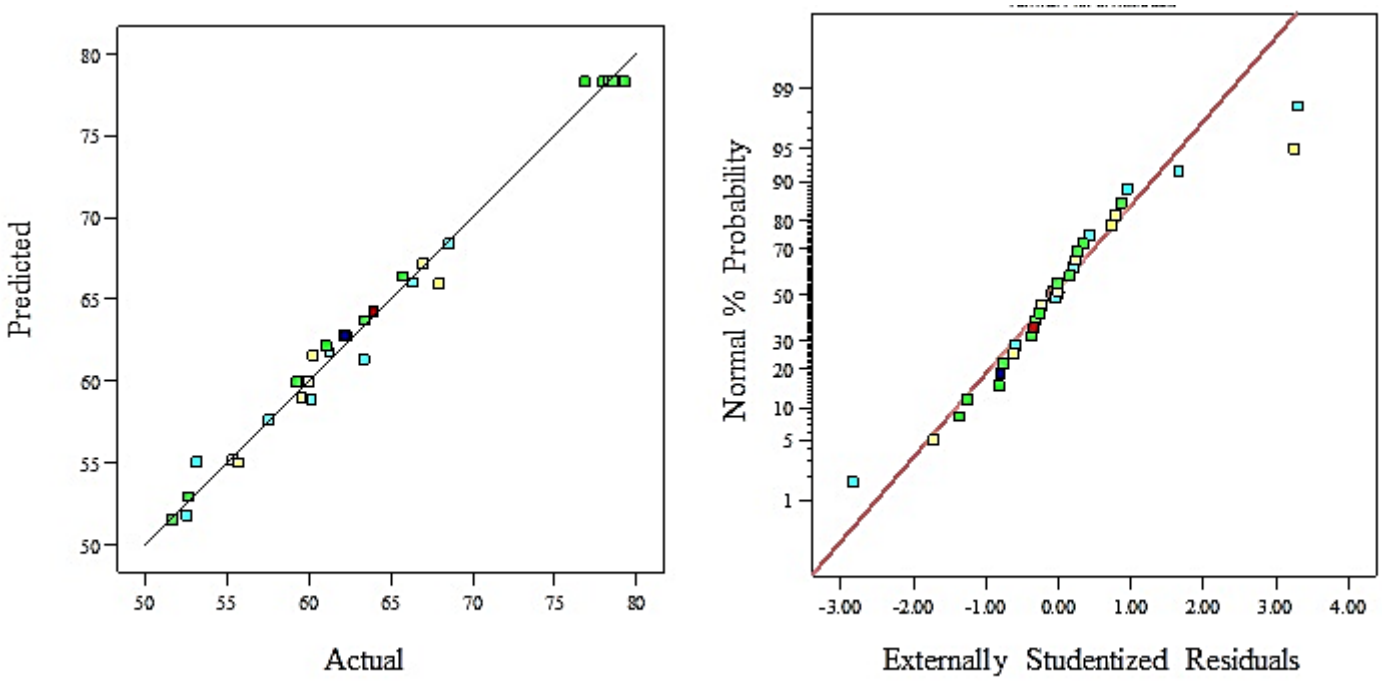

Figure 2

(i) Predicted vs Actual and (ii) Normal plot of residuals values for extraction.

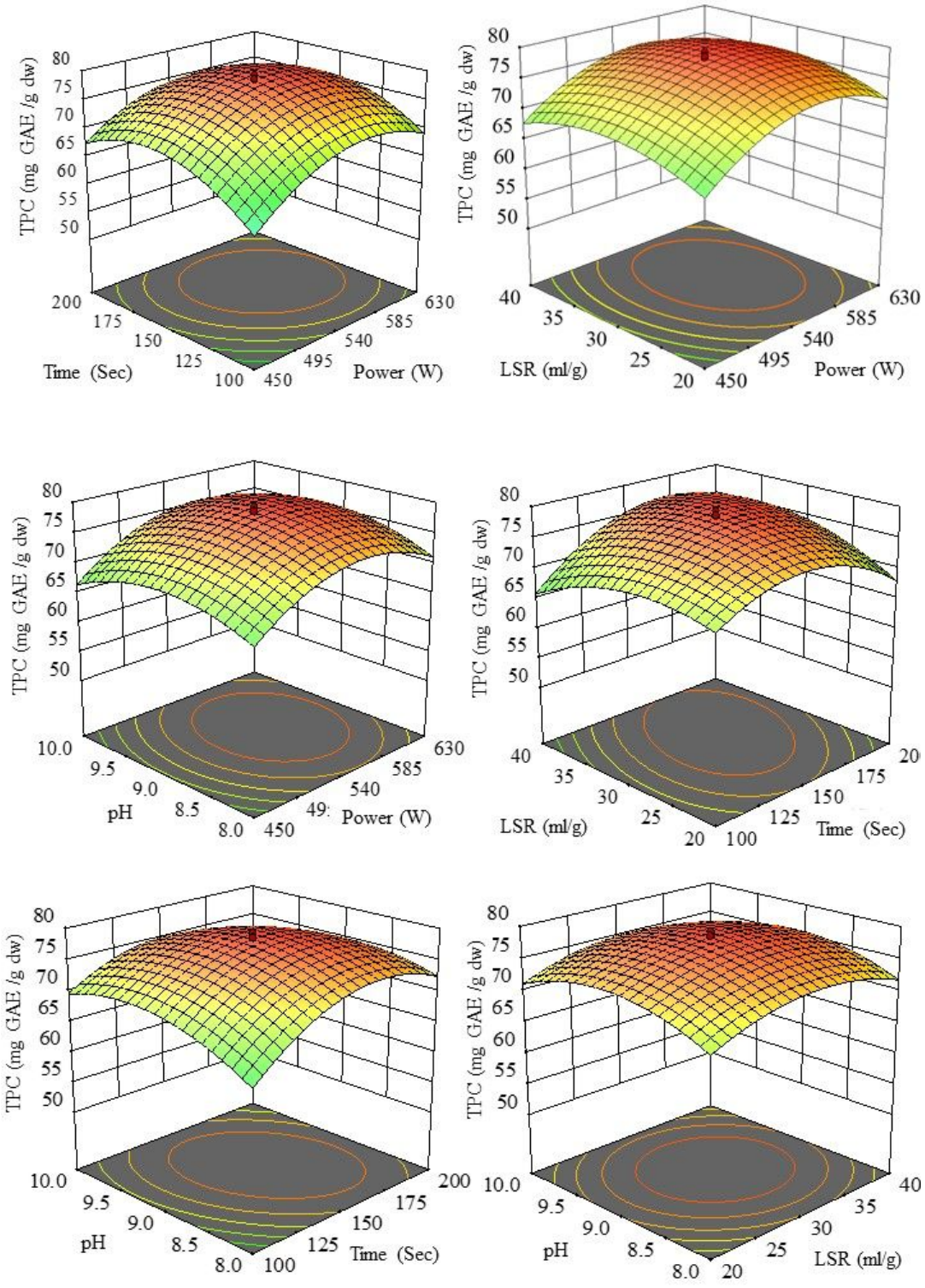

Figure 3

Response surface and contour plot showing the optimization of phenolic compounds extraction

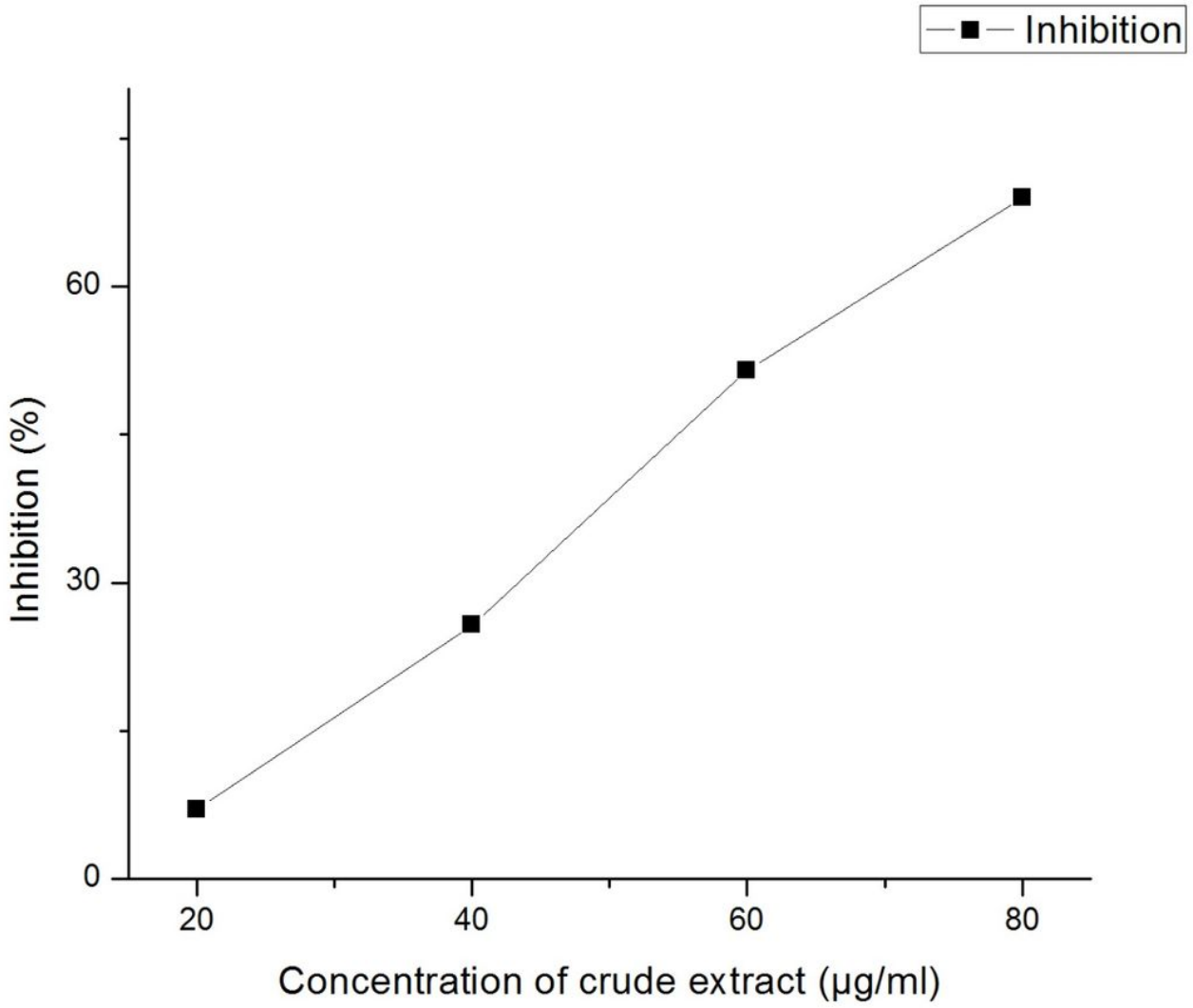

Figure 4

Dose-dependent antioxidant effect of polyphenol extract of *Pithecellobium dulce* on the free radicals. The percentage of inhibition was calculated from the DPPH assay.

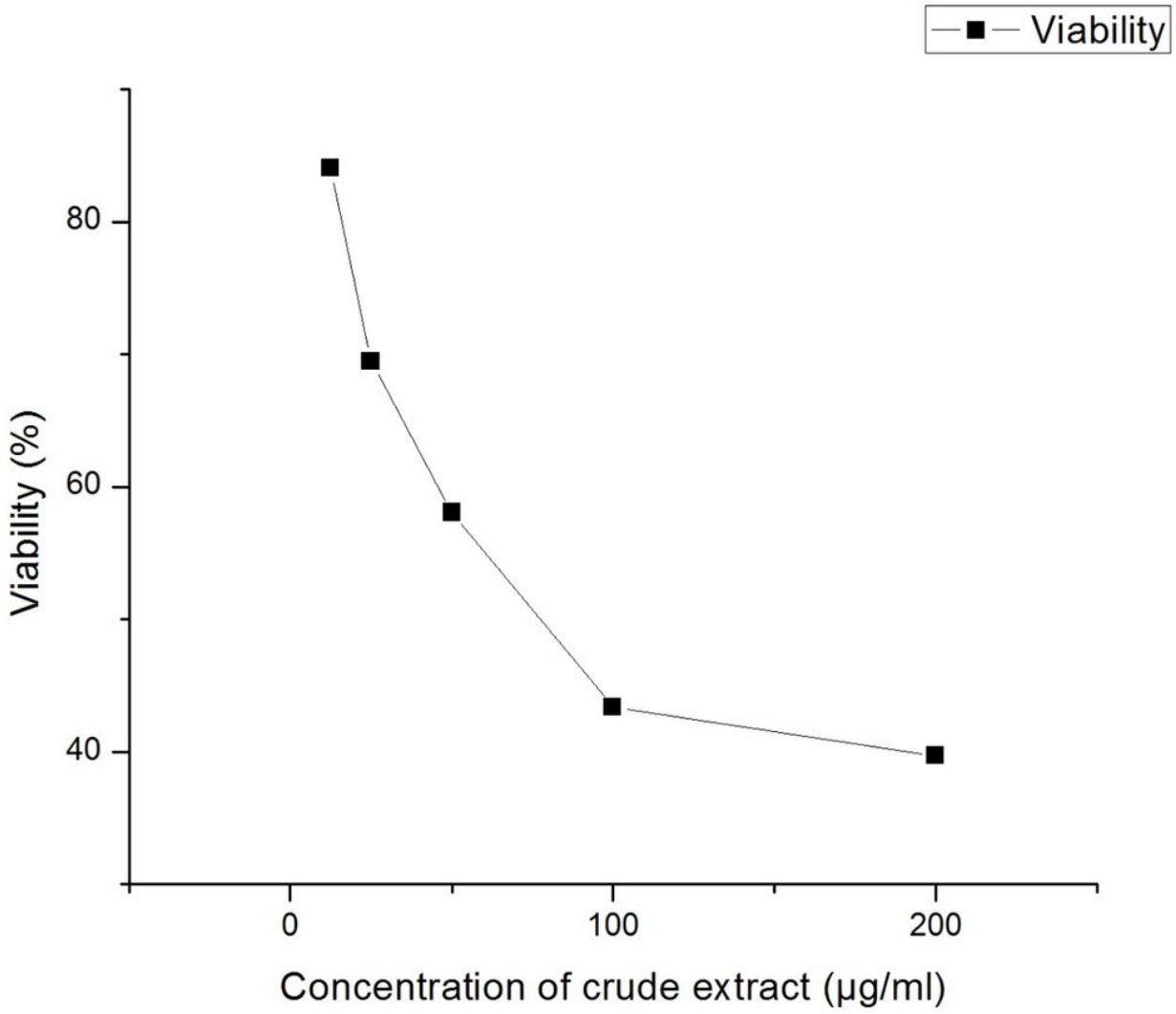

Figure 5

Dose-dependent effect of polyphenol of *Pithecellobium dulce* on the viability of human cervical cancer HeLa cells. The viability was determined using the MTT assay

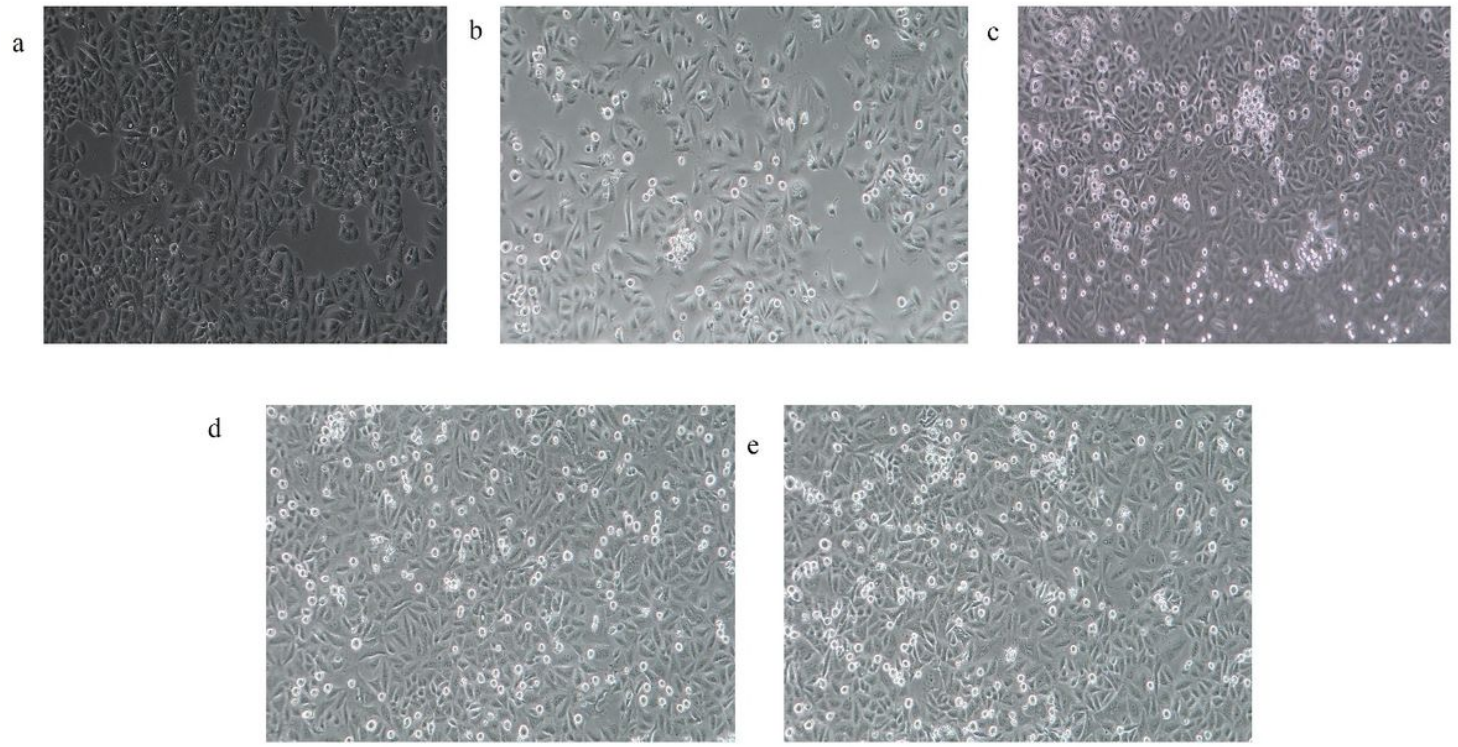

Figure 6

Characterization of induced cell death in human cervical cancer HeLa cells. The cells cultured without the extract were used as the control. Dose-dependent effect of the extract on the cells was observed under a phase contrast microscope a) control b) treated with 12.5 µg/ml of extract, c) 25 µg/ml of extract d) 50 µg/ml of extract and e) 100 µg/ml of extract.

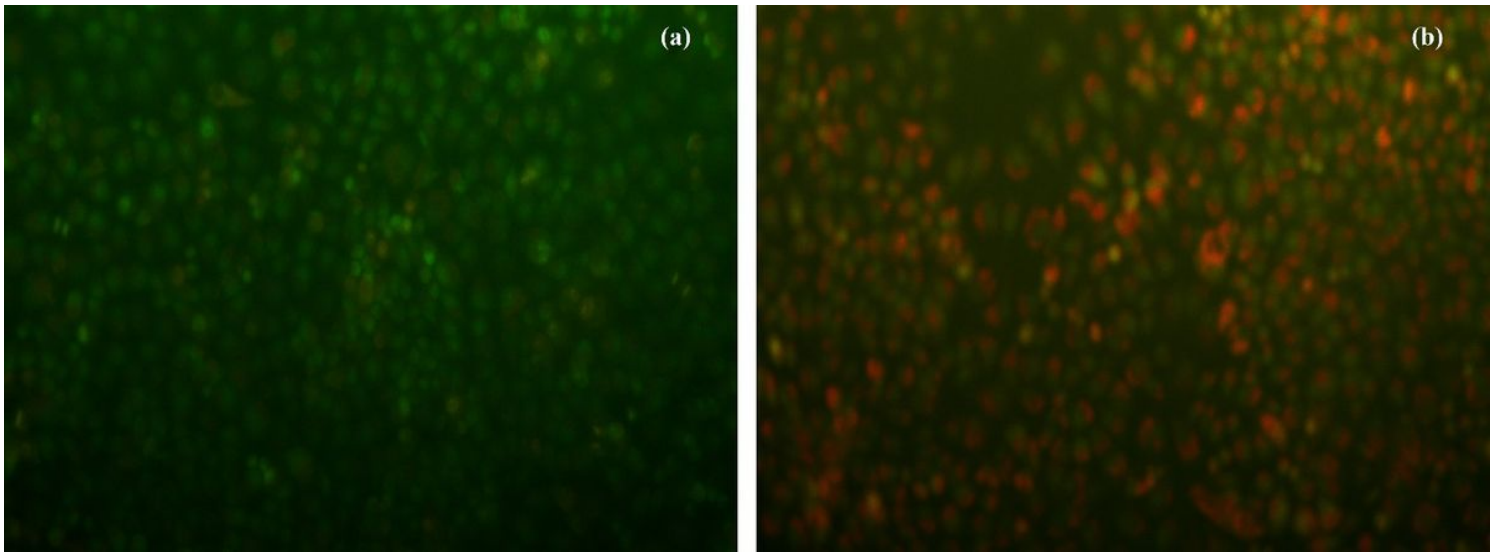

Figure 7

Direct Microscopic observation of apoptosis using acridine orange and ethidium bromide in HeLa cell lines a) control b) treated with 100 µg/ml of extract