Article

Effects of Time, Temperature, and Solvent Ratio on the Extraction of Non-Extractable Polyphenols with Anticancer Activity of Barhi Date Palm Kernels Extracts Using Response Surface Methodology

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Abstract: Dietary polyphenols exist in two forms; extractable polyphenols (EPP) or compounds solubilised by aqueous/organic solvents, and non-extractable polyphenols (NEPP) or compounds remain in the corresponding residues after the extraction. At present, most researchers focus on EEP fractions, while NEPP is neglected. Thus, this study aimed to release NEPP from the remaining powder residue of Barhi date palm kernels (BDPK) with acid hydrolysis. The related extraction conditions were determined and optimised using response surface methodology (RSM) for maximisation of NEPP with highest cytotoxic and antioxidant activities. The face-centred central composite design (FCCCD) was used to establish treatments based on three independent variables, namely; extraction temperature, time, and solvent/sample ratio. Under the optimal conditions, the experimental values for DPPH radical-scavenging capacity of NEPP (IC_{50}=57.52µg/mL), and cytotoxicity of NEPP against A549 and HT29 cells were IC_{50}=17.4 µg/mL and 31.4µg/mL, respectively. The experimental values were in agreement with those predicted by RSM models, confirming the suitability of the model employed and the success of RSM for optimisation of the extraction conditions for NEPP from BDPK. These results indicate that NEPP from industrial date fruit waste could be promising candidate as natural antioxidants with significant antiproliferation effect against A549 and HT29 cancer cells *in-vitro*.

Keywords: Barhi date palm kernels (BDPK); non-extractable polyphenols (NEPP); anticancer activity; antioxidant activity; response surface methodology; optimisation.

1. Introduction

The kernel of the date palm tree (*Phoenix dactylifera* L.), which constitutes 10% of the fruit weight, is the major by-product of date processing industry. Date palm kernels (DPK) could be considered an excellent source of many valuable substances such as dietary fibres, carbohydrates, protein, oil, minerals, vitamins, amino acids and bioactive polyphenols with potential applications of DPK and their constituents in the human nutrition, cosmetics, and pharmaceutical applications that may be beneficial to human health [1,2]. DPK, in general, contain higher concentration of secondary metabolites compared to the edible fruit [3]. Given the scale of the international date industry, a large quantity of DPK can easily be collected from the date processing industries or from the date palm groves [2].

Such inexpensive, largely abundant and low-value date palm fruit waste could, however, potentially be industrially exploited [4,5]. A healthy caffeine–free coffee, which can be produced from

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roasted or unroasted DPK powders are produced commercially and are widely consumed in Middle Eastern countries [6]. Recently, DPK have been recognised as a source of bioactive compounds including dietary fibre, high amounts of polyphenols and natural antioxidants [7]. Additionally, DPK extracts have shown some positive molecular activities, including prevention or management of neurodegenerative diseases [6], inflammation [8] and cancer [9,10]. Previous studies suggested that the polyphenolic compounds are primarily responsible for the antioxidant and biological activities of DPK [3,7,11], and this was experimentally confirmed in the lab [12,13].

Plant secondary metabolites such as polyphenols, play an important role in the defence against free radicals. Medicinal plant parts (roots, leaves, kernels, stems, flowers and fruits) are commonly rich in phenolic compounds, such as flavonoids, tannins, stilbenes, coumarins, lignans [14]. Polyphenolics exhibit a wide range of biological effects and such functions have been attributed to their free radical scavenging and antioxidant activities [15].

In view of these potential health benefits, there has been intensive research on natural antioxidants derived from plants. "Structurally, phenols comprise an aromatic ring bearing one or more hydroxyl substituents and range from simple molecules to highly polymerised compounds" [16]. It is well known that phenolic compounds exist in both extractable (free) and non-extractable (bound) forms in plant cells and that the extractable polyphenols (EPP) are solvent extractable. In contrast, the non-extractable polyphenols (NEEP), which are covalently bound to the plant matrix, cannot be extracted into water or aqueous/organic solvents mixtures [17].

Although the total phenolic contents and antioxidant activities of alcoholic DPK extracts have been investigated previously [3,9,11,18], these studies, only considered the extractable polyphenols (EPP) present in the DPK. Habib, *et al.* [19] and Ahmed, *et al.* [20] are the only ones who investigated the proanthocyanidins in their studies. "Proanthocyanidins are generally termed as NEPP when examining the TPC. NEPP represent the proportion of antioxidants remaining in the residue of the aqueous-organic extraction treatment of EPP, and this residue has been reported to contain large amounts of NEPP with specific biological activities" [2]. NEPP are large molecular weight, highly polymerised polyphenols, include hydrolysable tannins and proanthocyanidins covalently conjugated to cellulose, protein and polysaccharides through ester bonds, and can be difficult to hydrolyse [21,22]. NEPP are normally indigestible by intestinal enzymes [23]. It is anticipated that NEPP exert their antioxidant properties after being fermented by the colon microflora into bio-accessible phenolic compounds, which might be then beneficial to gastrointestinal health [24]. Furthermore, it is reported that the NEPP could contribute 60% to 90% to the total polyphenol content (TPC), which emphasises the fact that the main biological activities such as antioxidant and antiproliferative activities attributed to polyphenols would reflect the contribution of this fraction of polyphenols, which is usually neglected, to the content of total polyphenols in foodstuffs [2].

Alkaline, acidic or enzymatic hydrolysis methods can be used to release bound phenolic compound. Such extraction methods were previously mentioned in a few studies [25,26]. Acid catalysed oxidative depolymerisation using HCl-butanol and a metal catalyst is commonly used to depolymerise polymeric proanthocyanidins [27]. For polyphenols, most extractions are carried out under acidic conditions because they are generally manifested in low pH, and the acidic condition helps polyphenols to scavenge free radicals more effectively. Moreover, polyphenols at low PH stay neutral, thus readily extracted into organic solvents. This is done using weak acid or low concentrations of a strong acid [28]. Therefore, one of the objectives of this study was to extract the NEPP using the HCl-butanol oxidative depolymerisation of DPK polyphenols from a commercially important date cultivar (Barhi). This date variety was selected mainly based on agronomical factors that favoured commercial cultivation of the dates [1]. In our lab, extractable polyphenols (EPP) from Barhi date palm kernels (BDPK) showed potent *in-vitro* inhibitive effect against two human cancer cell lines, namely; lung (A549) and colon (HT29) [12]. To our knowledge, no studies exist to date on the optimisation of extraction conditions for NEEP in BDPK and findings from the current study would be helpful for appropriate analysis and quantification of this group of phenolic compounds in BDPK with anticancer activity.

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An appropriate experimental design is necessary for any optimisation study, and the two most common designs are one-factor-at-a-time (OFAT) experiments and response surface methodology (RSM). OFAT experiments were used here to provide data regarding extraction factors with significant effects on phenolic antioxidants from BDPK. Next, these factors were analysed by RSM using face-centred central composite design (FCCCD) to more precisely determine optimal extraction conditions, that enables the simultaneous evaluation of the effects of selected ranges of independent variables and their interactions on response variables [29,30].

The extraction of polyphenols from plant materials is strongly influenced by many factors; extraction time, temperature and solvent-to-sample ratio among other factors [31,32]. Since the previous results [13] indicated that the EPP from BDPK were strong antioxidants and showed cytotoxic effects in selected human cancer cells, this research was carried out with the aim of optimising critical process parameters (extraction temperature, time and solvent/sample ratio) affecting the extraction of NEPP from BDPK using RSM in order to simultaneously maximise possible extraction of the TPC, TFC, TPAC with high DPPH• scavenging capacity and anticancer effects of NEPP extracts on A549 and HT29 human cancer cells.

2. Materials and Methods

2.1 Chemicals

Folin-Ciocalteu phenol reagent, gallic acid, ascorbic acid, catechin, aluminium chloride (AlCl3) and 2,2 diphenyl 1-picrylhydrazyl (DPPH), extraction solvents (methanol, butanol and ethanol), ferric chloride hexahydrate, and hydrochloric acid, sodium carbonate, were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI 1640 medium, Penicillin-streptomycin solution and phosphate buffer saline (PBS) were from Nacalai Tesque INC. (Kyoto, Japan). Methanol and ethanol were from Merck Co. (Darmstadt, Germany). 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and trypan blue were from Sigma (St. Louis, MO, USA). The human lung (A549) and human colon cancer cells (HT29) were obtained from American Type Tissue Collection (ATCC, Manassas, Va., USA.).

2.2. Extraction Procedure

2.2.1. Preparation of Extractable Polyphenols (EPP) Fraction.

Extraction of extractable polyphenols (EPP) was carried out under optimised extraction conditions (by extracting for 2.37 hours at 43.23°C in 75.39% methanol/ethanol-to-water concentration and 54.57 mL/g of solvent/sample ratios). In this paper, which is based on unpublished data [12] was carried out following the method described by [12]. The collected EPP residues were used to extract the corresponding non-extractable (NEPP) from BDPK.

2.2.2. Preparation of Non-Extractable Polyphenols (NEPP).

For the NEPP acid hydrolysis, the residue post-EPP extraction process under optimised conditions was collected and was used for the extraction of NEPP following the method described by Sirisena, *et al.* [33]. The extraction process was conducted in the absence of a metal catalyst. Sirisena and her colleagues compared the yield of NEPP extracted from DPK in the presence and the absence of Fe^{3+} ions, and the results were obtained using spectrophotometric scan at absorption wavelength range 500 to 550 nm [21,33]. The scan for extraction without added Fe^{3+} showed a significant increase in absorbance at λ550 nm indicating extraction of proanthocyanin. Based on these results, the NEPP extraction method was modified and performed without Fe^{3+} that could interfere with total flavonoid and antioxidant assays.

The applied analytical method (acid butanol assay) is based on the ability of monomer and condensed 3–4, flavandiols to oxidise in acid and alcoholic medium at high temperature to give

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coloured procyanidins. From the EPP residue, 2 g (dry weight) was mixed with 25-ml butanol/HCl (97.5:2.5, *v*/*v*) and heated at 100°C in a conical flask placed in the centre of the magnetic stirrer, with stirring at a constant rate (at speed 7) for 3 hours (h). After the HCl-butanol depolymerisation, the mixture was centrifuged at 7000 rpm for 15 min, the supernatant was collected, and the residue was subjected to two times washings with 10 mL butanol. Supernatants were combined and evaporated at 60°C to a small volume, and the pH of the concentrated extract was adjusted to 4 with 1 M sodium hydroxide (NaOH). In a previous study [33], it was found that PH less than 4 and above 5 caused precipitation of the extract; hence PH ~4 was selected to maintain both intact extract and the compatibility with buffers. Using an amber reagent bottle, the dried extracts were collected, weighed to calculate the percent yield of the crude extract, capped tightly, freeze-dried and stored at -20°C until analysis. All the experiments were carried out in triplicate.

2.3. One-Factor-at-a-Time Experiments

Many factors can affect the extraction of NEPP with acid hydrolysis treatment. Three factors were selected, namely; extraction temperature, time and solvent/sample ratio. Firstly, the influence of the extraction time (1, 2, 3, 4, 5h) on the NEPP content was determined under the following fixed conditions butanol/HCl (97.5:2.5, *v*/*v*), solvent/sample ratio (12.5:1, ml/g) and extraction temperature 100°C. Secondly, the impact of the liquid/solid ratio (5:1, 10:1, 15:1, 20:1, 25:1 ml/g) on the NEPP content under the following fixed conditions: butanol/HCl (97.5:2.5, *v*/*v*), extraction time 3 h, temperature 100°C. Finally, the influence of temperature (40, 60, 80, 100°C) on the NEPP yield under the following fixed conditions: butanol/HCl (97.5:2.5, *v*/*v*), solvent/sample ratio (20:1, ml/g), extraction time 3 h.

2.4. Chemical Analysis

2.4.1. Determination of Non-Extractable TPC, TFC, TPAC Contents

The non-extractable TPC was determined by Folin-Ciocalteu colorimetric method [34]. The TFC was measured spectrophotometrically by using the aluminium chloride colorimetric assay [18]. While, total proanthocyanidin content (TPAC) in BDPK extract was determined using vanillin-HCl assay described in the previous study [35].

2.4.2 DPPH Free Radical Scavenging Assay

The electron donating ability of the obtained NEPP extracts was measured by bleaching a purple solution of 1,1- diphenyl-2-picrylhydrazyl (DPPH) radical and was estimated according to the method described previously [36]. Briefly, a solution of DPPH was freshly prepared by dissolving 6 mg DPPH in 50 mL methanol to obtain a final concentration (0.3 mM). 2.5 mL of DPPH solution was mixed together with the methanol extract with varying concentrations (20–100 µg/mL) in a test tube and vibrated using vortex mixer for 20 seconds (s). Then, the samples and controls were incubated in the dark at room temperature. After 30 min of incubation, the absorbance of the samples and control was read at 517 nm against the blank. The absorbance was recorded, and the antioxidant activity was expressed by the percent inhibition of DPPH radicals and calculated using the following equation;

$$\%\text{inhibition of DPPH activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

$A_{control}$: the absorbance of control (DPPH + methanol solution only, without test sample).
A_{sample}: the absorbance of the test sample (DPPH solution mixed with test sample extract/standard).
All measurements were performed in triplicate, and graphs were plotted using the average of three determinations.

2.4.3 Anti-Proliferative Effect of NEPP Extracts Against Cancer Cells

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The potential antiproliferative/cytotoxic effect of non-extractable (NEPP) extracts against two human cancer cells lines (A549 and HT29) was tested using MTT assay. For screening, the cells were treated with the NEPP extracts at 0-1000 µg/ml concentration [13]. The IC_{50} (inhibiting 50 % of cell growth) values of NEPP extracts were measured, and their direct antiproliferative/cytotoxic effects were determined.

2.5 Experimental Design and Statistical Analysis

After determining the preliminary range of extraction variables through the single-factor test, a three-level-three-factor, FCCCD was employed in this optimisation study. Dependent variables (responses) measured were (TPC, mg GAE/g DW), (TFC, mg CE/g DW), (TPAC, mg CE/g DW), (DPPH SAC, IC_{50}, µg/mL), (yield, %), (A549 IC_{50}, µg/mL) and (HT29 IC_{50}, µg/mL) of NEPP extract from BDPK, and represented as responses R_1, R_2, R_3, R_4, R_5, R_6 and R_7 respectively, were optimised using RSM. The independent variables of extraction temperature (°C), extraction time (h) and solvent/sample ratio (*v*/*w*, mL/g) were selected as three important factors to optimise the extraction of NEPP, and represented as variables A, B and C, and used to obtain the coefficients of the quadratic model using the aid of the Design-Expert software (version 7.0.2.; Stat-Ease, Inc., Minneapolis, MN, USA). The range and levels of the independent variables used in the experimental design in this study are shown in Table 1.

Table 1. Independent variable values of the Barhi date palm kernels NEPP extraction process and their corresponding levels

Independent variable	symbols	Coded independent variable levels		
		-1	**0**	**+1**
Temperature (°C)	[A]	70	85	100
Time (h)	[B]	2	3	4
Solvent /sample ratio (*v*/*w*, mL/g)	[C]	1:15	1:20	1:25

h, hour.

A total of 17 experiments were carried out as unveiled by the results in the single-factor experiments with three centre points to improve the precision of the method. Predicted (pred.) and adjusted (adj.) correlation coefficient (R^2) was calculated to evaluate the quality of the fitted model, and its statistical significance was checked by F-test and *p*-value test. Analysis of variance (ANOVA) was performed to evaluate the effect of independent variables on the responses, and $p < 0.05$ was statistically significant. All the experiments were carried out at random to minimise the influence of the unexplained variability in the observed responses due to systematic errors. Three-dimensional surface graphs were drawn to display the experimental region and the effects of independent variables on the responses.

2.6 Verification of the Experimental Model

Optimal conditions for the extraction of non-extractable TPC, TFC, TPAC and extract yield from BDPK with minimum IC_{50} of antioxidant capacity and cytotoxicity to A549 and HT29 cancer cells were obtained using the FCCCD. The adequacy of the model equation for predicting the response values was verified by conducting the extractions under the recommended optimal points. In this study, a numerical optimisation method was adopted to find a point that maximises/minimises the response. A series of solutions were generated and the solution to be employed for the verification would be selected based on its desirability and suitability [37]. The experimental and predicted values of responses (dependent variables) were compared in order to determine the validity of the model. To confirm the results, sets of experiments were carried out in triplicate under the selected optimised conditions. The percentage of the residual standard error (%) was calculated for each response.

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2.7 Statistical Analysis

The data analyses were performed with Minitab 17 (Minitab Inc., State College, PA, USA) software to analyse the data by ANOVA. The mean values were considered significantly different when p value is less than 0.05. All experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation (SD). The IC_{50} values were calculated from linear regression analysis. A Pearson correlation test was used to evaluate the relationship between the antioxidant activities, crude extract yield, total phenol content, total flavonoid content, total proanthocyanidin content and the anticancer activity of the NEPP crude extract from BDPK. The statistical significance level for correlation analysis was set up at $p < 0.05$. Optimal extraction conditions were estimated through three-dimensional response surface analyses of the three independent variables and the seven evaluated responses (dependent variables) using Design-Expert 7.0.1 analysis software.

3. Results and Discussion

Here we report for the first time a database regarding the levels of cytotoxic and antioxidant activities of the polyphenols (NEPP) from BDPK. A calibration curve of gallic acid was constructed to measure the content of phenolic compounds in the NEPP from BDPK. The calibration equation for gallic acid (TPC) was y = 0.0075x + 0.017 (R^2 = 0.9899). The calibration equation for catechin (TFC) was y = 0.0076x + 0.0442 (R^2 = 0.9755). While the calibration equation for catechin (TPAC) was y = 0.0082x + 0.0197 (R^2 = 0.9775). The calibration curve of ascorbic acid was also measured to determine the scavenging potential of TPC, TFC and TPAC by DPPH. The calibration equation for ascorbic acid was y = 0.0105x - 0.0102 (R^2 = 0.9802). All results in the present study were calculated based on the above calibration curve and expressed as gallic acid equivalent (GAE) in mg per g dry weight (DW); catechin equivalent (CE) in mg per g dry weight (DW); and IC_{50}, µg/ml scavenging effect of extract by DPPH.

3.1. One-Factor-at-a-time Experiments for Extraction NEPP From Barhi Date Palm Kernels

The initial step of the preliminary experiment was to investigate whether extraction temperature, extraction time and solvent/sample could be optimised for extraction of phenolic, flavonoid, proanthocyanidin with high antioxidant and cytotoxicity properties using one-factor-at-a-time (OFAT) experiments, to determine appropriate experimental ranges for subsequent optimisation analyses. This was performed by changing one factor while keeping the other two factors constant.

3.1.1. Evaluation of Extraction Time:

Extraction time is an important parameter in optimising the recovery of phenolic compounds and antioxidant capacity. From literature, extraction time can be as small as few minutes or very long extending up to 24 hours depending on the phenolic compounds present in the plant material [30,38].

In the present study, extraction time played an important role in extraction of phenolic compounds (TPC, TFC and TPAC), crude extract yield, and their antioxidant/cytotoxic properties of BDPK NEPPs' extract. With 12.5:1 mL/g solvent/sample ratio, extraction times from 1 to 5 hours and an extraction temperature of 100°C were studied. As illustrated in Figure (1A, B, C), the extraction time affected TPC, TFC and TPAC significantly ($p < 0.05$).

Non-extractable TPC, TFC and TPAC yield from BDPK extract was enhanced (5.80±0.44, 2.63±0.39, 3.11±0.19 mg) with a longer extraction time, peaking (8.26±0.67, 3.34±0.16, 6.95±0.38 mg) at 3h, after which values decreased slightly ($p > 0.05$). A similar trend was observed in the percentage yield of NEPP crude extract as that of TPC, TFC and TPAC (Figure 1E). This effect may be explained that a prolonged exposure of the sample in the solvent, enhanced the solubility process by allowing sufficient time for solvent penetration into the plant tissue dissolving the solute and subsequently migrate to the extraction medium [32]. Prolonging extraction times may allow recovered phenolic

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compounds to oxidise due to prolonged exposure of polyphenols to temperature, light and oxygen [39,40].

DPPH radical is a widely accepted tool to evaluate the free radical scavenging ability of natural compounds (Nagai, Inoue, Inoue, & Suzuki, 2003). The antioxidant potential is inversely proportional to the IC_{50} value, which was calculated from the linear regression of the antioxidant activity versus the extract concentration. The maximum scavenging capacity of DPPH radicals of NEPP extract from BDPK occurred at 3h (IC_{50} = 61.82 ± 4.53 μg/mL). However, these yields (TPC, TFC, TPAC) and antioxidant capacity decreased significantly ($p < 0.05$) at 5h (7.02 ±0.40, 2.64 ± 0.27, 5.96 ± 0.68) and (IC_{50} = 64.33 ± 3.58 μg/mL), respectively (Figure 1A, B, C, D).

Our previous work [13] confirmed that the NEPP extracts of BDPK demonstrated selective cytotoxicity towards human lung (A549) and colon cancer (HT29) cell lines while being less cytotoxic against the normal cells. Such selective cytotoxic activity suggested that the "active substances interacted with special cancer-associated receptors or cancer cell special molecule, thus triggering some mechanisms that cause cancer cell death" [41].

Data in Figure 1F, G revealed similar antiproliferation property pattern on both cancer cells as of DPPH radical scavenging capacity, in which the inhibitory effect of NEPP on the cell growth improved significantly ($p < 0.05$) at 3 h; IC_{50} = 43.15 ± 7.79 μg/ml and IC_{50} = 47.32± 13.63 μg/ml, in both cancer cells A549 and HT29, respectively.

However, this effect started to decrease beyond 3h, but insignificantly (Figure 1F, G). Therefore, a longer extraction time had no effect on the extraction of phenolic compounds [42]. Furthermore, inordinately long extraction process might lead to oxidation of phenolic compounds owing to prolonged light or oxygen exposure, which might lead towards the decreased of free radical scavenging ability of NEEP extract from BDPK and accordingly decreased its antiproliferative effect against cancer cells. The decomposition in polyphenolic compounds is also manifested by a decline in antioxidant activity [40].

Thus, taking into account the economic point of view and also depending upon the quantification (yield) of phenolic compounds and antioxidant/cytotoxic activities of BDPK NEPP extract, 3 h was selected as the optimal extraction time because at this time period all responses showed the highest values. So, an extraction time of 2–4 h was chosen for RSM optimisation.

(A)

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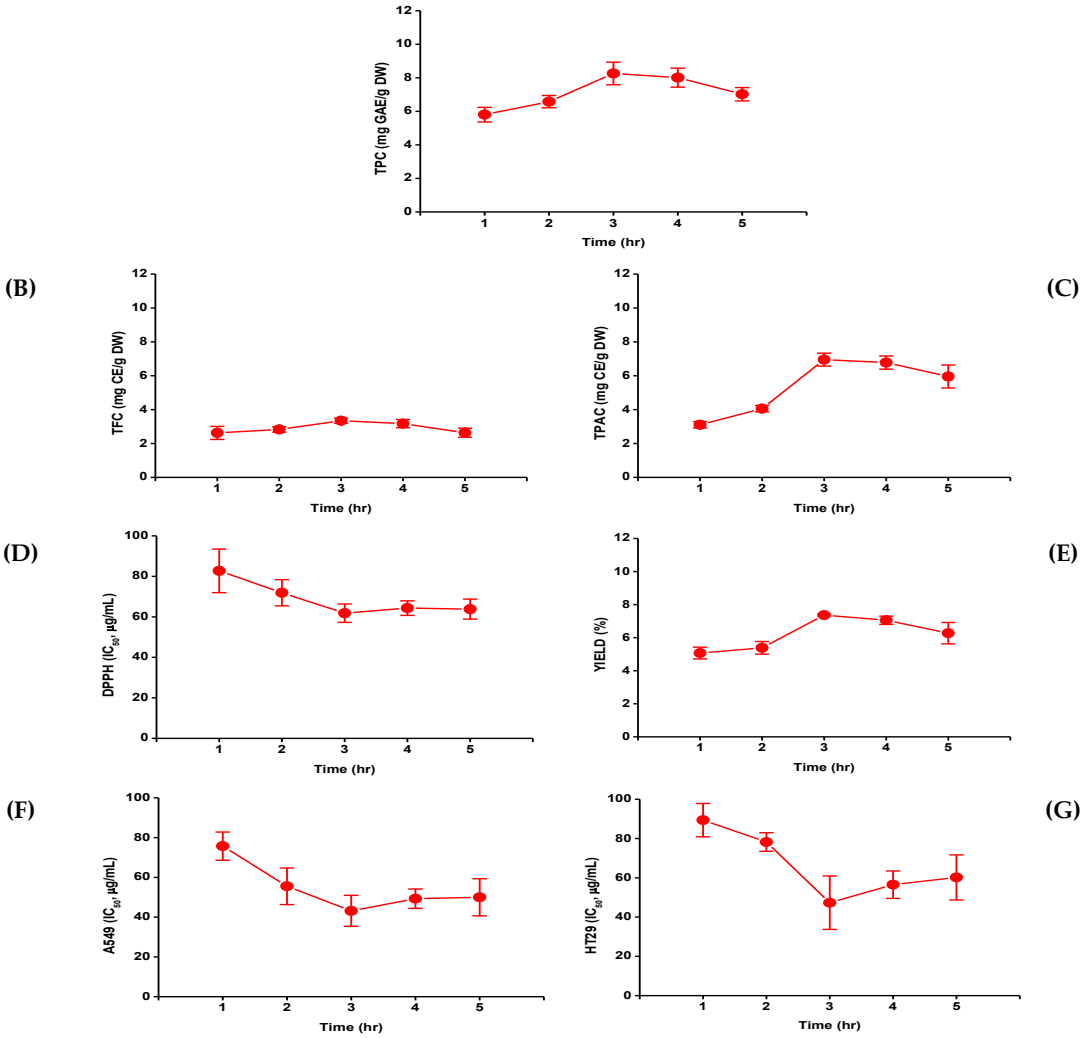

Figure 1. Effects of extraction time (h) on (A) TPC, (B) TFC, (C) TPAC, (D) DPPH scavenging capacity, (E) extracts yield and on the cytotoxicity of extracts in (F) A549 and (G) HT29 cells in non-extractable polyphenols extracts from Barhi date palm kernels. Extraction was conducted under these conditions; extraction temperature = 100°C and solvent/sample ratio = 12.5:1 mL/g.

3.1.2. Evaluation of Solvent/Sample Ratio:

Different solvent/sample ratio was needed for the maximum recovery of phenolic, flavonoids and proanthocyanidins compounds and antioxidant and antiproliferation activities against cancer cells. Figure 2 reveals that the total extraction yield (TPC, TFC, TPAC), crude extract yield, DPPH radical scavenging activity and antiproliferative capacity of NEPP significantly ($p < 0.05$) increased by the solvent/sample ratio until the ratio was 20:1 mL g^{-1}; after that, the NEPP yields and DPPH radical scavenging activity were almost unchanged ($p > 0.05$). A higher extraction solvent ratio can cause larger amounts of components from plant material to diffuse into solvent more effectively, bringing in a promoted extraction efficiency [43,44].

(A)

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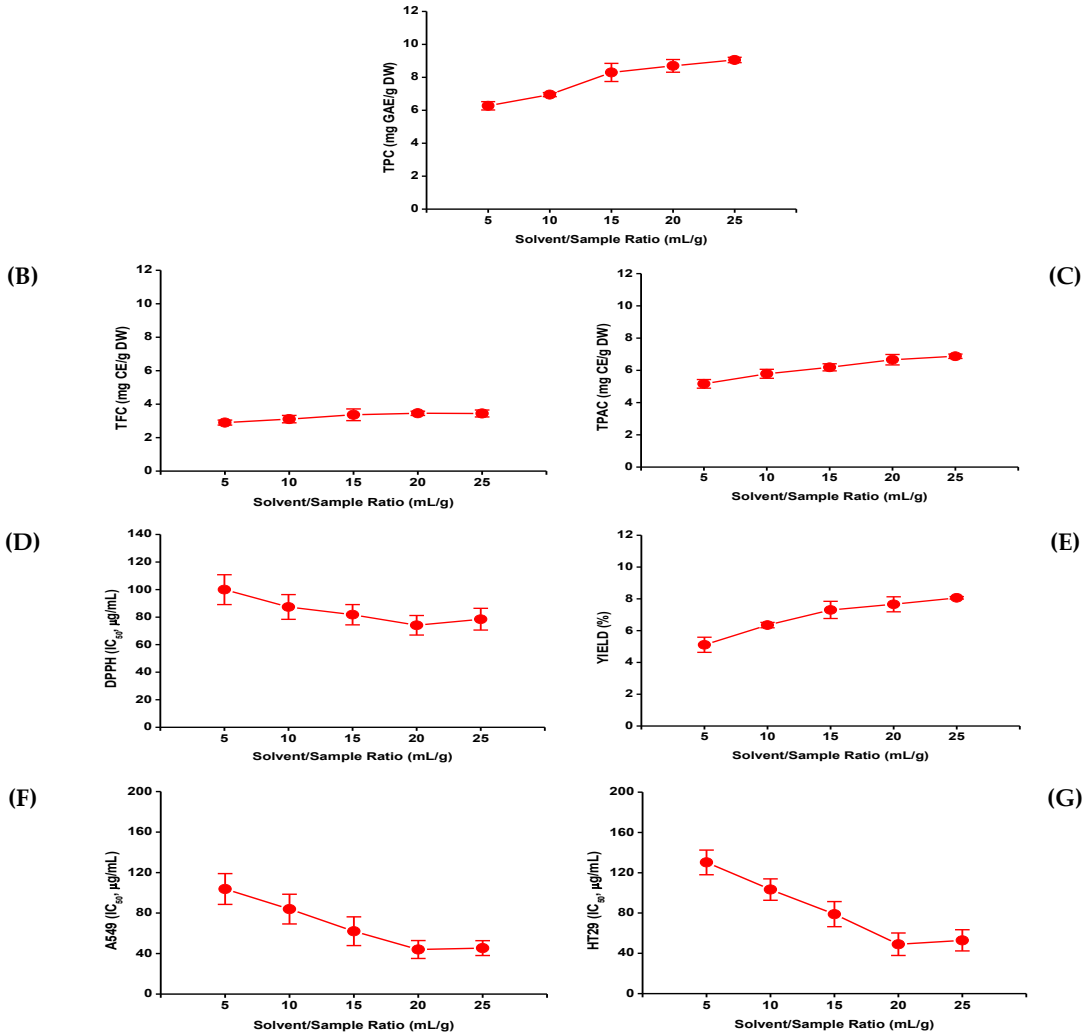

Figure 2. Effects of solvent/sample ratio (mL/g) on (A) TPC, (B) TFC, (C) TPAC, (D) DPPH scavenging capacity, (E) extracts yield and on the cytotoxicity of extracts in (F) A549 and (G) HT29 cells in non-extractable polyphenols extracts from Barhi date palm kernels. Extraction was conducted under these conditions; extraction temperature = 100°C and extraction time = 3h.

The anticancer capacity of NEPP from BDPK was evaluated against human lung (A549) and colon (HT29) cancer cell lines. Results presented in Figure 2F, G are expressed as concentration inhibiting fifty percent of cell growth (IC_{50}). Among the five NEPP extracts, extraction with 20:1 mL/g solvent-sample ratio exerted the most potent cytotoxic activity against human lung carcinoma A549 and colon cancer cells with IC_{50} values equal to 44.02 ± 8.854µg/ml and 49.03± 11.215µg/ml, respectively. However, these activities were less cytotoxic in lower or higher solvent ratio (Figure 2F, G). These data suggest that NEPP from BDPK reduces effectively human lung carcinoma and colon cancer cell viability. These results highlight for the first time the strong activity of BDPKs' NEPP against lung carcinoma (A549) and human colon cancer (HT29) cell lines.

Although, [45] showed that extractable polyphenols (EPP) from Ajwa date fruits were cytotoxic against other cancer cell lines such as hepatocellular carcinoma (HCT-116). Al-Sayyed, *et al.* [46] found that increasing consumption of dried date fruit reduced significantly the incidence rate of mammary cancer, palpable tumour multiplicity, tumour size and weight compared to the positive control group. In another study, Eid, *et al.* [47] studied the effect of the whole date fruit extract and its polyphenol-rich extract, which were prepared from methanol/water (4:1, *v/v*) containing 10% of 1 molar sodium fluoride (NaF) solution, on the Caco-2 cell lines. In the same study, they found that both extracts were able to inhibit Caco-2 cell growth, indicating that both were capable of probably

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acting as anti-proliferative agents *in-vitro* [47]. Based on the given results, 20:1 mL g^{-1} was ascertained as the solvent/sample ratio for RSM.

3.1.3. Evaluation of Extraction Temperature:

Heat can enhance the recovery of the phenolic compounds in some cases, as described by [7,42]. Generally, a high extraction temperature had a positive effect on the yield of phenolic compounds, but these increments are not consistent. Here, incubation temperatures for NEPP; TPC, TFC, TPAC recovery; crude extract yield, DPPH radical scavenging capacity and cytotoxicity were between 55–100°C (20:1 mL/g solve/sample ratio, 3 hours extraction time). A direct relationship was observed between the extraction temperature and TPC recovery, as shown in Figure 2A. With respect to TFC and TPAC recovery, DPPH radical-scavenging capacity, and NEPP crude extract yield, the extraction temperature was optimal at 85, 70, 85, and 85°C, respectively (Figure 2B, C, D, E).

Increased temperature led to enhance the extraction efficiency through increases of surface contact area and decreases viscosity and density of solvent media. These factors favoured the release of NEPP (bound phenolics) from plant material and weaken the cell wall integrity, thus, enhancing solubility and diffusion coefficients [48]. Increasing temperature may accelerate the transfer of NEPP and disrupt plant cellular constituents which may lead to increased cell membrane permeability, thus helped to release NEPP [49]. According to the equilibrium principle, the elevated temperature could improve the extraction rate and thereby reduce the extraction time required to reach the maximum recovery of phenolic compounds [50].

However, elevated temperatures may not be suitable for all phenolic compounds, especially those thermally sensitive, and thus easily oxidised as a function of temperature [39]. The TFC recovery was maximised at 85°C, while TPAC recovery was maximised at 70°C (Figure 3B, C). After these points, the recovery of TFC and TPAC were decreased, and this could be attributed to the decomposition of some thermally unstable flavonoids and proanthocyanidins [39]. Similar results were observed with respect to DPPH radical-scavenging capacity, which peaked at 85°C (Figure 3D). NEEP extract was able to reduce the blue DPPH radical solution into a yellow stable compound at IC_{50} =71.62 ± 9.31 μg/mL but then declined moderately ($p > 0.05$) with further increases in temperature (Figure 3D).

Phenolic compounds which are powerful antioxidants are known to have, in many cases, anti-proliferative activities against most cancer cell lines [51]. Several methods have been used to measure the antioxidant activities of EPP from date fruit (Amari and Hallawi) varieties and were reported to possess antioxidant activity comparable to vitamin C [52]. The earlier investigation reported that date fruits have the highest concentration of total polyphenols among the dried fruits due to the greater exposure to sunlight and extreme temperature for date fruits compared to other fruits [53].

Data in Figure 3F, G showed broadly similar pattern of antiproliferation activity of the tested NEPP extracts under various temperature ranges (55-100°C) against two human cancer (A549 and HT29) cell lines *in-vitro* and showed the highest cytotoxicity at 85°C (Figure 3F, G), therefore an optimal temperature of 85°C, was used for RSM optimisation.

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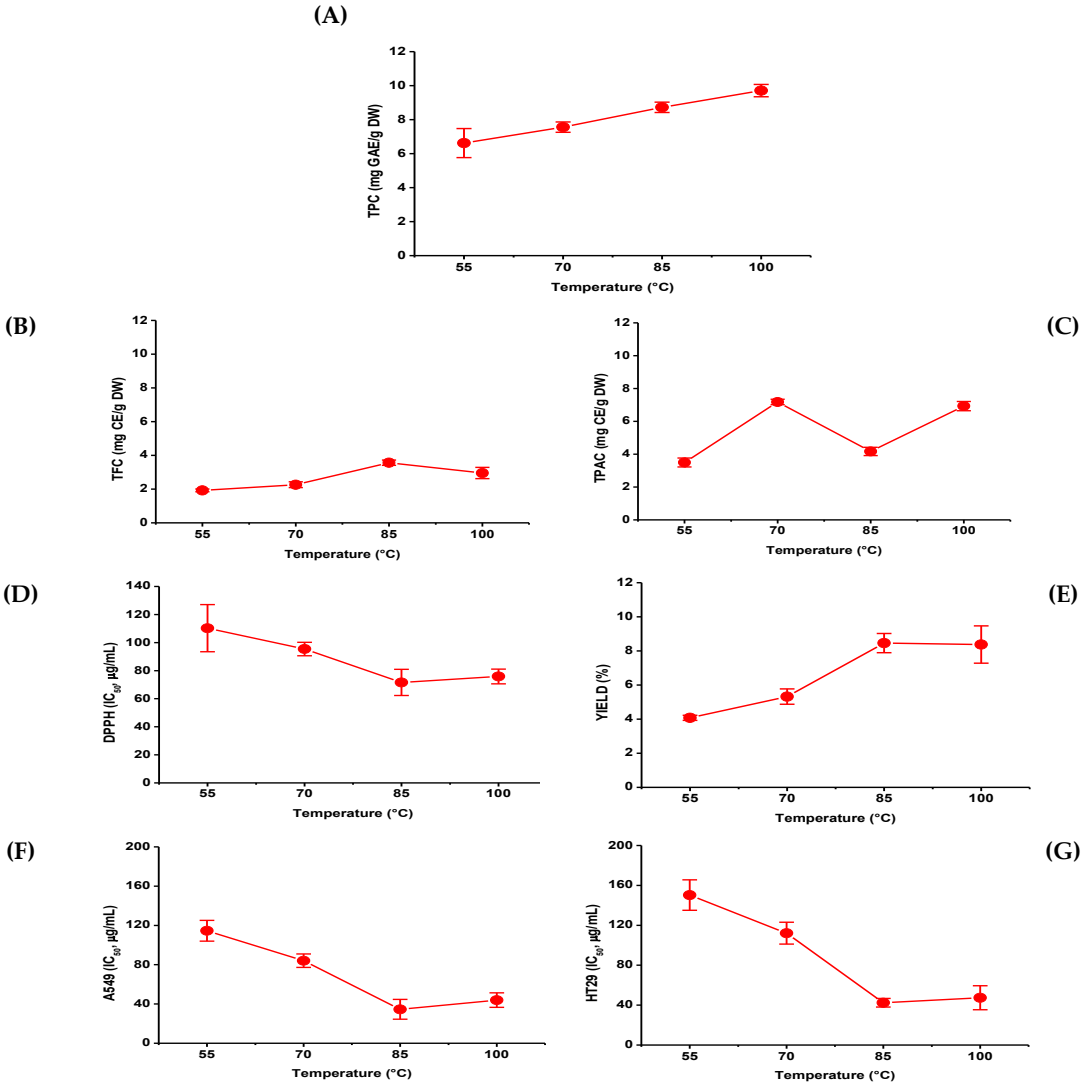

Figure 3. Effects of extraction temperature (°C) on (A) TPC, (B) TFC, (C) TPAC, (D) DPPH scavenging capacity, (E) extracts yield and on the cytotoxicity of extracts in (F) A549 and (G) HT29 cells in non-extractable polyphenols extracts from Barhi date palm kernels. Extraction was conducted under these conditions; extraction time = 3 h and solvent/sample ratio = 20:1 mL/g.

3.2. Fitting the Response Surface Models

This study aimed to determine the experimental conditions for the optimisation of TPC, TFC, and TPAC so as to maximise the bioactivity of the NEPP extract represented by the antioxidant capacity and antiproliferative property using response surface methodology. Based on the results of the one-factor-at-a time experiments (Figures; 1, 2 and 3), the levels of the three factors were determined; extraction temperatures (70°C, 85°C and 100°C), solvent-to-sample ratio (15:1 mL/g, 20:1 mL/g and 25:1 mL/g) and extraction time (2h, 3h, and 4h) were selected for the RSM experiment. The quadratic model from the FCCCD setup was used to generate a response surface image by identifying the relationship between each of the seven evaluation indices and process variables; extraction temperature (A), extraction time (B) and solvent/sample ratio (C), as well as to find out the conditions that optimised the extraction process for maximum value of TPC, TFC, TPAC, extract yield and minimum IC_{50} (µg/mL) of cytotoxicity and DPPH radical scavenging capacity of NEPP from BDPK. Hence, a quadratic model was selected and fitted well as suggested by the software for all three independent variables and the seven response variables. The experimental design and corresponding seven response variables are presented in Table 2.

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Table 2. Central composite design matrix and corresponding responses

Run	Independent variables			Responses (dependent variables)						
	A	B	C	R_1 (TPC)	R_2 (TFC)	R_3 (TPAC)	R_4 (DPPH)	R_5 (Y)	R_6 (A549)	R_7 (HT29)
1	70	4	25	10.66±0.3	3.75±0.12	7.14±0.34	70.2±4.65	8.71±0.93	21.3±3.26	33.43±5.54
2	100	4	25	10.61±0.07	3.76±0.03	7.24±0.25	69.22±6.97	9.57±1.04	20.14±4.6	33.22±4.16
3	85	3	20	10.83±0.78	4.12±0.06	8.45±0.93	59.3±5.44	13.2±1.3	18.58±2.77	31.71±3.75
4	70	4	15	10.75±0.52	3.64±0.01	7.11±0.98	70.5±9.66	10.7±0.84	21.37±1.54	33.56±6.89
5	85	3	25	10.79±0.36	4.05±0.03	8.25±1.2	58.5±6.41	13.09±1.22	19.14±2.3	32.56±6.44
6	85	3	15	10.77±0.11	4.03±0.12	8.23±1.07	58.7±1.55	13.4±2.03	19.22±1.09	32.6±3.32
7	100	2	15	10.67±0.9	3.73±0.05	7.33±0.85	70.1±4.62	7.95±0.54	22.49±2.87	34.25±2.76
8	70	2	15	10.59±0.47	3.63±0.03	7.11±0.4	70.2±8.83	8.62±0.66	21.33±0.98	33.23±6.39
9	70	2	25	10.69±0.25	3.62±0.22	7.22±0.72	64.21±9.6	7.13±0.26	22.06±3.22	33.68±7.51
10	70	3	20	10.72±0.66	3.77±0.06	7.58±0.45	64.14±1.16	11.5±0.99	19.95±3.15	32.37±4.99
11	85	3	20	10.83±0.08	4.18±0.05	8.74±1.52	58.12±3.32	14±0.64	18.5±4.3	31.65±2.6
12	100	3	20	10.75±0.1	3.83±0.17	7.89±0.9	64.15±7.42	11.3±1.35	20.46±2.74	33.19±3.37
13	85	4	20	10.79±0.36	4.02±0.09	8.17±0.74	59.4±6.08	13.5±1.43	18.08±2.11	31.6±4.57
14	85	3	20	10.8±0.55	4.17±0.28	8.78±0.66	58.7±7.32	14.2±2.6	17.84±0.9	31.48±5.5
15	100	4	15	10.68±0.45	3.71±0.04	7.4±0.31	63.39±5.5	9.2±1.2	20.72±3.04	33.95±2.96
16	85	2	20	10.78±0.52	4.05±0.14	8.15±0.94	59.12±2.29	11.5±1.73	19.33±2.48	32.26±5.03
17	100	2	25	10.79±0.76	3.68±0.05	7.38±0.95	69.09±5.22	8.43±0.22	22.54±5.21	34.43±2.76

IC_{50}, μg/ml was used to measure the capacity of non-extractable polyphenols (NEPP) from BDPK to neutralize 50% of DPPH free radicals. IC_{50}, μg/ml was also used to measure the ability of NEPP from BDPK to inhibit cell growth of A549 and HT29 by half (50%). Abbreviations: A, temperature; B, time; C, solvent/sample ratio; TPC, total phenolic content; TFC, total flavonoid content; TPAC, total proacnthocyanidin content; DPPH, (2,2-diphenyl-2-picrylhydrazyl) radical scavenging activity; and Y, yield.

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The statistical analysis of the quadratic models based on ANOVA is shown in Tables 3, 4, 5 and 6. The results indicated that the proposed models were significant ($p < 0.0001$). The coefficient of determination (R^2) were 0.9885, 0.9843, 0.9760, 0.9849, 0.9894, 0.9875 and 0.9887 for TPC, TFC, TPAC, yield, DPPH and cytotoxic effect on A549 and HT29, respectively, indicating that the model can predict ~99%, of the actual data for the responses. Whereas the adjusted coefficients of determination (Adj. R^2) were 0.9738, 0.9641, 0.9451, 0.9655, 0.9759, 0.9714, and 0.9742, respectively revealed the high degree of correlation between the empirical and predicted values. To ascertain the model further, the lack of fit showed statistically-insignificant ($p > 0.05$), indicating that all the established quadratic models were reliable and accurate for predicting the relevant responses.

Maximum recovery of TPC (10.83 ± 0.08mg GAE/g DW) and TFC (4.18 ± 0.05mg CE/g DW) was recorded during Run No. 11. Maximum extract yield of NEPP (14.2±2.6%) and TPAC (8.78±0.66mg CE/g DW) was recorded during Run No. 14. Minimum IC_{50} (µg/mL) of radical-scavenging capacity of DPPH was recorded at 58.12±3.32 µg/mL during Run No. 11. The minimum IC_{50} (µg/mL) of cytotoxicity of NEPP from BDPK on A549 and HT29 were recorded at 17.84±0.9µg/mL and 31.48±5.5 µg/mL during Run No. 5, respectively.

Multiple regression analysis was applied to the predicted data, and the software generated seven regression equations which demonstrated the predicted relationship between the responses and the three tested variables (Table 7), where R_1, R_2, R_3, R_4, R_5, R_6, R_7 and R_8 are the response values of TPC, TFC, TPAC, yield, DPPH and cytotoxicity on both A549 and HT29 cancer cells, respectively.

While A, B and C, are the coded values of the temperature, extraction time and solvent/sample ratio, respectively. A negative sign in each equation indicates an antagonistic effect of the variables, whereas a positive sign indicates synergistic effect of the independent variables [54].

For the extraction yield of TPC, TFC, TPAC, crude extract yield, DPPH radical scavenging activity and cytotoxicity, each response can be assigned a significant degree relative to the other responses. The results indicated that TPC (R_1), TFC (R_2) and TPAC (R_3) were significantly influenced at ($p < 0.05$) by temperature (A) and all quadratic terms (A^2, B^2, C^2) (Table 3, 4 and 5). Moreover, interactions of AB and BC were significant ($p < 0.001$) on TPC, while the three interactions of AB, AC and BC were not significant ($p > 0.05$) on TFC and TPAC.

DPPH (R_4) radical-scavenging capacity was not significantly influenced ($p > 0.05$) by all three linear terms (A, B, C), while all interaction parameters (AB, AC, BC) and quadratic parameters (A^2, B^2, D^2) were significant on (R_4) (Table 4). Extract yield was significantly affected at ($p < 0.05$) by linear (B, C), interaction parameter (AB) and all quadratic (A^2, B^2, C^2) parameters (Table 5). The inhibitory effect of BDPK extract on A549 cancer cells was highly significant influenced at ($p < 0.001$) by the linear (B), interaction parameter (AB) and quadratic parameters (A^2, B^2, D^2) (Table 6). Whereas the inhibitory effect on HT29 cancer cells was influenced significantly at ($p < 0.05$) by linear parameters (A and B). Additionally, two interactions of AB and BC were significant ($p < 0.01$) on the viability of HT29 cancer cells, while only two quadratic parameters (A^2 and C^2) had an effect ($p < 0.0001$) on the same cells (Table 6).

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Table 3. ANOVA results for responses R1 and R2 (TPC and TFC) in NEPP extract from BDPK.

Source	Sum of Squares	Df	Mean Square	F Value	p-value	Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	0.086	9	9.536E-003	67.08	< 0.0001	Model	0.64	9	0.071	48.81	< 0.0001
A-Temp	8.100E-004	1	8.100E-004	5.70	0.0484	A-Temp	9.000E-003	1	9.000E-003	6.14	0.0423
B-Time	9.000E-005	1	9.000E-005	0.63	0.4524	B-Time	2.890E-003	1	2.890E-003	1.97	0.2029
C-Solvent/Sample ratio	6.400E-004	1	6.400E-004	4.50	0.0715	C-solvent/sample ratio	1.440E-003	1	1.440E-003	0.98	0.3545
AB	0.011	1	0.011	79.13	< 0.0001	AB	8.000E-004	1	8.000E-004	0.55	0.4839
AC	2.000E-004	1	2.000E-004	1.41	0.2743	AC	1.250E-003	1	1.250E-003	0.85	0.3863
BC	0.018	1	0.018	126.96	< 0.0001	BC	6.050E-003	1	6.050E-003	4.13	0.0816
A^2	0.016	1	0.016	112.06	< 0.0001	A^2	0.25	1	0.25	171.33	< 0.0001
B^2	1.970E-003	1	1.970E-003	13.85	0.0074	B^2	0.014	1	0.014	9.23	0.0189
C^2	2.763E-003	1	2.763E-003	19.43	0.0031	C^2	0.012	1	0.012	7.98	0.0256
Residual	9.952E-004	7	1.422E-004			Residual	0.010	7	1.465E-003		
Lack of Fit	3.952E-004	5	7.904E-005	0.26	0.9006	Lack of Fit	8.187E-003	5	1.637E-003	1.58	0.4303
Pure Error	6.000E-004	2	3.000E-004			Pure Error	2.067E-003	2	1.033E-003		
Cor Total	0.087	16				Cor Total	0.65	16			

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R^2= 0.9885, adj. R^2= 0.9738 and pred. R^2= 0.9592 **(TPC)** R^2= 0.9843, adj. R^2= 0.9641 and pred. R^2= 0.9287 **(TFC)**adj., adjusted; pred., predicted; ANOVA, analysis of variance; TPC, total phenolic content; TFC, total flavonoid content; NEPP, non-extractable polyphenols.

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64 **Table 4.** ANOVA results for response R3 and R4 (TPAC and DPPH) of NEPP extract.
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Source	Sum of Squares	df	Mean Square	F Value	p-value	Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	5.42	9	0.60	31.61	< 0.0001	Model	391.96	9	43.55	50.70	< 0.0001
A-Temp	0.12	1	0.12	6.12	0.0426	A-Temp	1.09	1	1.09	1.27	0.2973
B-Time	1.690E-003	1	1.690E-003	0.089	0.7745	B-Time	1.000E-005	1	1.000E-005	1.164E-005	0.9974
C-solvent/sample ratio	2.500E-004	1	2.500E-004	0.013	0.9120	C-solvent/sample ratio	0.28	1	0.28	0.32	0.5866
AB	1.250E-005	1	1.250E-005	6.559E-004	0.9803	AB	20.70	1	20.70	24.10	0.0017
AC	7.813E-003	1	7.813E-003	0.41	0.5424	AC	15.43	1	15.43	17.96	0.0038
BC	0.011	1	0.011	0.55	0.4818	BC	19.63	1	19.63	22.85	0.0020
A^2	1.53	1	1.53	80.40	< 0.0001	A^2	128.52	1	128.52	149.62	< 0.0001
B^2	0.29	1	0.29	15.42	0.0057	B^2	11.16	1	11.16	12.99	0.0087
C^2	0.17	1	0.17	8.87	0.0206	C^2	5.11	1	5.11	5.95	0.0448
Residual	0.13	7	0.019			Residual	6.01	7	0.86		
Lack of Fit	0.069	5	0.014	0.42	0.8108	Lack of Fit	5.32	5	1.06	3.05	0.2648
Pure Error	0.065	2	0.032			Pure Error	0.70	2	0.35		
Cor Total	5.55	16				Cor Total	397.97	16			

66 R^2= 0.9760, adj. R^2= 0.9451, pred. R^2= 0.9222 (TPAC); R^2= 0.9849, adj. R^2= 0.9655, pred. R^2= 0.9341 (DPPH)

67 adj., adjusted; pred., predicted; ANOVA, analysis of variance; TPAC, total proanthocyanidin content; DPPH, (2,2-diphenyl-2-picrylhydrazyl) radical scavenging activity; IC50,

68 concentration of a substance/antioxidant required to inhibit DPPH radical by half (50%); NEPP, non-extractable polyphenols.

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Table 5. ANOVA results for response R5 (YIELD) of NEPP extract.

Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	87.23	9	9.69	72.85	< 0.0001
A-Temp	4.410E-003	1	4.410E-003	0.033	0.8607
B-Time	6.48	1	6.48	48.71	0.0002
C-solvent/sample ratio	0.86	1	0.86	6.50	0.0382
AB	0.20	1	0.20	1.52	0.2581
AC	2.34	1	2.34	17.62	0.0041
BC	0.047	1	0.047	0.35	0.5729
A^2	19.51	1	19.51	146.62	< 0.0001
B^2	6.84	1	6.84	51.44	0.0002
C^2	1.95	1	1.95	14.66	0.0065
Residual	0.93	7	0.13		
Lack of Fit	0.37	5	0.074	0.27	0.8996
Pure Error	0.56	2	0.28		
Cor Total	88.16	16			
Model	87.23	9	9.69	72.85	< 0.0001
A-Temp	4.410E-003	1	4.410E-003	0.033	0.8607
B-Time	6.48	1	6.48	48.71	0.0002
C-solvent/sample ratio	0.86	1	0.86	6.50	0.0382
AB	0.20	1	0.20	1.52	0.2581

R^2= 0.9894, adj. R^2= 0.9759and pred. R^2= 0.9622.

adj., adjusted; pred., predicted; ANOVA, analysis of variance; NEPP, non-extractable polyphenols.

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Table 6. ANOVA results for response R6 and R7 (IC50 concentration of NEPP on A549 and HT29 cancer cells).

Source	Sum of Squares	df	Mean Square	F Value	p-value	Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	36.39	9	4.04	61.39	< 0.0001	Model	14.30	9	1.59	68.18	< 0.0001
A-Temp	0.012	1	0.012	0.18	0.6878	A-Temp	0.77	1	0.77	32.92	0.0007
B-Time	3.77	1	3.77	57.24	0.0001	B-Time	0.44	1	0.44	18.74	0.0034
C-solvent/sample ratio	2.500E-004	1	2.500E-004	3.796E-003	0.9526	C-solvent/sample ratio	7.290E-003	1	7.290E-003	0.31	0.5934
AB	1.49	1	1.49	22.59	0.0021	AB	0.32	1	0.32	13.56	0.0078
AC	0.18	1	0.18	2.69	0.1451	AC	0.095	1	0.095	4.06	0.0838
BC	0.26	1	0.26	3.88	0.0895	BC	0.28	1	0.28	11.91	0.0107
A^2	9.72	1	9.72	147.63	< 0.0001	A^2	2.85	1	2.85	122.13	< 0.0001
B^2	0.44	1	0.44	6.67	0.0363	B^2	0.088	1	0.088	3.76	0.0938
C^2	2.08	1	2.08	31.51	0.0008	C^2	1.85	1	1.85	79.33	< 0.0001
Residual	0.46	7	0.066			Residual	0.16	7	0.023		
Lack of Fit	0.13	5	0.026	0.16	0.9568	Lack of Fit	0.13	5	0.027	1.89	0.3808
Pure Error	0.33	2	0.16			Pure Error	0.028	2	0.014		
Cor Total	36.85	1				Cor Total	14.47	1			
		6						6			
Model	36.39	9	4.04	61.39	< 0.0001	Model	14.30	9	1.59	68.18	< 0.0001
A-Temp	0.012	1	0.012	0.18	0.6878	A-Temp	0.77	1	0.77	32.92	0.0007
B-Time	3.77	1	3.77	57.24	0.0001	B-Time	0.44	1	0.44	18.74	0.0034
C-solvent/sample ratio	2.500E-004	1	2.500E-004	3.796E-003	0.9526	C-solvent/sample ratio	7.290E-003	1	7.290E-003	0.31	0.5934
AB	1.49	1	1.49	22.59	0.0021	AB	0.32	1	0.32	13.56	0.0078

R^2= 0.9875, adj. R^2= 0.9714 pred. R^2= 0.9560 **(A549)**; R^2= 0.9887, adj. R^2= 0.9742 pred. R^2= 0.9184 **(HT29);** adj., adjusted; pred., predicted; ANOVA, analysis of variance; IC_{50}, concentration of a substance/treatment required to inhibit cell growth by half (50%); NEPP, non-extractable polyphenols.

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Table 7. Quadratic equations for the seven responses in terms of coded factors.

	Responses	Equations	
(R_1)	TPC	+10.82 +9.000E-003*A - 3.000E-003*B + 8.000E-003* C -0.037*A* B + 5.000E-003*A*C - 0.047*B*C -0.077*A^2 - 0.027* B^2 - 0.032* C^2	(1)
(R_2)	TFC	+4.13 + 0.030*A + 0.017* B + 0.012*C - 0.010 *A*B - 0.013*A*C+ 0.027*B*C - 0.31 *A^2 - 0.071 *B^2 - 0.066* C^2	(2
(R_3)	TPAC	+8.56 + 0.11*A - 0.013*B + 5.000E - 003*C + 1.250E - 003*A*B - 0.031*A*C - 0.036*B*C - 0.76*A^2 - 0.33*B^2 - 0.25*C^2	(3)
(R_4)	DPPH SAC (IC_{50})	+57.86 - 0.33*A - 1.000E-003*B - 0.17*C - 1.61*A*B + 1.39*A*C + 1.57*B*C + 6.93 *A^2 + 2.04 *B^2 + 1.38*C^2	(4)
(R_5)	Extraction Yield	+13.97 - 0.021*A + 0.80*B - 0.29*C - 0.16*A*B + 0.54*A*C - 0.076*B*C - 2.70*A^2 - 1.60*B^2 - 0.85*C^2	(5)
(R_6)	A549 (IC_{50})	+18.30 + 0.034*A - 0.61*B + 5.000E - 003*C - 0.43*A*B - 0.15*A*C - 0.18*B*C + 1.91*A^2 + 0.41 *B^2 +0.88*C^2	(6)
(R_7)	HT29 (IC_{50})	+31.69 + 0.28*A - 0.21*B - 0.027*C - 0.20*A*B - 0.11*A*C - 0.19*B*C + 1.03*A^2 + 0.18* B^2 + 0.83* C^2	(7)

5 In these equations, *R* is the predicted response, A, B and C are the values of the independent variables, extraction temperature (°C), extraction time (h)
6 and solvent/sample ratio (mL/g), respectively.

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3.3. Optimisation by RSM

RSM plays a key role in efficiently identification of the optimum values of the independent variables. Three-dimensional response surface plots were proposed to depict the individual or interactive effects of these three selective parameters on the response variables, namely; extraction yield of NEPP (TPC, TFC, TPAC), crude extract yield, and its DPPH radical scavenging activity and its antiproliferative effect on two human cancer cells; A549 and HT29, respectively (Figure 4, 5, 6, 7, 8, 9, 10).

In the response surface plot and contour plot, the extraction yield of NEPP was obtained along with two continuous independent variables with their coded terms obtained from RSM, while the other independent variable was fixed constant at its zero level (the centre value of the test ranges). The centre value of the test ranges, namely; (3h, extraction time), (20:1 mL/g, solvent-to-sample ratio), (85°C, extraction temperature), and were chosen from previous single-factor experiments, and were selected for the RSM experiment.

To determine the optimum extraction conditions for NEPP; TPC, TFC, TPAC; percentage yield of crude extract; antioxidant and cytotoxic recovery from BDPK using acid hydrolysis method, the extraction process was conducted at different extraction time, temperature, and liquid/ solid ratio. The response surface plots predicting the specific surface area of NEPP extraction by the acid hydrolysis versus levels of the independent variables are presented in Table 2. The effect of investigated extraction parameters on each evaluated response was expressed as significant ($p < 0.05$) or insignificant ($p > 0.05$) according to P values for the regression coefficients in the quadratic model (Table 3, 4, 5, 6).

3.3.1. Response Surface Analysis of Total Phenolic Content

The analysis variance of total phenolic content (TPC) is noticed to be significantly ($p < 0.05$) affected by the linear and quadratic effects of the extraction temperature. It was found that the linear term of extraction temperature had a positive linear effect on TPC while showing a negative quadratic effect, contributing to a saddled shape. TPC increased when temperature increased to reach an optimum of 87°C. the beneficial effect of temperature on the extraction efficiency of phenolic compounds was reported in many studies [49,55,56]. It ameliorates the mass transfer, weakens the cell wall integrity, hydrolyses the bonds of bound phenolic compounds (phenol-protein or phenol polysaccharide), improves the solubilisation of the solutes in the solvent and reduces the surface tension and viscosity, thus more phenolics would distribute to the solvent [57,58].

Nevertheless, and beyond a certain value, some antioxidants like some phenolic compounds can be denatured by chemical reactions [59]. This limit temperature is different amongst extraction studies; it is 73.9°C° for some [26] or up to 63 °C [60], and 94 °C for others [55]. Regarding the duration of the extraction process, short [61,62] and long extraction periods can be proposed in the literature [26,63]. The present study showed a negative quadratic effect of time on the TPC as shown in Table 7, equation (1), which indicates that TPC increased with increasing extraction time to a certain level (approximately 2.7 h). However, further increase in extraction time shows decrease in TPC. The obtained results in this study were contrary to that obtained by El Hajj, *et al.* [64] who found that the extraction of total phenolics from cabernet sauvignon grapes (*Vitisvinifera* L.) increased with the increase of time. This contradiction is probably due to the working high temperatures employed in this study, which required short periods of time to avoid the possible polyphenols degradation.

The results in this study are similar to another study performed on grape by-products, and showed that the increase in the extraction temperature leaded to an increase in total phenolics from grape by-products and reduction of time [55]. In the current study, the interaction between the extraction time and extraction temperature had a highly significant ($p < 0.0001$) effect on TPC (Figure 4A). In other terms, extraction temperature influenced TPC synergistically with the extraction time. Similarly, there was an interaction between extraction time and sample/solvent ratio ($p < 0.0001$), which indicates that there was a synergistic interaction between extraction time and sample/solvent ratio on the yield of TPC (Figure 4C). However, the interaction between extraction temperature and

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sample/solvent ratio was not significant ($p > 0.05$) indicating that temperature worked independently of the sample/solvent ratio (Table 3 and Figure 4B).

3.3.2. Response Surface Analysis of Total Flavonoid and Proanthocyanidin Contents

Flavonoids and proanthocyanidins are the most abundant polyphenols in human diets. Experimental results of TFC and TPAC in Barhi date palm kernels extracts are presented in Table 2. TFC was between 3.62±0.22 mg catechin (CE) per g dry weight (DW), obtained with 70°C temperature, 2 h time and 25:1 (mL/g) solvent/sample ratio, and 4.18±0.05 mg CE per g DW, obtained with 85°C, 3 h and 20:1 mL/g sample/solvent ratio.

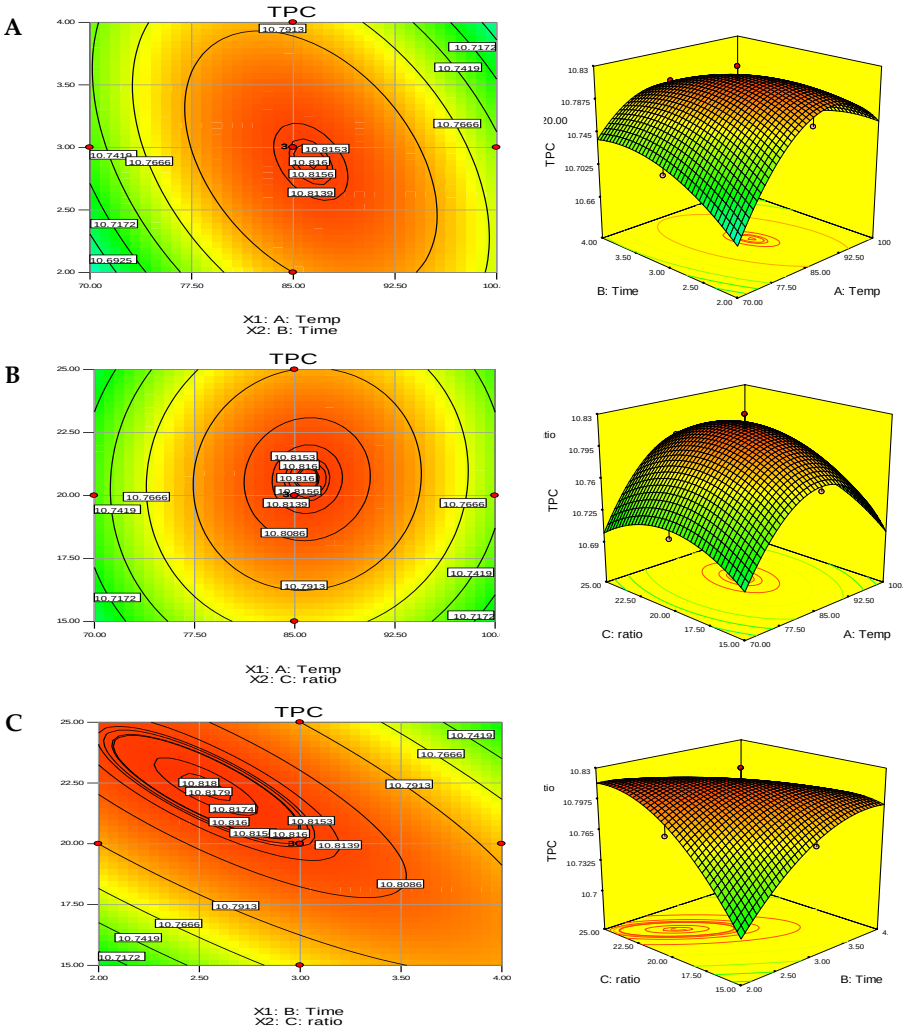

Figure 4. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the non-extractable total phenolic content (TPC, mg GAE/g DW) of BDPK.

Lower TPAC yields (7.11±0.4 mg CE per g DW) were obtained using 70°C, 2 h and 15:1 mL/g, while the highest TPAC yields (8.78±0.66 mg CE per g DW) were obtained with 85°C, 3 h and 20:1 mL/g sample/solvent ratio as was the case with TFC (Table 2).

There was a good correlation (r =0.748) and (r =0.807) respectively, observed between experimentally obtained TFC and TPC, and strong correlation (r =0.972) observed between TFC and TPAC suggesting that similar extraction parameters provide good extraction of both groups of compounds (Table 8). Moreover, the good negative correlation was observed between TPC, TFC, TPAC and (IC_{50}) of DPPH radical scavenging activity (0.735, 0.874, 0.907, respectively), which means

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that IC_{50} decrease, i.e. antioxidant activity increases, with increasing TPC, TFC and TPAC. This suggests that NEPP play an important role in the antioxidant activity of BDPK.

It can be seen that only the linear term of extraction temperature and the quadratic terms of extraction temperature, time and sample/solvent ratio had a significant influence ($p < 0.05$) on TFC, while all other effects were insignificant ($p > 0.05$). The same situation was observed for TPAC extractions, as confirmed by a strong correlation (r = 0.972) (Table 8).

Table 8. Correlation coefficients between the antioxidant and cytotoxicity capacity of NEPP extract from BDPK and the total content of phenolic, flavonoid, proanthocyanidin, and extract yield.

Factor	Responses						
	TPC	TFC	TPAC	DPPH	YIELD	A549 (IC_{50}, µg/mL)	HT29 (IC_{50}, µg/mL)
TPC	1.000	0.748*	0.807***	-0.735*	0.752*	-0.594*	-0.585*
TFC		1.000	0.972***	-0.874***	0.901***	-0.902***	-0.866***
TPAC			1.000	-0.907***	0.903***	-0.874***	-0.842***
DPPH				1.000	-0.816***	0.964***	0.789***
YIELD					1.000	-0.933***	-0.888***
A549						1.000	–
HT29							1.000

TPC, , total phenolic content; TFC, total flavonoid content; TPAC, total proanthocyanidin content; DPPH, (2,2-diphenyl-2-picrylhydrazyl) radical scavenging activity; IC_{50}, concentration of a substance/antioxidant required to inhibit DPPH radical by half (50%); NEPP, non-extractable polyphenols. $^{*}p < 0.01$, $^{***}p < 0.0001$.

The linear term of extraction temperature exhibited a positive effect on TFC and TPAC, which means that high temperature, is necessary for complete flavonoid and proanthocyanidin extractions (Figure 5, 6). The negative effect of the quadratic terms of temperature, time and solvent/sample ratio suggested that TFC and TPAC reaches a maximum near lower level of extraction parameters and then starts to decrease rapidly with more heating, prolonged time, increasing solvent ratio with a saddle point between lower and middle levels of these parameters.

3.3.3. Response Surface Analysis of Crude Extract Yield

The results in this study are similar to another study performed on grape by-products, and showed that the total phenolics from grape by-products increased with the increment of temperature and reduction of time [55]. In the current study, the interaction between the extraction time and extraction temperature had a highly significant ($p < 0.0001$) effect on TPC (Figure 4A). In other terms, extraction temperature influenced TPC synergistically with the extraction time. Similarly, there was an interaction between extraction time and sample/solvent ratio ($p < 0.0001$), which indicates that there was a synergistic interaction between extraction time and sample/solvent ratio on the yield of TPC (Figure 4C). However, the interaction between extraction temperature and sample/solvent ratio was not significant ($p > 0.05$) indicating that temperature worked independently of the sample/solvent ratio (Table 3 and Figure 4B).

The crude extract (NEPP) was weighted to determine the crude extract yield. The extraction yield (%, *w*/*w*) from each condition was estimated by the ratio of the weight of dry matter of BDPK after being extracted by acid hydrolysis to the weight of total dry matters before extraction.

Various factors showed significant effects on the NEPP extraction yield. Based on the experimental data illustrated in Table 5, total extracted yield ranged from 7.13±0.26 % (70°C, 2h, 25:1 mL/g) to 14.2±2.6 % (85°C, 3h, 20:1 mL/g) (Table 5). The effect of different independent variables on

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the yield was described in equation 5, Table 7. Based on the ANOVA test, the most significant factor ($p < 0.001$, $F = 48.71$) affecting the obtained yield was the positive effect of time in its linear term (Table 7, equation 5). It is concluded that longer extraction time had positive effects on the yield of extraction.

There were also significant ($p < 0.01$) positive interaction effects of extraction time and solvent to sample ratio (equation 5, Table 7), indicating that yield extraction increases considerably with an increase in a solvent to sample ratio and extraction time (Figure 7B). Table 7 and equation 5, demonstrated negative quadratic effects of temperature, time and solvent to sample ratio on extract yield.

These observations indicate that the extraction yield increases when the independent variables are in their middle values (Figure 7A, B, C), however, a further increase in these variables led to the decline of crude extract yield from BDPK. The linear negative effect of solvent to sample ratio was significant ($p < 0.05$), indicating that increasing the solvent to sample ratio favours extraction of NEPP only up to a certain value (20:1 mL/g). At higher solvent to sample ratio, the crude extract yield of NEPP decreased. Also, a high correlation between NEEP crude extract yield and TPC, TFC, TPAC and antioxidant and cytotoxic activities of the extracts in the DPPH and MTT assays were found. The Pearson's correlation coefficients (r) of extract yield with TPC, TFC, TPAC were 0.752, 0.901 and 0.903, respectively, and the coefficients (r) of extract yield with IC_{50} for DPPH and MTT for A549 and HT29 were -0.816, -0.933 and -0.888, respectively. These good correlations observed between experimentally obtained TPC, TFC, TPAC and NEPP crude extract yield, suggesting that similar extraction parameters provide good extraction of these groups of compounds (Table 8). Moreover, the good negative correlation was observed between crude extract yield and antioxidant and cytotoxic activities parameters (IC_{50}), which means that as IC_{50} decrease as antioxidant and cytotoxic activities increases, with increasing the yield of NEPP.

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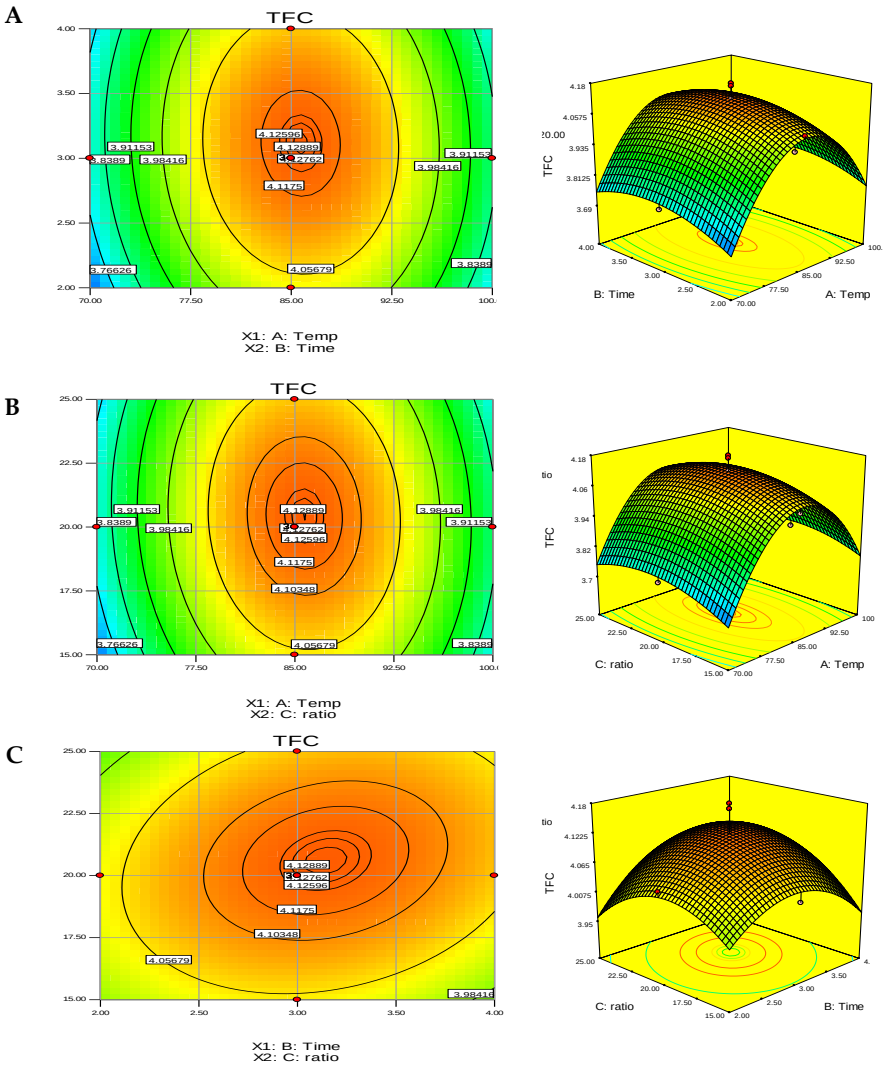

Figure 5. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the non-extractable total flavonoid content (TFC, mg CE/g DW) of BDPK.

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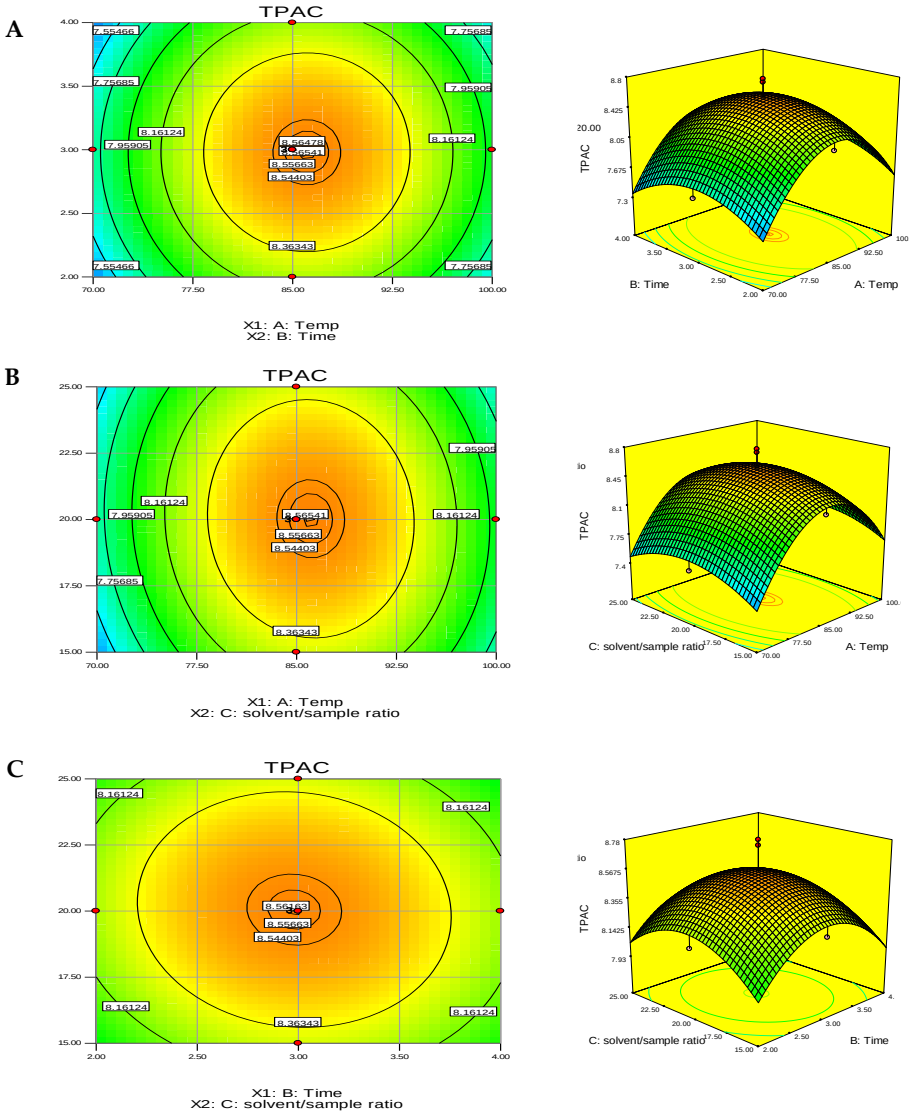

Figure 6. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the non-extractable total proanthocyanidin content (TPAC, mg CE/g DW) of BDPK.

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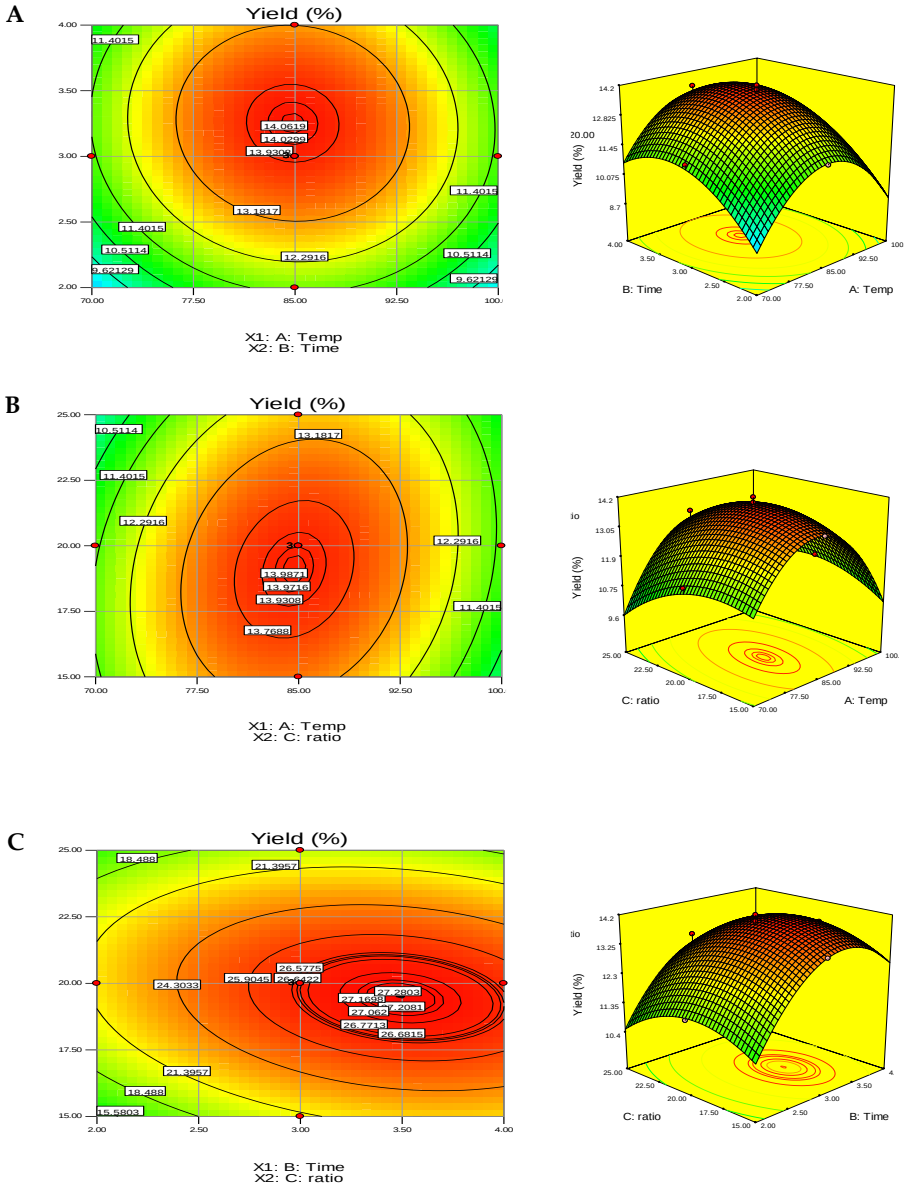

Figure 7. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the non-extractable polyphenols crude extract (%) of BDPK.

3.3.4. Response Surface Analysis of DPPH Radical Scavenging Activity

Current research on dietary antioxidants misses the so-called non-extractable polyphenols (NEPP), which are not significantly released from the food matrix either by mastication, acid pH in the stomach or action of digestive enzymes, reaching the colon nearly intact. NEPP, not detected by the usual analytical procedures, are made up of macromolecules and single phenolic compounds associated with macromolecules. Therefore, NEPP is not included in food and dietary intake data nor in bioavailability, intervention or observational studies. DPPH radical scavenging capacity of BDPK's NEPP extracts was evaluated by DPPH assay, which is widely used in evaluating the antioxidant activity of plant polyphenols *in-vitro* [65]. IC_{50}, as a reciprocal measure of DPPH radicals scavenging capacity of BDPK extracts, ranged between 58.12±3.32 and 70.5±9.66 µg/mL. The lowest IC_{50}, i.e. highest DPPH radical scavenging capacity of NEPP, was found to be a function of the positive quadratic effect ($p < 0.05$) of all independent variables (temperature, time, solvent/sample

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ratio). The positive effect of the quadratic terms of all parameters means that antioxidant activity will decrease significantly at a higher level of these variables.

Furthermore, none of the linear terms of independent variables had a significant effect ($p > 0.05$) on DPPH. On the other hand, the interaction between extraction temperature and time had a significant negative effect ($p < 0.05$) on IC_{50} suggesting that higher antioxidant activity will be preserved when temperature and time (85°C and 3 h, respectively) are in their middle levels (Figure 7A, B). Moreover, the interaction between extraction temperature and solvent/sample ratio and the interaction between time and solvent/sample ratio had a significant positive effect ($p < 0.05$) on IC_{50}, suggesting that DPPH radical scavenging activity will be decreased at a higher level of these variables (Figure 8A, B). There was a significant ($p < 0.01$) negative correlation between DPPH and NEPP (TPC, TFC, and TPAC) (Table 8). To the best of our knowledge, no other study has been conducted on the DPPH radical scavenging activity of NEPP in date palm kernels using acidic hydrolysis.

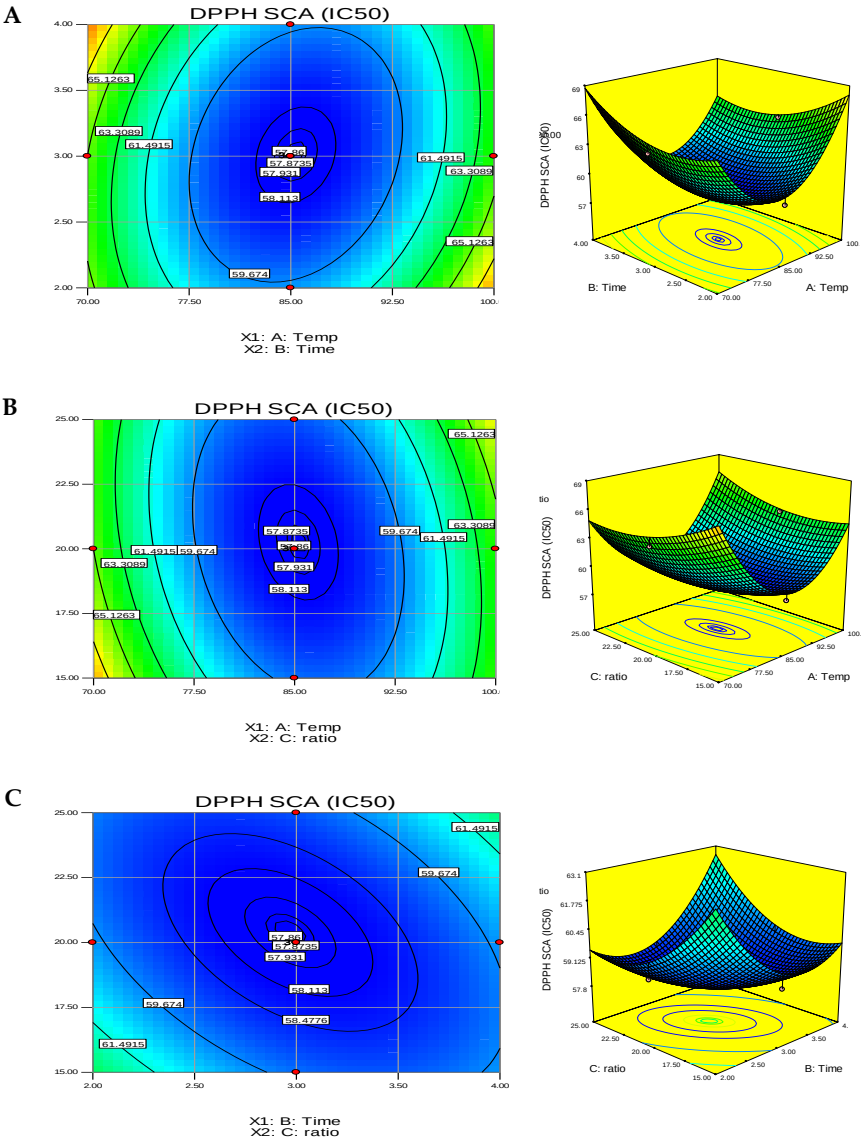

Figure 8. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the DPPH radical scavenging effect of NEPP from BDPK.

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3.3.5. Response Surface Analysis of Cytotoxic Activity

MTT assay was used to assess the cytotoxic properties of the NEPP extracts, which provides a simple method for determination of the cell's viability based on the activity of mitochondria in living cells. The NEPP extracts showed a selective antiproliferative effect against the two human cancer cell lines, A549 and HT29. Interestingly, the normal cell lines, 3T3 showed good resistance with median inhibitory concentration (IC_{50}) values of higher than 100 µg/mL of NEPP extracts of BDPK [13]. The IC_{50} values for the active extracts and the respective standard reference drug (paclitaxel) were calculated for the two tested cell lines after 24 and 72 h of incubations [13].

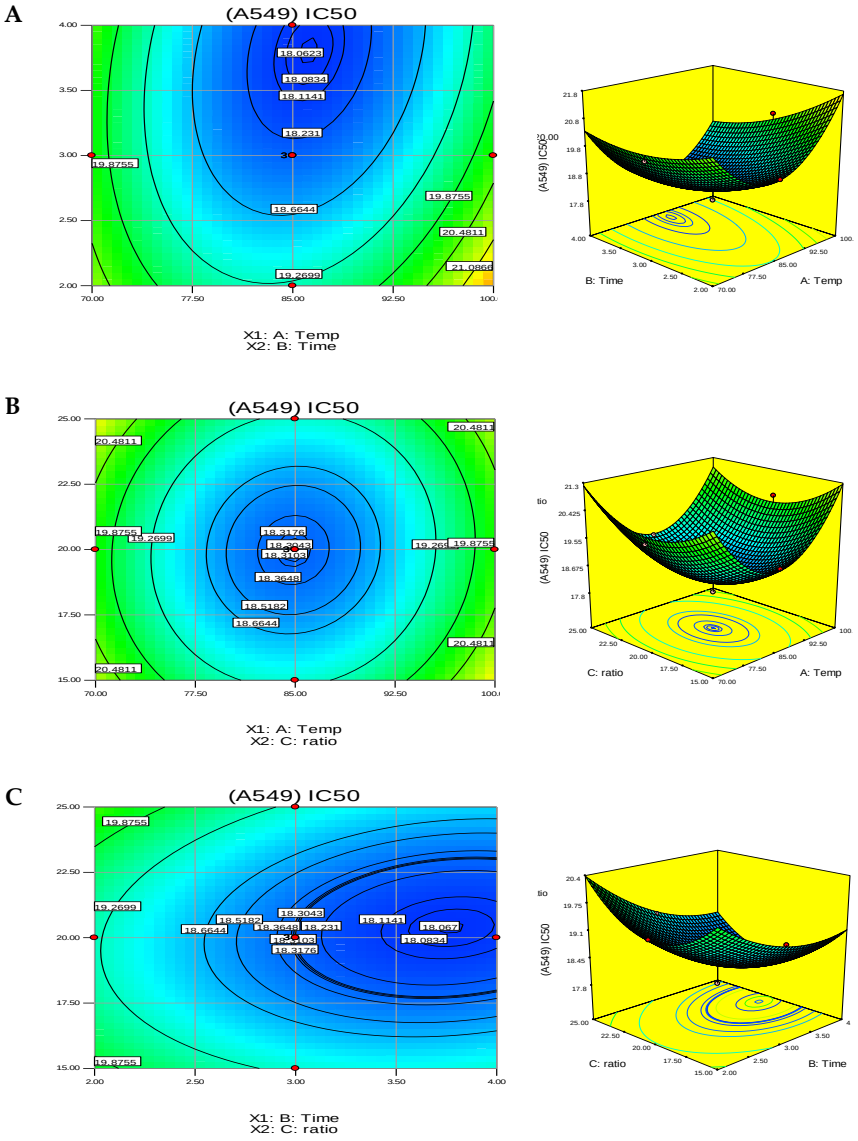

Figure 9. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the cytotoxic effect of NEPP from BDPK in human lung (A549) cancer cells.

Experimental results of cytotoxic effects of NEPP in BDPK on cancer cells are presented in Table 6. The antiproliferative/cytotoxic effect, which is represented by IC_{50} ranged between 17.84 ± 0.9 and 22.54 ± 5.21 µg/mL in A549, and between 31.48 ± 5.5 and 34.43 ± 2.76 µg/mL in HT29. The lowest IC_{50} (17.84 ± 0.9 µg/mL and 31.48 ± 5.5 µg/mL), i.e. highest cytotoxic activity, of NEPP on A549 and HT29, respectively was recorded under experimental parameters of 85°C temperature, 3h time and solvent/sample ratio of 20:1 mL/g. There is a close agreement between the observed values of IC_{50} in

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A549 and HT29 and the theoretical values predicted by the design (Figure 11). The model predicted a minimum IC_{50} (highest cytotoxic effect) of NEPP on A549 and HT29 (18.30 and 31.69, respectively) under the same factor levels combination (85°C temperature, 3 h time and 20:1 mL/g solvent/sample ratio) as obtained from the empirical experiments (Figure 11). The good correlation between these results confirmed that the design was adequate to reflect the expected optimisation. The predictive quadratic model equations (6 and 7) for extraction conditions with IC_{50} as target response of the antiproliferative effect of NEPP on A549 and HT29 are illustrated in Table 7.

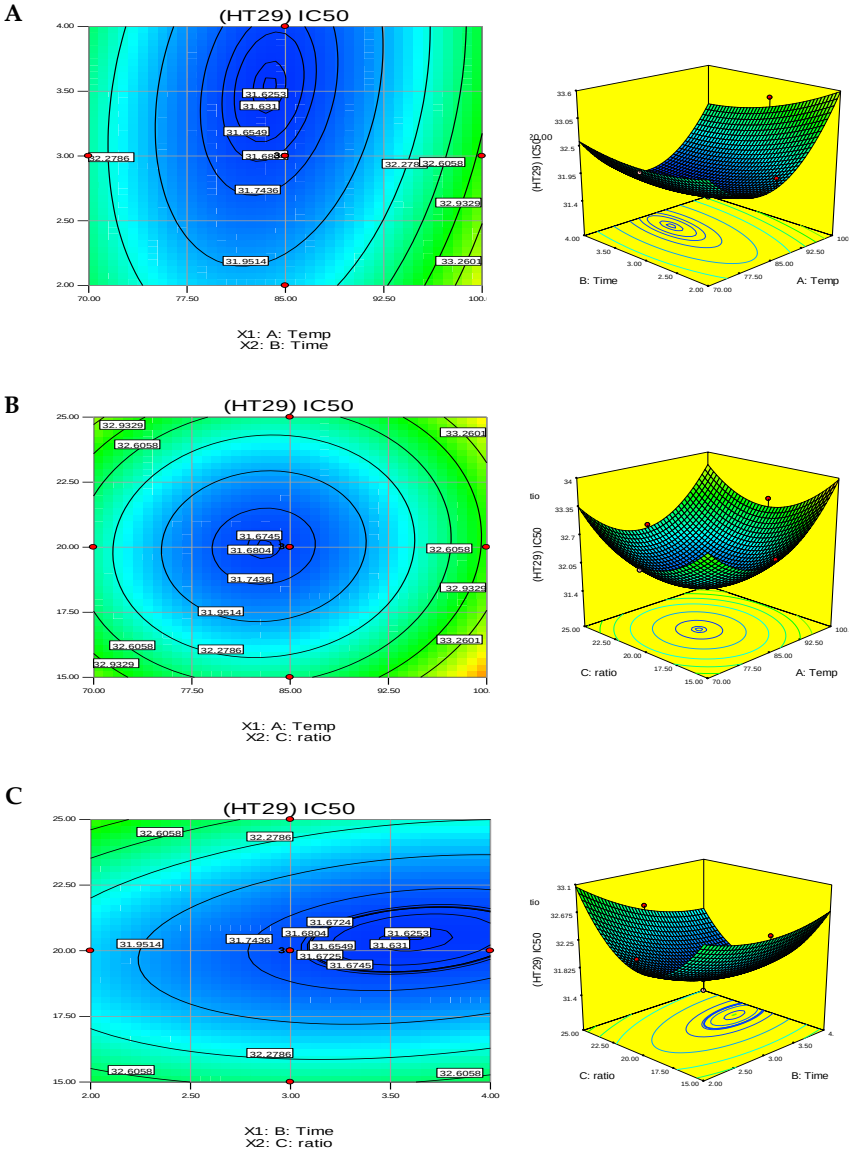

Figure 10. Response surface plots and contour plots for the effect of temperature and time (A); temperature and solvent/sample ratio (B); time and solvent/sample ratio (C) on the cytotoxic effect of NEPP from BDPK in human colon (HT29) cancer cells.

It can be seen that the linear term of extraction temperature exhibited a positive effect ($p < 0.05$) on IC_{50} in HT29, which means that IC_{50} increases, i.e. cytotoxic activity decreases, with increasing extraction temperature. However, the linear term of extraction temperature was not significant ($p > 0.05$) in the case of A549.

Moreover, extraction time showed significant negative effect ($p < 0.01$) on IC_{50} in both A549 and HT29 (Table 6; Table 7, equations 6 and 7), which means that the cytotoxic activity of NEPP on both cells will be best at 3 h. On the other hand, the interaction between extraction temperature and extraction time had a significant negative influence on IC_{50} in both cells. This means that higher

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cytotoxic activity will be preserved by prolonged extraction time only if the temperature at the middle level (85°C) is applied as extraction temperature (Figure 9A and 10A).

It is the same case with application of longer time (4 h), which demands the application of 20:1 mL/g, solvent/sample ratio for the preservation of relatively high cytotoxic activity of NEPP in HT29 (Figure 11C). Moreover, all quadratic terms of NEPP extraction parameters had a significant effect ($p < 0.05$) on IC_{50}, which was positive. The positive effect of the quadratic terms of extraction parameters means that cytotoxic activity of NEPP will decrease significantly at a higher level of these variables., which is clearly visible in Figure 9A, B, C and Figure 10A, B. The model equations for the empirically determined IC_{50} are presented in Table 7. These findings were further supported by the results obtained from DPPH scavenging activity test.

3.4. Verification of the Predictive Model

In order to verify the predictive mathematical model of the investigated process, extraction of NEPP from BDPK was performed at optimal conditions for maximised TPC, TFC, TPAC and extract yield and minimised IC_{50}. Results of investigated responses in optimised NEPP extract were 10.78±0.11 mg GAE/ g DW, 4.14±0.03 mg CE/g DW, 8.61±0.14 mg CE/g DW, 14.20±0.14%, 57.52± 4.52µg/mL, 17.4±0.6 µg/mL and 31.4±0.54 µg/mL for TPC, TFC, TPAC, IC_{50} of DPPH and IC_{50} of MTT for A549 and HT29, respectively. Comparison of predicted and experimental results showed that the experimental values of the all seven responses were obtained and differed only minimally from the predicted values with residual standard error of < 4%, indicating that the established model was effective.

TPC obtained in the optimised extract was lower than TPC as predicted by the model. On the other hand, TFC and TPAC results from optimisation process were higher than TFC and TPAC predicted model (Table 9). Interestingly, the IC_{50} (DPPH, A549 and HT29) of optimised NEPP extracts from BDPK were lower when compared with their predicted values (-1.13, -3.53 and -0.85, respectively). It has been confirmed that, besides increased NEPP yield, optimised extraction condition provides extracts with highest cytotoxic capacity and antioxidant activity.

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Table 9. Validation of the experimental model.

Variable investigated		Responses						
		TPC (mg GAE/g DW)	TFC (mg CE/g DW)	TPAC (mg CE/g DW)	DPPH SCA (IC_{50} µg/mL)	Yield (%)	A549 (IC_{50} µg/mL)	HT29 (IC_{50} µg/mL)
Temperature (85.17°C)	**Actual**	10.78± 0.11	4.14±0.03	8.61± 0.14	57.52± 4.52	14.20±0.14	17.4±0.6	31.4±0.54
Time (3.20h)	**Predicted**	10.81	4.13	8.55	57.93	14.06	18.00	31.66
Solvent/sample ratio (20:1 mL/g)								
Predicted error (%)		-0.32	0.16	0.68	-1.13	0.93	-3.53	-0.85

Responses are means ±SD (n=3)

TPC, total phenolic content; TFC, total flavonoid content;

DPPH, (2,2-diphenyl-2-picrylhydrazyl) radical scavenging activity; IC50, concentration of a substance/treatment required to inhibit DPPH radical/cell growth by half (50%); NEPP, non-extractable polyphenols.

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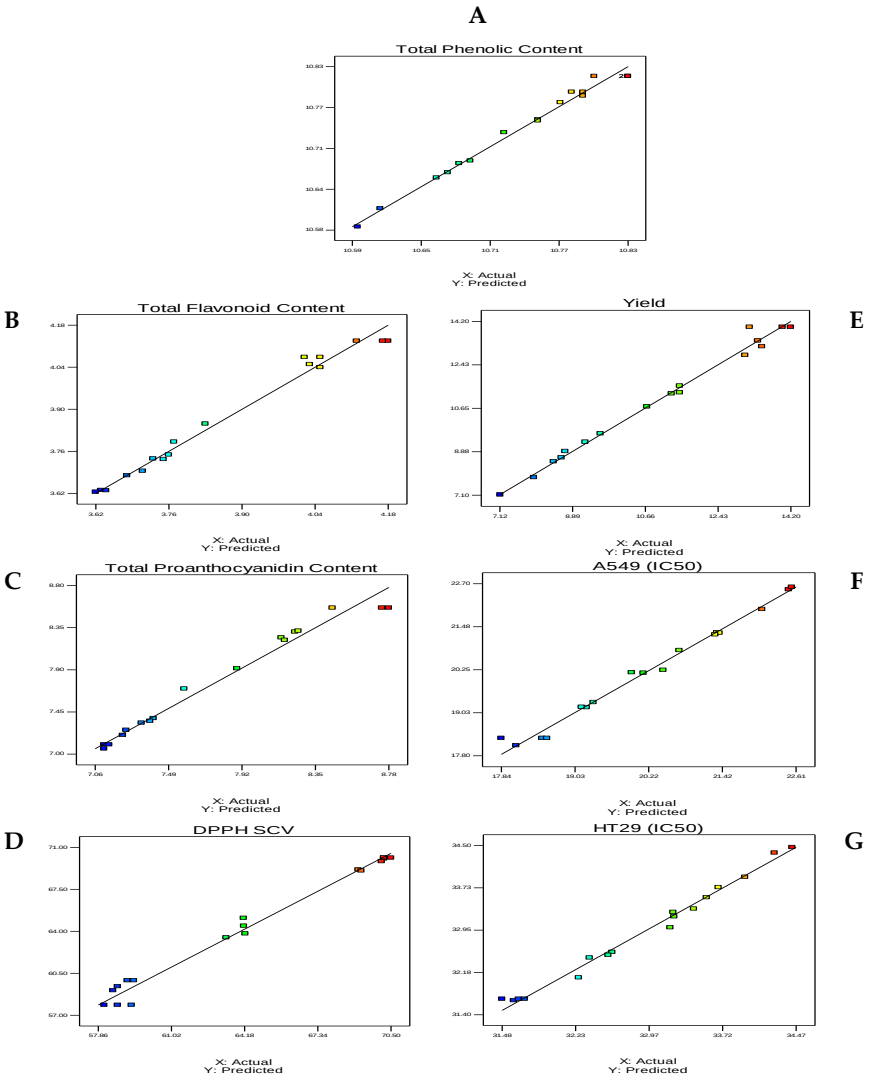

Figure 11. Correlation graph between the predicted and experimental yield values. (A) The correlation graph of total phenolic content; (B) The correlation graph of total flavonoid content; (C) The correlation graph of total proanthocyanindin content; (D) The correlation graph of DPPH scavenging capacity; (E) The correlation graph of extracts yield (F) The correlation graph of cytotoxicity of NEPP extracts on A549 and (G) HT29 cells.

4. Conclusions

This study was conducted to model and optimise the extraction conditions with acid hydrolysis for maximising the recovery of bound or NEPP (TPC, TFC and TPAC), DPPH radical scavenging activity and the inhibitory potential of the NEPP extract from BDPK on the proliferation of A549 and HT29 cells by employing RSM. Quadratic models for the seven evaluation indices were obtaine with R^2 in the range of 0.9760–0.9894. The simultaneous optimisation of the multi-response system by desirability function indicated that the desirability of 93.4% can be possible under these conditions: extraction time, 3.20 h; solvent/sample ratio, (20:1mL/g) and extraction temperature (85.17^{0}C). Acid hydrolysis treatment effectively promoted the recovery of NEPP in BDPK remaining in the residue, which is usually missed out during conventional solvent extraction. The acid cleaved the interflavan bonds, resulting in a conversion of NEPP into red anthocyanidins.

Notably, to the best of our knowledge, this is the first report on DPPH radical scavenging activity and cytotoxicity screening of NEPP from DPK, Barhi variety. However, BDPK was found to contain a higher EPP fraction [12,13], compared to the NEPP fraction, and the ability of EPP to scavenge DPPH radicals and to inhibit the cancer cells' growth was better than of NEPP [12,13]. Here, it is

important to remember that this hydrolysis was acidic (PH ~4), and this is necessary, particularly "when the glycosylation patterns are extremely complex, and when standard reference materials of polyphenol glycosides are unavailable. The hydrolysis can simplify the chromatographic profile during separation, and aid quantification and structural identification of the polyphenols" [28]. However, this acidic environment could have allowed additional compounds to be released that were not polyphenols or could have broken down some phenolic compounds occurring as glycosides into aglycones [17].

Also, when acid hydrolysis is performed at a high temperature, it can lead to a loss of phenolic compounds [66]. This possibility could also have led to a false reading in the NEPP detection, which could have been misleading in the overall data collection. The non-extractable polyphenol is rarely studied, however, with further research, it has the potential to be utilised as an anticancer and antioxidant agent in disease prevention studies. For future research studies, HPLC-MS results on NEPP extract will allow researchers to understand the exact compounds present in the extract. In summary, the response surface methodology could be successfully employed to optimise the extraction of NEPP fraction from BDPK and the results demonstrate that the extract has a significant inhibitory effect on lung and colorectal cancer cells *in-vitro*.

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