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Research Article

Antioxidant and cytotoxic activities of protein hydrolysates from shrimp shell wastes, germinated soybean and pigeon pea flour blends: A mixture response surface methodology approach

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Abstract

Cancer has high prevalence rate and mortality with conventional chemotherapy and other management protocols being both expensive and inaccessible especially in low/medium income countries (LMIC). Sourcing alternative cheaper and easily accessible treatment from blends of antioxidants sources can reduce the burden of cancer on patients. This work therefore seeks to produce a blend from the protein hydrolysates of shrimp shell waste, germinated soybean and germinated pigeon pea which not only has high antioxidant activity but also can inhibit cervical cancer cell proliferation. *In vitro* antioxidant and cytotoxic activities of the mixtures of germinated pigeon pea, germinated soybean, and shrimp shell waste hydrolysates were evaluated using the mixture response surface methodology (MRSM). Fourteen blends were obtained using the simplex centroid design. Total phenolic content (TPC), 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) and % cytotoxicity of the samples were analysed. Numerical optimization was conducted with the goal of simultaneously maximizing the DPPH scavenging activity and TPC while minimizing % cytotoxicity. The optimized blend consisted of 91.06 % pigeon pea, 8.94 % shrimp shell waste and 0 % soybean. The predicted responses obtained were 74.28 % DPPH scavenging activity, 39.6 GAE mg/dL TPC and 21 % cytotoxicity. The IC_{50} values for the optimized blend and a standard chemotherapeutic drug were 0.260 mmol/mL and 0.013 mmol / mL respectively. This *in vitro* study revealed that the pigeon pea - shrimp shell waste blend, as generated by MRSM, was comparable to a standard anticarcinogenic drug with respect to potency.

Keywords: pigeon pea, soybean, hydrolysates, shrimp shell waste, cytotoxicity, antioxidants, DPPH

1. INTRODUCTION

Epidemiological studies have shown that 75 - 80% of all fatal cancers in the USA can be prevented because they are caused by extrinsic factors[1,2]. Diet, smoking, alcohol consumption, reproductive behavior, infections, geophysical factors such as sunlight and prolonged exposure to extrinsic agents such as fossil fuel combustion products, radioactive waste, dust and fumes, pesticide residues, and food additives are the avoidable environmental factors attributable to cancer increase[2]. The free radicals emanating from most of these environmental factors act on body tissues and cause DNA mutations. The impact of these radicals becomes overwhelming in the absence of formidable antioxidant systems or repair mechanisms, triggering a cascade of signal transduction that results in carcinogenesis [3,4].

Cancer of the cervix is a preventable disease that is global in occurrence. Global cervical cancer incidence increased by 76,000 cases between 1980 and 2010, which implies an annual increase of 0.6%[5]. The management of this disease requires huge resources for surgery, chemotherapy, radiotherapy or a combination of these approaches. Apart from the financial implications involved, most of these treatments not only lead to the destruction of tumour cells but also normal cells. This has necessitated research on the use of commonly consumed natural foods, rich in bioactive compounds such as antioxidants, which have cytotoxic activity and yet no harmful side effects. Zulfafamy *et al*[6]. reported that the antioxidant and antiproliferative activity of black rice bran can be increased by fermentation. Other studies have revealed that some mango cultivars (*Mangifera indica*) have anticancer properties and this effect has been attributed to the amount of polyphenolics in the fruit[7].

Protein hydrolysates from animal and plant sources like shrimps, oysters, fish, soybeans, pigeon peas, tea, etc. have the ability to mop up free radicals/reactive oxygen species (ROSs)[8,9]. Hydrolysates derived by enzyme hydrolysis of plant proteins such as soybeans, wheat, chickpea, maize, canola, hempseed, pea seed, flaxseed, etc., have been shown to remove free radicals and therefore exhibit antioxidant properties [8-12]. Studies suggest that plant proteins such as soybeans, if eaten, can ameliorate the risk of developing several cancers. A review by Messina *et al.* [13] on animal experiments conducted between

1975 and 1993 indicates that 65% of reported studies show that soy supplementation, has chemo-protective action on cancers[13].

Pigeon peas (*Cajanus cajan*) are important legume crops grown in tropical and semitropical regions of the world. Pigeon peas are good sources of protein[21]. The constituents of mature pigeon peas are carbohydrates, protein, minerals, vitamins, and amino acids, but immature pigeon peas have higher quality of proteins and significantly higher vitamin C[22]. These immature ones also contain phenolic compounds that are known for their antioxidant properties as good electron donors [23,24]. These compounds prevent oxidation in foods and also protect the body from damage by free radicals[25–27]. Just like in soybeans, germination also affects the concentration of phenolic compounds and other compounds in pigeon pea which have an antioxidant effect and this is due to the activation of hydrolases and polyphenoloxydases[28]. Germinated pigeon pea contains different concentrations of phenolics and other biologically active substances such as cajaninstilbene acid (CSA) and cajanone[29,30]. These phenolic acids, polyphenols and bioactive compounds may interact synergistically to exert an increased antioxidant activity.

Germination affects the types and levels of amino acids in soybeans and also leads to the production of relatively smaller peptides[14,15]. These changes vary with time, implying that at different germination times, the soy composition varies. Consequently, the anticarcinogenic properties of soybean will also depend on the germination time.

The exoskeleton (shell) removed from shrimp is called shrimp shell waste. Shrimp shell waste is a rich source of protein and studies have shown that peptides can be produced from them when they are hydrolysed[16,17]. Unfortunately, such peptides are underutilized, especially as bioactive peptides. Shrimp shell waste has been reported to be a good source of natural antioxidants[18,19]. Studies have also confirmed the remarkable antioxidant activities of shrimp shell waste[17], including its inhibition of human cancer cell proliferation[16,20].

Since diets rich in fruits and vegetables provide prophylactic antioxidant micronutrients, hydrolysates of their proteins with proven antioxidant and antitumor activities should be of immense help to vulnerable indigent multiparous women of developing countries. Using cheap and easily available sources of such bioactive peptides from plant sources such as legumes like soybean and pigeon pea or from animal sources e.g. shrimp shell waste, may be an antidote to the increasing incidence and prevalence of cervical cancer morbidity and mortality in low/middle income countries (LMIC), like those found in Africa.

When antioxidants from different food sources are in combination, the net antioxidant effect may not necessarily be the sum of individual antioxidants. This is due to the possibility of other interactions that could be synergistic and/or antagonistic in nature. Therefore, this work seeks to use the optimization technique of mixture response surface methodology to produce a blend from the protein hydrolysates of shrimp shell waste, germinated soybean, and germinated pigeon pea that not only has high antioxidant activity but also can inhibit cervical cancer cell proliferation.

2. MATERIALS AND METHODS

2.1 Materials

Soybean seeds (*Glycine max*) and pigeon pea seeds (C*ajanus cajan*) were purchased from a retail outlet in Abakaliki, Ebonyi State, South East Nigeria. Shrimp shell waste (*Penaeusnotialis*) was obtained from shrimp purchased at Akpoha Bridge Fish Market, Akpoha, Afikpo North LGA, Ebonyi State, South East Nigeria. Human cervical cell lines C-33A were obtained from the American Type Culture Collection (ATCC, USA). All reagents used in this study were of analytical grade.

2.2 Sample Preparation

2.2.1 Germination of soybean and pigeon pea

Exactly 500 g each of the soybean and pigeon pea seeds were soaked separately in 1500 mL of distilled water for 16 h at room temperature. The distilled water was changed every 6 h, to avoid microbial growth. The two sets of seeds were individually covered with jute bags that were sprinkled with water every 3 h, to maintain humidity. Based on preliminary investigations, soybeans were allowed to germinate for 48 h in light, while pigeon beans were allowed to germinate for 48 h in dark conditions to obtain optimal antioxidant activity. Portions of the germinating seeds were removed, air-dried, ground to pass 40 µm size mesh and stored in air-tight containers in a refrigerator until needed for analysis according to the method described by Mora-Escabedo *et al.* [31]

2.2.2 Preparation of the shrimp shell waste

For the sample preparation of the shrimp shell waste, the head was severed from the body and its internal contents removed leaving only the exoskeleton. Similarly, the shell was removed from the body. This shell waste was washed thoroughly with tap water and then air-dried. The dried shells were ground using laboratory electric blender and the ground powder was sieved to pass through a 40 µm size mesh. The finely ground powder was placed in an airtight container and stored in a refrigerator for further analysis.

2.2.3 Preparation of hydrolyzed germinated soybeans, hydrolyzed germinated pigeon beans and shrimp shell waste hydrolysates

To obtain the protein hydrolysate, the germinated soybean, and germinated pigeon pea samples were further hydrolysed according to the method described by Lo *et al.*[34 32] as follows: a batch of the refrigerated stored dried protein source (germinated pigeon pea and germinated soybean) was dissolved in distilled water at the ratio 1:5 w/w. To this mixture was added 0.5 mL of 1 % pepsin at pH 2.0 and allowed to react for 30 min. The pepsin reaction was stopped by increasing the pH to 7.0 using 0.1 mol/L NaOH, after which, 0.5 mL of 2 % pancreatin (pH 7.0) was added at 37 °C in a water bath(Lab-Line Barnstead 18050A) for another 30 min. Proteases were inactivated and hydrolysis stopped by heating the mixture in a thermostatically controlled water bath for 15 min. The hydrolyzed samples formed were cooled to room temperature, centrifuged (MPW-260 Laboratory Centrifuge) at 4,000 x g for 10 min and the supernatant stored at -20 ° C for separation and further analysis.

For the shrimp shell waste sample, further hydrolysis was carried out according to the method described by Kannan *et al.* [20] as follows: ground shrimp shell waste powder was dissolved in water in the 1: 5 w / w ratio. 100 mL of the mixture was added 0.032 g/kg pepsin, stirred at 37 ° C before adding the cryotin-F enzyme. The mixture was incubated for one hour at 37 ° C before heating the mixture at 85 ^{0}C for 3 min to inactivate the enzyme. To obtain the hydrolysate, the mixture was centrifuged at 3,000 x g for 15 min and the supernatant stored at -20 °C for separation and further analysis.

2.2.4 Separation of fraction of the Shrimp Shell Hydrolysates

The protein hydrolysate from the shrimp shell waste was subsequently fractionated using Amicon ultra filtrate apparatus (Amicon^R Ultra-15 Centrifugal Filter 10 kDa MWCO Millipore) which had molecular membrane cut-offs of 30 and 10 kDa. Compressed air at a constant pressure of 10 psi was fed

into the Amicon cell, and the peptide hydrolysates were first filtered through the 30 kDa membrane and then followed by filtration with the 10 kDa membrane. Based on preliminary investigations, the optimal antioxidant activities for shrimp shell waste was the <10kDa size range fraction of the hydrolyzed sample. This fraction was stored in a refrigerator in plastic airtight containers until needed for further analysis.

2.3 Total Phenolic Content (TPC)

This method was determined using the Singleton-Rossi method [33]. Exactly 5 mL of 85% phosphoric acid and 10 mL concentrated hydrochloric acid were added to Folin-Ciocalteu`s reagent. This was refluxed for 10 h and then 15 g of lithium sulfate, 5 mL of water and 1 drop of bromine solution were added. This was refluxed for 15 min, cooled to room temperature and made up to 100 mL with distilled water. The enzyme extract was prepared by adding 100 mg of the hydrolyzed sample flour into a tube containing 4 mL of 70 % aqueous ethanol with 0.1 % acetic acid. A calibration curve was prepared using 10 mM gallic acid as a working solution with 5 dilution points (0.2, 0.4, 0.6, 0.8 and 1.0 mM), with distilled water as a negative control. One hundred microliters (100 µL) of sample was placed in a tube containing 100 µL of Folin-Ciocalteu reagent with a pipette. This was well mixed and allowed to stand for 3 min, after which 100 µL of saturated sodium carbonate solution and 700 µL of distilled water were added. The reaction was allowed to stand for about 90 min in the dark and the absorbance read at 725 nm using spectrophotometer (Hewlett Packard 8452). The results were calculated by extrapolation using the gallic acid curve.

2.4 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical-scavenging activity

DPPH radical-scavenging activity was measured based on the method described by Sefatie *et al.* [34]. The reaction mixture was made up of 1 mL of hydrolyzed sample, 1 mL of 0.02 mol/L phosphate buffered saline, and 1 mL of 0.2 mM DPPH in 95 % ethanol. This mixture was shaken and allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was read at 517 nm against the blank, using a spectrophotometer (Hewlett Packard 8452).

DPPH scavenging effect (%)/%Inhibition=

AO-A1/A0 X100

where A0=Absorbance of Control

A1= absorbance of sample

IC_{50}= 50% of DPPH inhibition and in this case, using the optimized blend.

2.5 Cytotoxicity Screening of Samples

For cytotoxicity assay, two critical steps were involved. First, the compound was prepared and then the cell was prepared. For the preparation of the compound, a batch of the hydrolyzed samples from the various formulations (test samples), was dissolved with 100 % (v/v) Dimethyl sulphoxide (DMSO) to 10 mg/mL. The dissolved sample was diluted to 1 mg/mL with sterile deionized water, for primary screening. For the determination of IC_{50}, a twofold serial dilution was conducted from 15.625 µg/mL to 1 mg/mL in 10% (v /v) DMSO. In both cases, the compound solution was mixed by pipetting many times thoroughly. Positive controls were prepared by using serial twofold dilutions in 10% (v/v) DMSO, to prepare six concentrations from 5 µg/mL to 160 mg/ mL . To each compound well of the 96-well tissue-culture plate, 10 µL of the test sample in 10% (v/v) DMSO, was added. This was done in triplicates. To each of the negative-control wells, 10 µL of 10% (v/v) DMSO was also added, while to each of the positive-control wells, 10 µL of a well-known anti-cancer drug in 10% (v/v) DMSO was added.

The cells were prepared prior to assay. For the preparation, the cells were washed once using sterilized Phosphate Buffered Saline (PBS), after removing the medium from cell monolayers. The PBS was removed and 0.25% (w/v) trypsin in versene-EDTA was added. The cell-growth surface was covered. The cells were allowed to grow, until they started to dissociate, when sterilized plastic was used to disperse them from the culture surface using 10 volumes of culture medium containing fetal bovine serum (FBS). The medium was mixed until a homogeneous cell suspension was obtained.

Cell suspension was transferred to a sterile polypropylene tube, where cells were counted in a hematocytometer chamber under a microscope to determine cell concentration, using a 1:1 mixture of cell suspension and 0.4 % trypan blue solution (w / v). The growth medium was adjusted, until a healthy cell seeding density of 1.9×10^4 cells per tissue-culture well that can stain with trypan blue dye, was obtained.

To the assay plates, 190 µL of the cell suspension was added. This was mixed occasionally to ensure that the cells were evenly distributed. The cytotoxicity of the test compounds was then determined as explained below.

2.6 Determination of the Cytotoxicity

The cell density was first determined using the sulphurhodamine B (SRB) assay, which was based on the measurement of the cellular protein content, according to the method described by Vichai and Kirtikara [35]. Cervical cancer cell lines were seeded in 96-well plates in triplicates and incubated for 4 h. Then 10 µL of the test sample in 10 % (v/v) dimethyl sulphoxide (DMSO) was added to each well of the 96-well tissue-culture plate. Similarly, 10 µL of 10 % (v/v) DMSO was also added to each negative control well, 10 µL of Roswell Park Memorial Institute (RPMI) culture medium in 10 % (v/v) DMSO to each negative control well and 10 µL of the anticancer drug in 10 % (v/v) DMSO to each positive control well. The 96-well plate for the test sample, DMSO negative control, RPMI culture medium negative control, and anticancer drug positive control were put in the incubator for 12 h. The cervical cell monolayers were fixed by pipetting 100 µL of 10 % (w/v) trichloroacetic acid (TCA) into each well, incubated at 4 °C for 1 h for staining to take place. The TCA fixed the cervical cancer cells to the tissue culture plates while the SRB was the bright-pink aminoxanthene dye with two sulphonic groups that bound to protein component of the cervical cancer cells under mild acidic conditions. The excess dye was removed by washing each well four times with distilled water and then blow-dried. Since SRB binding dissociates under basic conditions, and the amount of dye removed from stained cells was directly proportional to cervical cell mass, protein bound dye was dissolved by putting 200 µL of 10 mM Tris base solution in each well, swirled around and allowed to stand for 5 min before reading at 510 nm on the spectrophotometer for determination of determination of determination of OD using microplate reader.

$$\%\ Cell\ death\ (\%\ cytotoxicity) = \frac{(A-B)}{A} x\ 100$$

Where: A =OD of untreated cells (control)

B =OD of treated cells (with extracts)

2.7 Optimization

The numerical optimization technique of the Design-Expert software was used for the simultaneous optimization of multiple responses. The procedure was carried out by maximizing the DPPH and TPC values while minimizing the

percentage of cytotoxicity (maximizing anti-proliferative rate), of the extracts in the cervical cancer cell lines *in vitro*.

2.8 Comparison of the Potency of the Optimized Blend with an Anticancer Drug

To establish the efficacy of the optimized blend extract, a comparative analysis was done between different concentrations of the extract and a well-known standard anticancer drug by determining the IC_{50} (which defines the concentration at which 50 % of the cervical cancer cells were killed). A statistical analysis software, GraphPad Prism, version 7.0, was used for the analysis. The results obtained were expressed as % control cell growth, % growth inhibition and % cell killed while the IC_{50} was expressed in mmol/mL of the extract.

2.9 Experimental Design

A three-component augmented simplex centroid design was used [36]. Hydrolysed germinated soybean flour (*SB*), hydrolysed germinated pigeon pea flour (*PP*) and shrimp shell waste hydrolysates (*SS*) were the three mixture components evaluated for antioxidants, antioxidant activities and cytotoxicity in this study. Each mixture component was expressed as a fraction of the mixture in percentage, with each treatment combination summing up to 100 %, such that:

$$\sum X_i = X_1 + X_2 + X_3 = 100$$

The number of points (n) needed for a mixture experiment in this design was:

$$n = 2^q - 1$$

Where q is equivalent to the number of components studied (3). This implies that the number of flour mixtures, n = 7. Augmentation of the simplex centroid resulted in three additional points being added to bring the number of mixtures to ten. Four runs were replicated to provide for an internal estimate of error. This resulted in a total of fourteen flour mixtures.

2.10 Data Analysis

The model search was started with the special cubic equation:

$$y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$$

where Y represents the predicted responses, DPPH, TPC and cytotoxicity, βs are the parameter estimates for each linear and nonlinear term for the prediction model. X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, and $X_1X_2X_3$ are the linear terms of soybean, pigeon pea, and shrimp shell and the nonlinear terms of soybean x pigeon pea, soybean x shrimp shell, pigeon pea x shrimp shell, soybean x pigeon pea x shrimp shell flours, respectively. Regression analysis was carried out on values obtained for DPPH, TPC, and cytotoxicity using Design-Expert Version 9.0.6.2 (Stat-Ease, Minneapolis Inc. 2015). The model to be chosen was based on a significant model ($p < 0.05$), adjusted R^2 above 60 % [37] and reasonable agreement between predicted R^2 and adjusted R^2, that is, the difference between the two was less than 0.2. Non-significant terms were removed from the model.

3 RESULTS AND DISCUSSION

3.2 Antioxidant activity of mixtures of hydrolysed germinated soybean, hydrolysed germinated pigeon pea and shrimp shell waste hydrolysates.

The antioxidant and cytotoxic activities of blends of hydrolysed germinated soybean (SB), germinated hydrolysed pigeon pea (PP) and shrimp shell waste hydrolysates (SS) is presented in Table 1. DPPH scavenging ability is a representation of the free radical reducing activity of antioxidants. It was observed that 100 % PP which had the highest DPPH activity of 94 % had a TPC of 14.04 GAE mg/ dL while the blend of 66.6 % SB: 16.7 % PP: 16.7 % SS which had the least DPPH scavenging activity of 12 %, had a TPC of 86.04 GAE mg/ dL . This shows that having higher levels of TPC does not necessarily translate to having higher DPPH scavenging ability. This therefore suggests that other factors such as the presence of other bioactive compounds in the sample could have contributed to antioxidant activity.

When different antioxidant extracts are in a mixture, synergy or antagonism could occur, but this depends on the type and concentration of the antioxidants. Formulation 9, which is 100 % PP, was found to have the best DPPH scavenging activity of 94 % with TPC of 14.04 GAE mg/ dL (Table 1) while Formulation 5 which contains 66.6 % PP: 16.7 % SB: 16.7 % SS had DPPH scavenging activity of 47 % but TPC of 175.32 GAE mg/dL. Apart from the possibility of other bioactive compounds being present, it is also possible that the best antioxidant activity may be at a particular concentration of antioxidants and not necessarily at the highest concentration of TPC. Such optimal concentrations could be the best point where the free hydroxyl groups sum up to mop up the free radicals, or where regeneration of reduced groups is at best or even where antagonism is minimal. Relating the high DPPH scavenging activity in Formulations 3, 5 and 9, with the corresponding TPC levels (Table 1), revealed that TPC levels as low as 14.04 GAE mg/dL can still exert high antioxidant activity (DPPH scavenging activity), suggesting that at a certain TPC threshold, antioxidant activity is exerted.

The sample with least percentage (%) cytotoxicity activity refers to the sample that resulted in the lowest cancer cell proliferation. Formulation 9 (100 % PP) was found to have the least cytotoxicity with a value of 13.83 %. This was followed by other formulations, which had varying levels of pigeon pea. Pigeon pea hydrolysates have been reported to demonstrate antioxidant activities [38]. The presence of saturated double bonds and benzene rings joined by a planar unsaturated C2 structure in the structure of bioactive compounds enhances this antioxidant effect [39]. Similarly, the presence of a 5-hydroxy group in the A ring and methoxy groups at the 3-position is responsible for the strong antioxidant activities of cajaninstilbene acid [40]. These chemicals especially cajaninstilbene acid have also been reported to exhibit anti-inflammatory properties by suppressing the production of inflammatory cytokines in the macrophages [41].

Table 1: Antioxidant and Cytotoxic activities of the various blends

Formulations	SB (%)	PP (%)	SS (%)	DPPH (%)	TPC (GAE mg/dL)	Cytotoxicity (%)
1	50	50	0	27	101.70	28.59
2	0	50	50	34	46.08	30.30
3	50	0	50	50	129.06	31.23
4	33.3	33.3	33.3	21	105.30	28.72
5	16.7	66.6	16.7	47	175.32	30.83
6	16.7	16.7	66.6	14	87.48	37.02
7	66.6	16.7	16.7	12	86.04	33.33
8	100	0	0	61	6.12	35.84
9	0	100	0	94	14.04	15.22
10	0	0	100	63.5	1.80	37.02
11	100	0	0	59	5.76	37.15
12	0	0	100	62.3	1.98	32.41
13	0	100	0	90	12.60	13.83
14	50	0	50	50	126.00	28.19

SB = Hydrolyzed Germinated Soybean; PP= Hydrolyzed Germinated Pigeon pea; SS = Shrimp shell waste Hydrolysates; DPPH = 2,2-Diphenyl-1-Picrylhydrazyl activity test; TPC = Total Phenolic Content

3.2 Modelling Process

Regression analysis carried out on the data obtained, revealed that the quadratic model was significant in predicting the DPPH scavenging activity (Table 2). The prediction equation obtained for DPPH scavenging activity after removing the nonsignificant terms was:

$$DPPH = 0.584SB + 0.950PP + 0.617SS - (0.0214SB * PP) - (0.0194PP * SS)$$

Where *SB*= Soybean; *PP*= Pigeon pea; *SS*= Shrimp shell waste.

The quadratic model had a *p*-value of < 0.001, which implies that it was very significant [42] and could explain the 83 % of the observed variations. There was a very close agreement between the adjusted and predicted coefficients of determination (R^2). These all are indicators of the fitness of the model. The negative sign (-) before the coefficients implies that the blending of the hydrolysates had antagonistic effects on the DPPH. The positive sign (+) implies synergistic effects on the response. Among the single components, the pigeon pea had the highest positive coefficient, which means that it had the highest effect of increasing the DPPH, while the soybean with the lowest coefficient had the lowest effect on the DPPH. This agrees with what can be seen in Table 1.

The regression equation obtained for TPC was:

$$TPC = (0.0023SB * PP) + (0.0027SB * SS)$$

The quadratic model was significant in predicting TPC because it had a *p*-value less than 0.001, adjusted R^2 was 71 % and the difference between adjusted and predicted R^2 was less than 0.2. The combination of soybean hydrolysates with pigeon pea and soybean hydrolysates with shrimp shell waste had synergistic effects on TPC. The results showed that the linear and tertiary blends did not have any significant effect ($p > 0.05$) on the TPC.

The linear model was adequate to predict the cytotoxicity of the blends. The equation obtained was:

$$Cytotoxicity = 0.33SB + 0.20PP + 0.37SS$$

The model could explain 65 % of the observed variations with a *p*- value of 0.001 and there was close agreement between the predicted and adjusted R^2. Since the lowest% of cytotoxicity activity implies the highest level of inhibition or the lowest cancer cell proliferation, this means that shrimp shell waste having the highest coefficient actually resulted in the highest cancer cell proliferation while the pigeon pea with the lowest coefficient resulted in the lowest cancer cell proliferation. The non-significance of quadratic and special cubic terms implies that blending of the components had no significant effect on cancer cell proliferation.

Table 2: Coefficient estimates, model significance, adjusted R squared (Adj. R^2), and predicted R squared (Pred. R^2) values for DPPH, total phenolic content (TPC) and cytotoxicity

Variables	DPPH	TPC	Cytotoxicity
SB	0.58	n.s	0.33
PP	0.95	n.s	0.20
SS	0.62	n.s	0.37
SB*PP	-0.021	0.0023	n.s
SB*SS	n.s	0.0027	n.s
PP*SS	-0.019	n.s	n.s
SB*PP*SS	n.s	n.s	n.s
Model	0.0009[a]	0.0075[a]	0.0013[a]
(Prob > F)			
Adj.R^2	0.83	0.71	0.65
Pred.R^2	0.78	0.56	0.51

The quadratic model was used n.s= non -significant

3.3 Optimization

Numerical optimization was conducted with the goal of simultaneously maximizing the DPPH scavenging activity and TPC while minimizing % cytotoxicity. The optimized blend consisted of 91.06 % pigeon pea, 8.94 % shrimp shell waste and 0 % soybean (Figure 1). The predicted responses were 74.28 % DPPH scavenging activity, 39.6 GAE mg/dL TPC and 21 % cytotoxicity (anti-proliferative rate of 79 %).

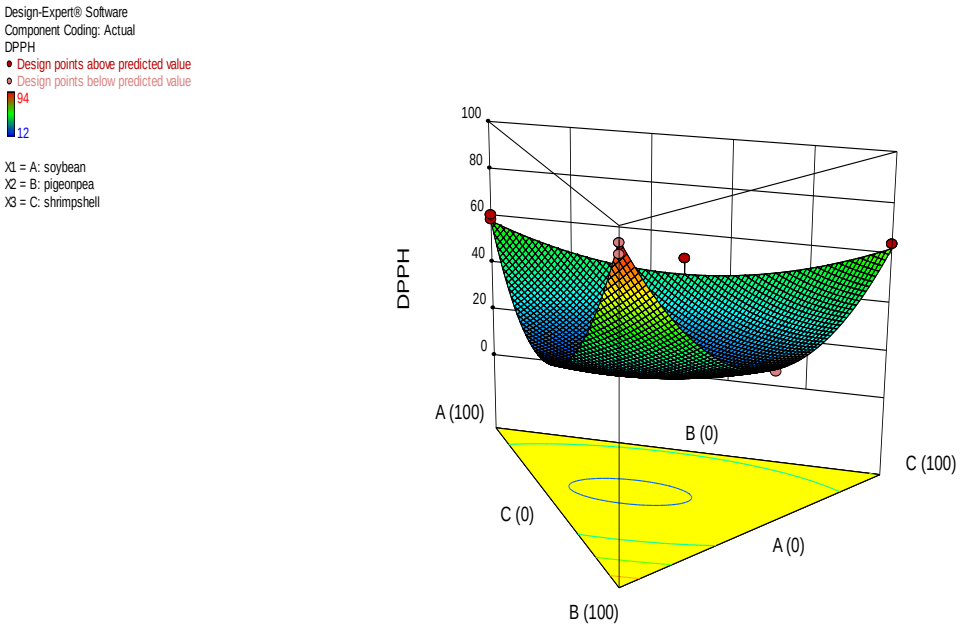

Figure 1: 3D- surface plot of optimized blend from soybean, pigeon pea and shrimp shell waste (Source: Design-Expert(R) version 9.0.6.2, State-Ease, Inc. Minneapolis, 2015)

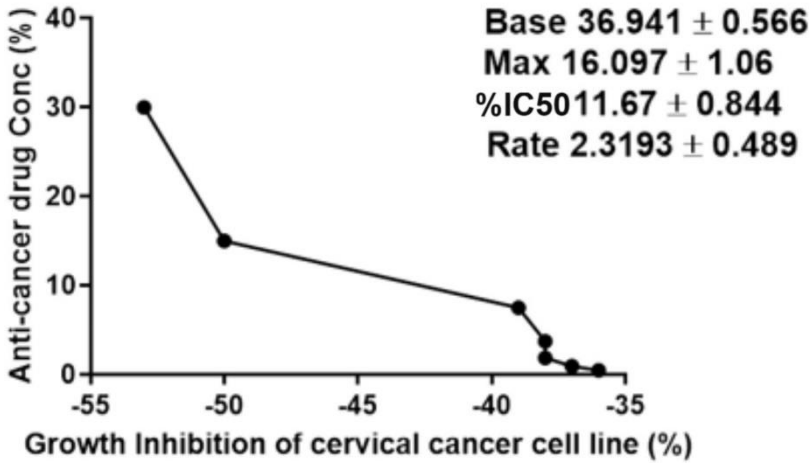

Figure 2: Dose-Response curve between the anti-cancer drug concentration and percent growth inhibition of cancer cell lines

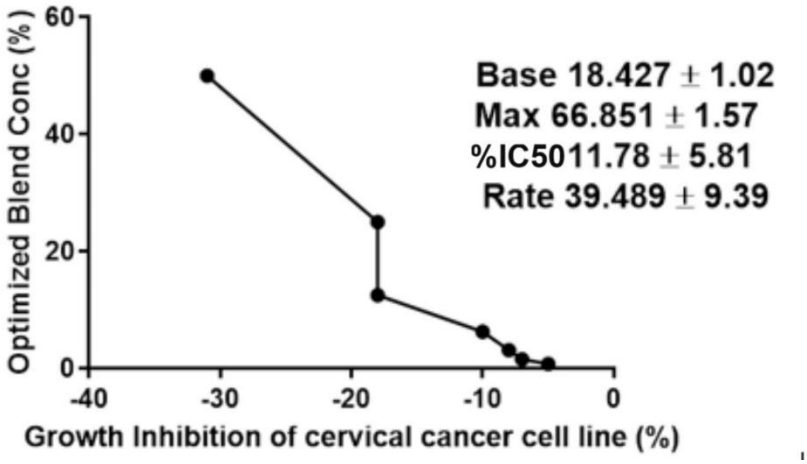

Figure 3: Dose-response curve between the optimized blend concentration and percent growth inhibition of cancer cell lines

3.4 Potency of the Optimized Blend and Anti -Cancer Drug

A similar behaviour was observed with a standard anticancer drug and the extract obtained from the optimized blend; in that the percentage of cancer cell growth inhibition and cancer cells killed increased as the concentration of the extract and the drugs increased and *vice versa*. Consequently, the IC_{50}, a threshold of 50 % cell-growth inhibition, which is a standard cut-off for compound toxicity against cell lines was derived.

From Figures 2 and 3, the percentage IC_{50} were 11.67 % (0.013 mmol/ mL) and 11.78 % (0.260 mmol/mL) for the anti-cancer drug and the optimized blend extract respectively. These were the concentrations at which 50 % of the cervical cancer cell lines were killed *in vitro*. Serial dilutions of the blend extract and the drug were each used and the % growth inhibition and % cells killed were determined. The maximum dose required to achieve the IC_{50} for the optimized blend extract was 66.85 % provided that the loading dose of 18.43 % was maintained. For the anticancer drug to achieve the IC_{50} of 11.67 %, the maximum dose required was 16.10 % provided the loading dose of 36.94% was maintained.

The closeness in the IC_{50} values obtained for the optimized blend and anticancer drug suggests that the optimized blend extract is potent and in terms of cost-benefit analysis, side effects, affordability and accessibility, the extract can be said to have very great potential and long-term advantage.

4.0 CONCLUSION

The results have demonstrated that combinations of the different antioxidant sources produced varying antioxidant and cytotoxic activities. The study showed that the germinated hydrolysed pigeon pea - shrimp shell waste hydrolysate blend, as generated by the response surface methodology of the mixture, was comparable to a standard anticarcinogenic drug with respect to potency. The use of this blend has great potential and advantages compared to the anticancer drug, which is not only very expensive as a chemotherapeutic agent, but also poorly tolerated by patients, with myriads of side effects, some of which are life threatening.

Conflict of Interest: The authors declares that no conflict of interest.

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