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Interaction Effect of Polyherbal Formulations with Antioxidant Activity

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Abstract: Plant extracts individually contain a complex mixture of bioactive compound and various combinations of plant extracts might give interaction effect on antioxidant activity. Possible interactions among antioxidant component can be synergistic, antagonistic, additive and indifferent which is importance on determining the effectiveness of plant extract combinations on this activity. Therefore, in this study, the interaction effect towards antioxidant activity of total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and lipid peroxidation inhibition (LPI) presence in aqueous extract of leaves of *Nephrodiuminophyllum* (pakismerah), *Polygonum minus* (kesum), *Annona squamosal L.*(nona fruit) and *Stevia rebudiana* has been evaluated. The *in-vitro* antioxidant activity of the extracts and their different combinations were conducted by TPC, TFC, DPPH and LPI assays. Meanwhile, the interaction effect was assessed by comparing the experimental antioxidant activity of the mixtures with calculated theoretical values and the interactions of the compounds were determined. The results suggest that *P. minus* individually has highest phenolic content and percentage inhibition of DPPH while *N. inophyllum* has highest flavonoid compound and lipid peroxidation inhibition. Indifferent effect was exhibited in the majority of all combinations on all assays but only one antagonistic interaction between *P. minus* and *A. squamosal L* on DPPH assay. However, there were only four synergistic effects evaluated on all assays with the highest one was shown on DPPH assay for combination of *N. inophyllum* and *A. squamosal L.* In conclusion, this present study justifies these polyherbal formulations have influenced antioxidant properties by validating their interaction effects.

INTRODUCTION

Medicinal plants are widely used in the development of herbal supplements due to its high margin of safety, cost-effective, eco-friendly and readily availability [1]. These medicinal plants have rich sources of phytochemical constituents such as flavonoids, phenolic, tannins and other compounds. These plants might exert beneficial effects such that radical scavenging activity inhibitor, known as antioxidant.

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Antioxidants are responsible to prevent and delay the oxidation of an oxidizable substrate that helps to reduce the risk of oxidative stress-related diseases and enhance resistant effect on human [2, 3].

The principle of an antioxidant is its ability to trap free radicals which referred to the oxygen-centered molecules that contain a single electron at the outermost orbit [4]. In healthy individuals, free radical production is continuously balanced by natural antioxidative deference systems. Reactive oxygen species (ROS) and free radicals cause the deterioration of foods and as inducers of lipid peroxidation [5]. Lipid peroxidation is used to measure the inhibitory activity of the plant extracts caused by the hydrogen peroxides [6]. Therefore, the study of biological activity and chemical composition of medicinal plant extracts as a potential source of natural antioxidants are becoming a trend in development of product.

P. minus (kesum) which from Polygonaceae family exhibit high antioxidant activity due to its high content of gallic acid, reducing power and total phenolic compound [7]. Its leaves can prevent the oxidative damage on the fatty tissues and eliminate the unnecessary free radicals [6]. Meanwhile, *N. inophyllum* (pakismerah) is belonging to Polipodiaceae family. Its leaves possess good sources of natural antioxidant and can neutralise the free radicals due to its potential in giving a good reducing power [6]. Another plant is *A. squamosa* or locally known as "buahnona" is from family of Annonaceae. The use of *S. rebudiana* belonging to the family Composita *e* is mostly to act as natural sweetener. Many studies have showed the potential of *A. squamosa* and *S. rebudiana* on antioxidant activity [8, 9].

In general, combination of plant extracts might modify antioxidant activity through the interaction effect of synergistic, antagonistic, additive or indifferent [10]. Synergistic effect is defined as a combination of two or more substances shows higher effects than the sum of the single substances while antagonistic is the opposite of synergistic. Meanwhile, additive is the combined effect of two or more components is equal to the sum of the effect of each component given alone but indifferent is no significant different are showing when combination plant extracts [11]. In other word, a good antioxidant activity can be established if plant extract combinations have synergistic effect. Thus, this study aims to investigate the antioxidant activity of aqueous extracts of leaves of N. *inophyllum, P. minus, A. squamosal L* and *S. rebudiana* for polyherbal formulation. Then, it followed by evaluation of their interaction effect towards the antioxidant activity.

MATERIALS AND METHODS

Chemicals and Instruments

DPPH, methanol, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, sodium nitrate, aluminium chloride hexahydrate, rutin, ethanol 95%, linoleic acid, phosphate buffer (pH7), ferrous chloride, hydrochloric acid and butylated hydroxytoluene (BHT) were analytical grade.

The instrument used was UV-Vis spectrophotometer (BIOMATE 3S) located in Food Analysis Laboratory UniversitiTun Hussein Onn Malaysia.

Collection and Preparation of Plant Materials

Leaves of *P. minus, A. squamosa* and *N. inophyllum* were obtained from the local market while *S. rebaudiana* was obtained from Ethno ResoucesSdnBhd, Sungai Buloh, Selangor. Those plants were prepared according to [12] with slight modification.

The leaves were washed thoroughly, cut into small pieces, dried and grinded into powdered form. Then, the extract was filtered through eight-layer of muslin cloth.

The filtered extracts were then centrifuge at 5000 rpm for 10 minutes. The supernatant was collected and further drying. For process of drying extract, freeze dryer were used and stored in freezer of -80°C overnight.

DPPH Radical Scavenging Activity Assay

The DDPH assay was done as described by [13] with slight modifications where 200µl of plant extract was mixed with 1 ml of 0.2 mM DPPH solution. The solution was mixed by using vortex for 5 minutes. Then, the solution was allowed to stand in the dark for 30 minutes at room temperature. As for blank, 60% of methanol was prepared. The absorbance of the solution was determined at 517 nm. The % inhibition rate of DPPH was calculated as below:

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

Where: $A_{control}$ = absorbance value of control, A_{sample} = absorbance value of sample

Total Phenolic Content (TPC) Assay

Total phenolic content was determined by using Folin-Ciocalteu assay by referring to method [14] with modifications where 300 µl of sample was placed into the test tube. Next, 1.5 ml of Folin-Ciocalteu (diluted into 10 times of dilution) was added and followed by 1.2 ml of 7.5% sodium carbonate. The solutions were allowed to stand at room temperature for 30 minutes. Then the absorbance of the solution was determined at 765 nm. TPC was expressed as gallic acid equivalent in mg/gm. The calibration equation for the gallic acid was y=0.009x–0.0217. The total phenolic content equivalent to the gallic acid was calculated as follows:

$$C=cV/m \quad (2)$$

Where: C = Total phenolic content, c= concentration of gallic acid, V= volume of extract, m= mass of the extract.

Total Flavonoid Content (TFC) Assay

The total flavonoid content was determined by using aluminium chloride calorimetric assay as mentioned by [15] with modifications when 1 ml of extract was mixed with 4 ml of distilled water. At 0 min, 0.3 ml of 5% sodium nitrite was added and the solution was allowed to stand for 5 minutes. Then, 0.6 ml of 10% aluminium chloride hexahydrate was added and then 2 ml of 1 M NaOH was added followed by mixing using vortex. The absorbance of solution was determined at 510 nm. Rutin was used as comparison in standard curve where it expressed as milligram of rutin equivalents per gram of extract. The standard equation was y=0.0021x+0.004. The total flavonoid equivalent to rutin concentration was calculated and compared with standard curve.

Lipid Peroxidation Assay

Lipid peroxidation inhibition assay was done according to [16] with modifications by using ferric thiocyanante method where 2 mg of extract was mixed with 95% ethanol, then followed by 2.05 ml of 2.51% linoleic acid in 95% ethanol. Then, 4 ml of 0.05 M phosphate buffer (pH 7.0) was added including 1.95 ml of distilled water. Then, the solutions were kept in the dark at 60°C for 12 hours. After that, 0.1 ml of this solution was added 4.85 ml of 75% ethanol, followed by 0.1 ml of 30% thiocyanate solution. After 3 minutes, the 0.02 M of ferrous chloride and 0.1 ml of 3.5% hydrochloric acid was added. BHT was used as positive control. Next, the absorbance of solution was measured at 500 nm and % of inhibition was calculated as follows:

$$\text{Inhibition } \% = \left(\frac{A_0 - A_1}{A_0}\right) \times 100 \quad (3)$$

Where: A_0 = Absorbance of control reaction, A_1 = Absorbance of sample extract.

Calculation of Synergistic Effects of Antioxidant Mixtures

The experimental antioxidant activity was determined analytically using the method described while theoretical values were calculated as the sum of the antioxidant values of each compound. If the experimental antioxidant activity was greater than the theoretical antioxidant activity, it was considered as synergistic effect and if it was lower than the theoretical antioxidant activity it was interpreted as antagonistic effect [17].

Statistical Analysis

Data that have been obtained in this study were analysed as described by [18] where each determination were conducted in triplicate and the results obtained as an average value in which mean ± standard deviations. Data will be analysed by using a one-way Analysis (ANOVA) of IBM SPSS Statistics version 20 models. The differences between the mixtures will be determined by using Tukey tests. The statistical significance will be set at $p<0.05$.

RESULT AND DISCUSSION

In general, DPPH radical scavenging activity is mainly used to screen the ability of compounds as hydrogen donors or free-radical scavengers and to know the anti-oxidative activity of the plant extracts. Hydro-peroxides radical or lipid peroxides were major propagators from the chain of autoxidation of lipids resulting to the formation of non-radical products. However, DPPH radical scavenging could help in inhibit the propagation phase of the lipid peroxidation [19]. Also, total phenolic and total flavonoids content are correlated with the antioxidant activity [20].

Based on the results obtained (Table 1), it showed that butylated hydroxytoluene (BHT) has the highest total phenolic and total flavonoid content as well as lipid peroxidation inhibition among the other

plant extracts individually. Meanwhile, *P. minus* has the highest percentage inhibition of DPPH. However, plants extracts are preferred over the synthetic antioxidants (BHT) as natural antioxidants have multiple beneficial effects which are produced by the presence of a wide spectrum of antioxidant components [11].

Table 1: Phytochemical Content and Antioxidant Activity of BHT and Plant Extracts Individually

Samples	Phytochemical content		Antioxidant activity	
	TPC	TFC	DPPH	LPI
BHT (control)	94.84 ± 0.068[a]	39.84 ± 0.052[a]	28.79 ± 0.046[a]	78.92 ± 0.59[a]
N. inophyllum	56.79 ± 0.058[e]	34.76 ± 0.050[b]	29.32 ± 1.92[ab]	74.23 ± 0.65[b]
P. minus	88.38 ± 0.24[b]	30.52 ± 0.00[c]	62.21± 0.62[e]	71.15 ± 0.60[c]
S. rebaudiana	39.78 ± 0.10[i]	22.86 ± 0.00[h]	38.87 ± 1.75[c]	68.10 ± 1.32[d]
A. squamosal L.	47.50 ± 0.07[h]	20.05 ± 0.00[i]	36.64 ± 1.38[bc]	68.05 ± 0.53[d]

Values of antioxidant activity of BHT and Plant extracts are mean ± standard deviation (n= 3)
BHT = Butylated Hydroxytoluene.

Different letter at each line indicates significant different at ($p<0.05$) as measured by TukeyHSD[a] test.

However, when the plant extracts were combining with the other plant extracts, the combinations showed a few possible interaction effects as shown in Table 2. Based on the results, it indicates that majority of the plant combinations were revealed with indifferent effects. This means that these combinations of mixture between both samples exhibit neither good nor bad [21]. For DPPH assay, there is only one antagonistic interaction is evaluated. This might be because of the antioxidant activity degrades during the storage of plant extract resulting from the exposure of light and fluctuation temperature of storage [22].

Table 2: Phytochemical content, antioxidant activity and interaction effect of combinations of plant extracts

Combination	Predicted value	Experimental value	Types of interaction
Total phenolic content (TPC)			
50% A + 50% C	52.15	52.18	Indifferent
50% A + 50% D	48.29	49.44	Indifferent
50% B + 50% C	67.94	67.95	Indifferent
50% B + 50% D	64.08	66.75	Synergistic
Total flavonoid content (TFC)			
50% A + 50% C	27.41	27.29	Indifferent
50% A + 50% D	28.81	28.52	Indifferent
50% B + 50% C	25.29	25.24	Indifferent
50% B + 50% D	26.69	26.21	Indifferent
DPPH scavenging assay			
50% A + 50% C	32.98	77.26	Synergistic
50% A + 50% D	34.10	47.04	Synergistic
50% B + 50% C	49.43	29.84	Antagonistic
50% B + 50% D	50.54	61.19	Synergistic
Lipid peroxidation inhibition (LPI)			
50% A + 50% C	71.14	70.83	Indifferent
50% A + 50% D	71.17	69.30	Indifferent
50% B + 50% C	69.60	68.22	Indifferent
50% B + 50% D	69.63	68.08	Indifferent

(A) N. inophyllum (B) *P. minus* (C) *A. squamosal L.* (D) *S. rebaudiana*

From the overall phytochemical content evaluation and antioxidant activity conducted, there are only four synergistic interactions are evaluated. The highest synergistic effect is showed on the combination of *N. inophyllum* and *A. squamosal L* on the percentage inhibition of DPPH. Meanwhile, the lowest synergism is presented on the combination of *P. minus* and *S. rebudiana* for its total phenolic content. It is believed that phenolic compounds also have the ability in preventing the autoxidation of lipids [23]. Basically, this synergistic interaction may reduce their adverse side effects which are caused by the higher concentrations of a single herb [11]. The increase in mechanism leads to optimum antioxidant activity [24].

In previous study, it has been reported that some antioxidants in combination act in a regenerating manner. This means that either the stronger regenerating the weaker, antagonistic effect or the weaker regenerating the stronger, synergistic effect [25]. This can be explained from as study that stated selected individual plants contained abundant quantity of phenolics and flavanoids and their polyherbal combination with green tea was found to produce best antioxidant activity among all individual extracts. This will help in avoiding undesirable side effects due to higher doses of single herb [26].Therefore, it is applicable to mix the plant extracts to have good antioxidant activity.

CONCLUSIONS

In conclusion, most of the polyherbal formulations of the aqueous extracts of the studied leaves of plant extracts showed indifferent interaction towards antioxidant activity. However, there are four synergistic interactions which obtained by significant increased when combining plant extracts. Thus, this finding may leads to the understandable product development in the future particularly in the studied herbs.

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