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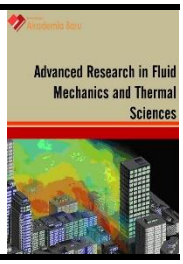

Antioxidant Activity and its Interaction Effect on Polyherbal Formulations of *Nephrodium inophyllum, Polygonum minus Annona squamosal L.* and *Stevia rebaudiana*

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ABSTRACT

Antioxidant activities have a great potential on various plant extracts due to the complexity of the composition of bioactive compounds present in the plants. Besides that, various combinations of plants can give possible interactions such that synergistic, antagonistic and indifferent between particular compounds which is importance in development of food products. Therefore, the objective of this study is to determine and evaluate the antioxidant activity and the possible interaction effect on aqueous extracts of leaves of *Nephrodium inophyllum* (pakis merah), *Polygonum minus* (kesum), *Annona squamosal L.* (nona) and *Stevia rebaudiana* through in-vitro assay. In this study, the antioxidant activity of the extracts individually and their different combinations were analyzed by total phenolic content (TPC), total flavonoid content (TFC), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and lipid peroxidation inhibition (LPI) assay. Meanwhile, the interaction effect was evaluated by comparing the experimental antioxidant activity of the mixtures with calculated theoretical values and the interactions of the compounds were determined. The results depicted that individual extract of *N. inophyllum* had significant flavonoid content and lipid peroxidation inhibition while P. minus has the highest phenolic content and percentage inhibition DPPH compared to other plant extracts. For the interaction effect, synergism was exhibited on only four combinations of plant extracts for all assays conducted with the highest one was shown on DPPH assay for combination of *N. inophyllum* and *A. squamosal L.*. This proved that there was a little molecular interaction between particular phenolics or flavonoids compound found in plant extracts. Out of all studied plant extracts, there was only one antagonistic interaction between P. minus and A. squamosal L on DPPH assay. However, indifferent effect on other compounds was revealed in the majority of all plant extract combinations for all assays. Hence, this present study justifies the aqueous extracts have significant antioxidant properties individually thus it gave possible interaction effects that could contribute for future design in food industries.

Keywords:
DPPH; TPC; TFC; antioxidant; interaction

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1. Introduction

In this current era of modernization, World Health Organization has been reported that around 80% of the populations of developing countries including Malaysia are still depends on herbal medicines to treat various diseases [1, 2]. This might be due to its high margin of safety, cost-effective, eco-friendly and readily availability of the herbal plants [3]. These plants are well known to have rich sources of phytochemical content such that phenols, flavonoids, tannin, fatty acids and other compound which believe might exert high content of antioxidant compounds and also have been recognized as having potential in reducing variety of diseases risk [4, 5]. 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 [6, 7].

Basically, 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 [8]. In healthy individuals, free radical production is continuously balanced by natural anti-oxidative deference systems. The production of antioxidant compound is due to counteract of reactive oxygen species (ROS). ROS and free radicals could cause lipid peroxidation in which could lead to the spoilage and deterioration of food [9]. Lipid peroxidation is used to measure the inhibitory activity of the plant extracts caused by the hydrogen peroxides [10]. 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.

In this study, there are four different types of Malaysian herbs from different families were selected to develop the formulation. *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 [11]. Its leaves can prevent the oxidative damage on the fatty tissues and eliminate the unnecessary free radicals [10]. Meanwhile, *N. inophyllum* (pakis merah) 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 effect [10]. Another plant is *A. squamosal L.* or locally known as “nona” is from family of Annonaceae. It was found that this plant did not show any toxic reactions or lethality when consumed it [12]. The use of S. rebaudiana belonging to the family Compositae is mostly to act as natural sweetener. Many studies have showed the potential of *A. squamosal L.* and *S. rebaudiana* on antioxidant activity [12, 13].

Antioxidant activity can have limitation on its effectiveness only at high concentration of certain substances or plant extracts as it could alter the physical properties such as taste, colour and texture of the herbal extracts. This is because these herbal extracts are known to act as natural antioxidant agents which can prevent food spoilage caused by oxidation action. Therefore, the new therapy to modify the antioxidant activity is through the interaction effect of synergistic, antagonistic, additive or indifferent which may help in reducing their adverse side effects which are caused by the higher concentrations of a single herb [14, 15]. Synergism effect is very significant in the mixture of the plant extracts to have a good antioxidant activity. In general, synergistic (positive interaction) is defined as a combination of two or more substances shows higher effects than the sum of the single substances while antagonistic (negative interaction) 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 plant extracts are combined together [15]. Thus, this study aims to evaluate the antioxidant activity of aqueous extracts of leaves of *N. inophyllum, P. minus, A. squamosal L.* and *S. rebaudiana* as well as it random combinations of two different herbal extracts towards total phenolic content (TPC), total flavonoid content (TFC), 2,2-

diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and lipid peroxidation inhibition (LPI) assay. Then, it was followed by evaluation of their interaction effect towards the antioxidant activity.

2. Methodology

2.1 Chemicals and Instruments

DPPH (SIGMA ALRIDCH, Malaysia), Gallic acid (SIGMA ALRIDCH, Malaysia), Folin-ciocalteu reagent (SIGMA ALRIDCH, Malaysia), rutin (SIGMA ALRIDCH, Malaysia), linoleic acid (SIGMA ALRIDCH, Malaysia), methanol (QRec, Malaysia), aluminium chloride hexahydrate (QRec, Malaysia), sodium carbonate (Bendosen, Malaysia), sodium nitrate (Bendosen, Malaysia), ethanol 95 % (HmBG, Germany), phosphate buffer (pH7), ferrous chloride, hydrochloric acid and butylated hydroxytoluene (BHT) were analytical grade. The instrument used was UV-Vis spectrophotometer (BIOMATE 3S), vortex mixer (LABMART 3000, Malaysia), Ultra-low temperature freezer (-80°C) (New Brunswick, Germany), freeze dryer (Labconco, United States), Centrifuge (MPW351R, India) and blender (Waring Commercial, United States of America). All the chemicals and instruments were provided at Food analysis and Food Microbiology laboratory Universiti Tun Hussein Onn Malaysia (UTHM).

2.2 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 Resouces Sdn Bhd, Sungai Buloh, Selangor. Those plants were prepared according to Gowdhami, Sarkar and Ayyasamy with slight modification [16]. 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.

2.3 DPPH Radical Scavenging Activity Assay

The DDPH assay was done as described by Afsharnezhad *et al.,* with slight modifications [17]. This is 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.

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

where $A_{control}$ = absorbance value of control, A_{sample} = absorbance value of sample

2.4 Total Phenolic Content (TPC) Assay

Total phenolic content was determined by using Folin-Ciocalteu assay by referring to method [18] 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.

2.5 Total Flavonoid Content (TFC) Assay

The total flavonoid content was determined by using aluminium chloride calorimetric assay as mentioned by [19] 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.

2.6 Lipid Peroxidation Assay

Lipid peroxidation inhibition assay was done 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 60oC 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 [20].

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

where A_0 = Absorbance of control reaction, A_1 = Absorbance of sample extract.

2.7 Calculation of Synergistic Effects of Antioxidant Mixtures

The experimental antioxidant activity was determined analytically using the method described in previous sections while theoretical values were calculated as the sum of the antioxidant values of each compound. If the value of experimental antioxidant activity was greater than the value of 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. Meanwhile, indifferent effect was claimed when there is no effect or significant different between experimental values and theoretical values [21].

2.8 Statistical Analysis

Data that have been obtained in this study were analysed where each determination were conducted in triplicate and the results obtained as an average value which is expressed in mean ± standard deviations [22]. 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 $p<0.05$.

3. Results and Discussion

Based on the results obtained (Figure 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 [12]. There were study reported that antioxidant activities of plat materials have strong relationship with the phenolic and flavonoid content in the plant extracts [2, 23]. In previous study, *Ortosiphon aristatus* aqueous extract has higher properties of antioxidant of 78.84 % compared to *Phyllanthus niruri, Strobilanthes crispus* and *S. rebudiana* which might be because of its total phenolic content in the plant is in compatible range [24]. Therefore, effectiveness of antioxidant activities for each plant extracts was mainly depending on its phenolic and flavonoids content.

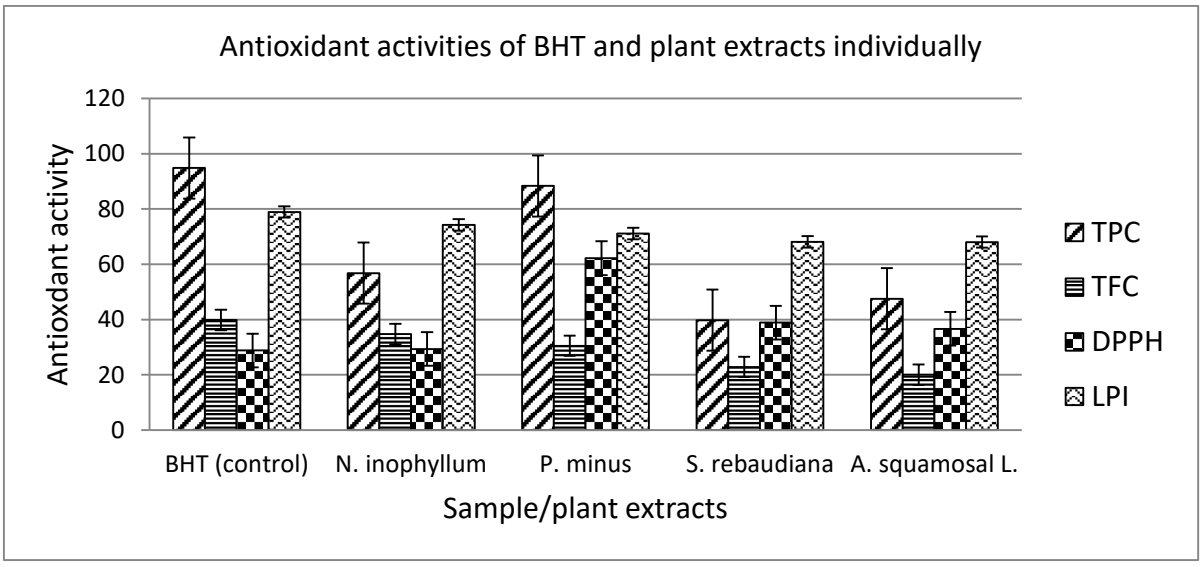

Fig. 1. Antioxidant activity of BHT and plant extracts individually

However, in food industry, single herb or substance can contribute to a good antioxidant activity only at high concentrations as it can lead to alteration in the taste, colour, odour and texture of foods and indirectly give constraint on preservation system [25]. In this study, herbal plant extracts that were studied are having different secondary metabolites which mainly compose of phenolics and flavonoids with considerable antioxidant activity. They are the main components in many of the food products in the industry and sometimes are used in combinations [20, 26]. Thus, an approach has been identified by combining the plant extracts to enhance the antioxidant activity through possible synergistic interaction although at low concentration of the plant extracts [14]. For this study, the

combination of the plant extracts were randomly mixed of only two herbal extracts for each formulation on all assays as shown in Table 1.

Table 1
Interaction effect of combinations of plant extracts on antioxidant activity

Assays	Combination	Predicted value	Experimental value	Types of interaction
Total Phenolic Content (TPC)	50% A + 50% C	52.15	52.18 ± 0.051^{c}	Indifferent
	50% A + 50% D	48.29	49.44 ± 0.068^{d}	Indifferent
	50% B + 50% C	67.94	67.95 ± 0.13^{b}	Indifferent
	50% B + 50% D	64.08	66.75 ± 1.16^{b}	Synergistic
Total Flavonoid Content (TFC)	50% A + 50% C	27.41	27.29 ± 0.00^{c}	Indifferent
	50% A + 50% D	28.81	28.52 ± 0.00^{b}	Indifferent
	50% B + 50% C	25.29	25.24 ± 0.00^{e}	Indifferent
	50% B + 50% D	26.69	26.21 ± 0.029^{d}	Indifferent
DPPH scavenging assay	50% A + 50% C	32.98	77.26 ± 3.53^{a}	Synergistic
	50% A + 50% D	34.10	47.04 ± 1.54^{c}	Synergistic
	50% B + 50% C	49.43	29.84 ± 0.21^{d}	Antagonistic
	50% B + 50% D	50.54	61.19 ± 6.24^{b}	Synergistic
Lipid peroxidation inhibition (LPI)	50% A + 50% C	71.14	70.83 ± 1.16^{b,c}	Indifferent
	50% A + 50% D	71.17	69.30 ± 0.39^{b}	Indifferent
	50% B + 50% C	69.60	68.22 ± 0.74^{c}	Indifferent
	50% B + 50% D	69.63	68.08 ± 1.05^{c}	Indifferent

(A) N. inophyllum (B) *P. minus* (C) *A. squamosal L.* (D) *S. rebaudiana*
Values of TPC, TFC, DPPH and LPI of polyphyto extracts are mean ± standard deviation (n = 3)
Different letter (a-d) in the same column indicates significantly different at ($p < 0.05$) as measured by TukeyHSD^{a} multiple test.

Based on the results obtained, it indicated 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 [27]. This might be because of the plant extracts were randomly mixed without using any tools such as statistical software for optimization process that could contribute to different percentage of concentration of plant extracts instead of equally mixed of two plant extracts. There was study reported that combination of aqueous extract of 23.96% of *S. crispus*, 0.62% of *P. niruri* and 75.42% of *O. aristatus* on antioxidant properties which shown synergistic effect that were conducted using statistical software package [4]. Besides that, synergistic effect was also shown in proportion of 40%:60% for combination of *Pterospartum tridentatum* and *Cymbopogon citratus* or *Gomphrena globosa* and *C. citratus.* Therefore, a right proportion and combination of plant extracts could contribute to synergistic interaction.

From the overall antioxidant activities conducted, there were only four synergistic interactions were evaluated. The highest synergistic effect was showed on the combination of *N. inophyllum* and *A. squamosal L.* on the percentage inhibition of DPPH. Meanwhile, the lowest synergism was presented on the combination of *P. minus* and *S. rebaudiana* for its total phenolic content. It was believed that phenolic compounds also have the ability in preventing the autoxidation of lipids [28]. A study had stated plant materials derived polyphenols displayed characteristics inhibitory patterns towards the antioxidant capacity assays [29]. Basically, this synergistic interaction may reduce their adverse side effects which are caused by the higher concentrations of a single herb [14]. The increase in mechanism leads to optimum antioxidant activity [2]. For DPPH assay, there was only one antagonistic interaction was 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 [30, 31]. Antagonistic interaction indicated that the plant extracts combination was not

suitable for development of food or supplement industries as it was declined the food preservation systems.

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 [32]. This can be explained from a 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 [33]. Therefore, it is applicable to mix the plant extracts to have good antioxidant activity.

4. Conclusions

In conclusion, each of the studied plant extracts showed high antioxidant activity for all assays tested. Furthermore, 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 design of food or supplement industry particularly in the studied herbs.

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