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Abstract

Introduction: Kacangma (*Leonurus sibiricus*), turmeric (*Curcuma longa*) and black pepper (*Piper nigrum*) are the oldest and most important spice crops used for culinary purposes in Malaysia for centuries. Although many biological activities including antioxidant properties (AP) have been reported, there are no confirmed reports on the activities of this cocktail, nutri-Pepper enhancer.

Purpose: The study evaluated the AP of the Nutri-Pepper enhancer *in vitro.*

Methodology: The AP were evaluated using *in vitro* antioxidant assays where the total polyphenolic content (TPC), DPPH radical scavenging activity, tyrosinase inhibition activity, xanthine oxidase superoxide scavenging activity (XOD), and oxygen radical absorbance capacity (ORAC) were measured.

Results: Based on the antioxidant activity range, extract of Nutri pepper enhancer was high in tyrosinase inhibition activity, 87.16%, moderate in xanthine oxidase superoxide scavenging activity (XOD) 69.72% and low in 1,2-diphenyl-2- picrylhydrazyl radical scavenging activity (DPPH) 46.78%. On the other hand, the extract showed high ORAC and total phenolic content with the value of 236,00 µmol TE/100g and 282 mg/100 GAE.

Conclusion: This finding proven that the consumption of this product can reduce or even eliminate the harmful effects on humans from contaminants in foods and from the environment; this is even more important for people living in polluted cities.

Keywords: *Antioxidant, Nutri pepper enhancer, xanthine oxidase superoxide scavenging (XOD) activity, oxygen radical absorbance capacity (ORAC), 1, 2-diphenyl-2- picrylhydrazyl radical scavenging activities (DPPH).*

1.0 INTRODUCTION

The complex biochemical reactions of the body and increased exposure to environmental toxicants and dietary xenobiotics result in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to oxidative stress under different pathophysiological conditions (Nimse and Pal, 2016). Antioxidants prevent oxidative damage through one-electron reactions with free radicals [superoxide radicals ($0_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($O^{\bullet}$), and hydrogen peroxide (H_2O_2)] that adversely alter cellular lipids, protein, DNA, and polysaccharides (Nimse and Pal, 2016; Lobo *et al*., 2010). Therefore, a balance between free radical and antioxidant concentrations is necessary to maintain proper physiological functions (Lobo *et al*., 2010).

Many people consume antioxidants as a defense against oxidative stress. Antioxidants in the form of commercial food additives have been manufactured synthetically and may contain high amounts of preservatives (Tanvir *et al*., 2015). Some synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ), have been reported to produce toxins or act as carcinogens (Lobo *et al*., 2010; Shasha, 2014). Therefore, identifying potential natural antioxidant sources can be a useful alternative to ensure sound health (Afroz *et al*., 2016). Food is the source of essential nutrients for growth and maintenance, but other bioactive compounds of plant origin promote health by slowing the aging process and preventing disease (Denre, 2014). As a result, antioxidant constituents in plant material have piqued the interest of scientists, food manufacturers, cultivators, and consumers for their roles in the maintenance of human health (Tanvir *et al*., 2015).

Recently, attention has been paid to the role of medicinal plants as a source of natural antioxidants. Curcumin and leonurine are the main constituent of turmeric and kacangma (*Leonurus sibiricus*) with antioxidant, anti-inflammatory, and anti-carcinogenic activities. Several reports have documented the protective effect of curcumin and leonurine against the oxidative damage inflicted by paracetamol (Aycan *et al*., 2014) and lead (Mabrouk *et al*., 2016) in the liver; Curcumin could also alleviate the inflammation associated with Alzheimer's disease (Abulfadl *et al*., 2018).

Piperine is the bioactive alkaloid ingredient of black pepper (*Piper nigrum*). It has various pharmacological potencies including antioxidant and anti-inflammatory actions (Srinivasan, 2007; Vijayakumar and Nalini, 2006). Recently, co-administration of plant extracts has gained great attention, whereas using two or more components may enhance their clinical efficacy (Liang *et al*., 2014). Lambert et al., (2004) have demonstrated that the bioavailability of (-)-epigallocatechin-3-gallate was increased by 1.7-fold when it was co-administrated with piperine in a mouse model. Despite the proven antioxidant activity of curcumin and leonurine, it has poor bioavailability due to its lower water solubility, poor absorption when given orally, and rapid elimination from plasma (Alkharfy *et al*., 2015). Accordingly, we hypothesize that piperine may potentially enhance the antioxidant efficacy of curcumin and leonurine by increasing its bioavailability. However, several investigations have shown the capacity of only a few kinds of pepper formulas (Zorica *et al*., 2019). Moreover, the antioxidant property of pepper based nutraceutical in itself has not been done systemically (Jeena *et al*., 2014). In view of the situation

in which the ORAC value of food is now known, it may be important to also evaluate the antioxidant capacity of the newly developed pepper based nutraceutical, Nutri pepper enhancer with ORAC, DPPH and XOD assay.

2.0 OBJECTIVE

The objective of this study was to evaluate the antioxidant properties of nutri-pepper enhancer by three bioassay systems, namely xanthine oxidase superoxide scavenging system (XOD) and 1,2-diphenyl-2-picrylhydrazyl radical scavenging system (DPPH) and Oxygen radical absorbance capacity (ORAC) assay.

3.0 MATERIAL AND METHOD

3.1. Subjects

Newly developed pepper based nutraceutical, nutria-pepper enhancer were investigated. These nutri-pepper enhancers were obtained from Malaysian Pepper Board whose manufacturing is governed by the regulations of the Pharmaceutical Affairs Law, and strictly controlled by other government regulations including Good Manufacturing Practice. As a result, products are assured of quality and safety at the highest level.

3.2. Total Phenolic Content Determination

Folin–Ciocalteu method was used to determine the total phenolics content of sample (Singleton and Rossi, 1965).

3.3 Antioxidant Activity Determination

3.3.1 Xanthine Oxidase Superoxide Scavenging System

The inhibitory effect on XO was measured spectrophotometrically at 295 nm under aerobic condition, with some modifications, following the method reported by Chang *et al*., 1996. A well-known XOI, allopurinol (100 µg/ml) was used as a positive control for the inhibition test. The reaction mixture consisted of 300 µl of 50 mM sodium phosphate buffer (pH 7.5), 100 µl of sample solution dissolved in distilled water or DMSO, 100 µl of freshly prepared enzyme solution (0.2 units/ml of xanthine oxidase in phosphate buffer) and 100 µl of distilled water. The assay mixture was pre-incubated at 37°C for 15 min. Then, 200 µl of substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37°C for 30 min. Next, the reaction was stopped with the addition of 200 µl of 0.5 M HCl. The absorbance was measured using UV/VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having 100 µl of DMSO instead of test compounds in order to have maximum uric acid formation. The XOI activity was calculated using as follow, in which α is the activity of XO without test extract and β is the activity of XO with test extract. % XO inhibition = $(1 – \beta/\alpha) \times 100$(1).

3.3.2 DPPH Radical Scavenging Activity

The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts (Blois, 1985; Peng *et al*., 2007). Approximately 40 µg/ml of the extract was added at an equal volume (2.5 ml) to methanol solution of DPPH (0.3 mM, 1 ml). After 30 min at room temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation: DPPH antiradical scavenging capacity (%) = [1 − ($Ab_{of\ sample}$ − $Ab_{of\ blank}$)/$Ab_{of\ control}$] × 100. Methanol (1.0 ml) plus plant extract solution (2.5 ml) was used as a blank, while DPPH solution plus methanol was used as a control.

3.3.3 Tyrosinase Inhibition Assay

The assay was determined spectrophotometrically as described by Tomita *et al*., 1990 with minor modification. L-DOPA was used as substrate. Each well contained 40 µg/ml L of sample added with 80 µL of phosphate buffer (0.1 M, pH 6.8), 40 µL of tyrosinase (1000 units/m), and 40 µL of L-DOPA (2.5 mM). Each sample was accompanied by a blank that had all the components except tyrosinase. Kojic acid was used in place of sample as positive control. Absorbance was measured at 515 nm with 655 nm as a reference. Percentage of inhibition was calculated using the following formula:

Percentage of tyrosinase inhibition (%): (Control OD − Sample OD/Control OD), × 100

Where Control OD is absorbance of control and Sample OD is absorbance of test sample.

3.3.4 ORAC Determination

ORAC is the direct capacity of chain-breaking antioxidant based on the hydrogen atom transfer mechanism. In this assay system, β-phycoerythrin (β-PE) was originally used as a fluorescent probe, changed to Fluorescein, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analog) as a control standard. Fluorescein is added to sample or Trolox. And then, after AAPH is added, active oxygen appears and the fluorescence intensity of Fluorescein decreases. If there is any antioxidant, decrease of the fluorescence intensity is delayed. The antioxidant capacity is obtained by calculating the difference of the decrease of the fluorescence intensity between sample and Trolox. Results are expressed as ORAC units, where 1 ORAC unit equals the net protection produced by 1 μM Trolox. In this study, samples were prepared in triplicate, and the results were expressed as mean.

The oxygen radical absorbance capacity (ORAC) assay was carried out according to the method described by Huang et al., (2002). AAPH, Fluorescein and Trolox were completely dissolved in 75 mM disodium phosphate buffer (pH 7.4). At first, 150 μL of 167 nM Fluorescein was added to each well of a 96-well polypropylene plate. After 25 μL of the blank solution, 50, 25, 12.5, and 6.25 μM of Trolox standard solution, and the sample solution was added to the wells, the plate was covered with a lid and incubated in the preheated (37°C) Fluorescence reader for 10 min with a 3 min shaking during this time. Then, followed by the addition of 25 μL of AAPH kept in an ice bath to each well of the plate, the fluorescence was measured every 5 min for 30–45 min. Approximately (1 g) of sample extract was completely dissolved in 5 mL of hot water by a supersonic wave for 2 h. Furthermore, it was mixed in vortex and centrifuged at room

temperature for 3000 r.p.m., 10 min. A supernatant was collected and diluted 10 000 times in a 75 mM disodium phosphate buffer (pH 7.4) for the ORAC measurement. ORAC value was expressed as the capacity of 100 g in each object.

3.4 Statistical Analysis

All data were expressed as means ± standard errors of triplicate measurements and were subjected to statistical analysis of ANOVA (SPSS 15.0). A value of $P < 0.05$ was considered as significant value of the data.

4.0 RESULTS AND DISCUSION:

The evaluation of XO inhibitory activity of nutria-pepper enhancer was conducted at a concentration of 100 µg/ml, at which 85.81% of the extracts were found to have XO inhibitory activity. This justified the fact that Malaysian medicinal plants have well diverse chemical structures from their secondary metabolite and chemical diversity (Abd Aziz *et al*., 2011) which makes them promising remedies for antioxidant supplement in humans. To our knowledge, there have been no studies or reports to date regarding inhibition of XO activity by pepper based herbs, but a small number of studies of plant, vegetable, and flavor extracts have been reported to possess XO-activity inhibition (Ong *et al*., 2017). The direct antioxidant activities of this product further enhance the capacity of this product as good antioxidant agents.

The DPPH free radical scavenging activity is a widely used model for evaluating the free radical scavenging ability of various compounds. The absorbance decreased at 517 nm, resulting in a color change from purple to yellow, as radicals were scavenged by antioxidants through the donation of hydrogen to form the stable DPPH molecule. In DPPH assay system, antioxidants will either transfer an electron or hydrogen atom to the stable DPPH radical, thus neutralizing its free radical character. The ability of an extract or any other agent to neutralize DPPH can be considered, at least as part of the antioxidant properties. Results in Table 1 showed free radical scavenging activity of this extracts. The *in vitro* antioxidant activities of nutri-pepper enhancer at 40µg/ml were found to be 80.12, with reference to standard (Gallic acid) 97.5±0.5 (Table 1). This finding indicated that the polyphenol compound the presented in this product can react rapidly with these free radicals and retard or alleviate the extent of oxidative deterioration.

Normally, antioxidant agent poses tyrosinase inhibition activity. Hence, further investigations on tyrosinase inhibition mechanism of these extracts were conducted. Table 1 showed anti-tyrosinase activities of Nutri pepper enhancer extracts. At concentration of 150 µg/mL, the tyrosinase inhibition activity of the sample was 73.16%. The tyrosinase inhibition of extracts was comparable to a well-known powerful tyrosinase inhibitor, kojic acid (78.84%) at same concentration. The result demonstrated that antioxidant activity of this extracts by tyrosinase inhibition assay had similar trend with DPPH assay with strong antioxidant properties.

Plant bioactive compounds such as phenolic acids abundantly present in fruit and vegetables have received considerable attention because of their wide range beneficial effects which includes; antioxidant, anti-cancer, and anti-inflammatory (Yang et al., 2001). In this study, total phenolic content was measured using the Folin–Ciocalteu method. The linear calibration curve was plotted by the series of gallic acid concentrations against their absorbance. This gave a coefficient of correlation (r) value of >0.95. The result showed that the phenolic content of Nutri

Pepper Enhancer was found to be 282±5.8 GAE/mg (Table 1). This value is considered high when compared with other established herb for example *osmarinus officinalis*, *Salvia officinalis*, *Thymus vulgaris*, *Origanum vulgare*, *Ocimum basilicum*, *Melissa officinalis*, *Mentha piperita*, *Origanum majorana*, *Satureja hortensis* and *Hyssopus officinalis* with the total phenolic contents ranged between 100-150 mg GA/100g (Beata and Marek, 2019).

ORAC assay is used to measure the relative antioxidant activity of sample using fluorescence-based technology of detection. The presence of antioxidant in sample will inhibit the fluorescence decay by a hydrogen atom transfer mechanism. In general, food that that shows high ORAC value is high in it measure of antioxidant capacity. Table 1 shows the ORAC value of Nutri pepper enhancer extracts which was expressed as mmol TE/g with the ORAC value of 236,000 mmol of TE/g of extract weight. This finding is significant higher than positive control which further support the hypothesis that this product is a good antioxidant agent. The phenolic compound which was detected in the samples after phytochemical screening might also contribute to the termination of chain radical reactions by donating hydrogen atoms to the peroxy radical. The therapeutic effects of product were further supported by the present of high ORAC value of this product.

Due to high antioxidant activity, this pepper based product enable to suppress harmful effects of carcinogenic pollutants that may be present in foods and beverages, especially aflatoxins, heterocyclic amines, acrylamide, 1,2-Dimethylhydrazine and cadmium. Besides, this product also can neutralize the harmful effects of hazardous solvents and motor exhaust emissions from road transport in urban areas. Therefore, it is important and reasonable to encourage people to consume this pepper based nutraceutical product regularly in order to protect them from harmful environmental impacts, especially in large, polluted cities.

Table 1: Antioxidant activities of Nutri pepper enhancer determine by different experiment

No	Sample	Sample Description	DPPH free scavenging activity (%)	Superoxide free radical scavenging activity (%)	Tyrosinase inhibition activity (%)	ORAC µmol TE/100g	Total phenolic content mg/100 GAE
	Sample concentration		40 µg/ml	100 µg/ml	40 µg/ml	100 g	200 µg/ml
1	Nutri pepper enhancer	capsule	80.12±6.71	75.81±7.11	87.16±6.71	236,000	282±5.8
2	Galic acid (standard)		97.75± 5.56				
3	Allopurinol (standard)			97.05±7.22			
4	Ascorbic acid (standard)			84.36±0.14			
5	kojic acid (standard)				78.84		
6	Catechin (green tea)					13,690	

5.0 CONCLUSION

Nutri pepper enhancer exhibited superior and potent antioxidant capacities in terms of the total, DPPH radical scavenging activity, superoxide free radical scavenging activity, tyrosine inhibition activity, and these results were in consistent with high total phenolic, contents values. The present study demonstrates high phenolic content and antioxidant potential of nutri pepper enhancer that could contribute to sustain antioxidant status and protect against free radical damage. Nutri pepper enhancer is thought to be used as food supplement against free radicals generated in response to oxidative stress and served as strong anti-oxidant agent to protect people from acute and chronic diseases. Also, it is thought that these data will be reference for future studies.

6.0 RECOMMENDATIONS

This finding also presents a strong body of evidence that the consumption of this product can reduce or even eliminate the harmful effects on humans from contaminants in foods and from the environment; this is even more important for people living in polluted cities. All of this information will hopefully add to an already high level of interest toward spices and culinary herbs. Spices and herbs should certainly be incorporated as integral parts of healthy, nutritious eating, and as functional food ingredients.

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