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FULL PAPER

Antioxidant and antimicrobial activity of lemuni noodle

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Abstract

Vitex negundo Linn. commonly known as the five-leaved chaste tree in English, lemuni in Malay and nirgundi in Hindi. In Malaysia, 'Nasi lemuni' is popular food that is based on the leaves of this plant. In this study, the noodles were made from lemuni leaves, and the antioxidant and antimicrobial properties of this noodle were determined. The methanol extract of flavonoids produced the highest flavonoid content (2130.77±1.00 µg QE/g of sample), whereas the distilled water extract had the highest tannin content (2961.40±0.58 µg CE/g of sample) in lemuni noodle. For evaluating antioxidant capacity of lemuni noodle, Ferric Reducing Antioxidant Power (FRAP) and 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) assays were used. Both assays showed the highest capacity in methanol extract: DPPH (63.06±0.44%) and FRAP (3236.67±1.53 (µM Fe(II)/g). The disc diffusion method was used to determine the antimicrobial properties of lemuni noodle against *Escherichia coli, Salmonella* spp., *Staphylococcus aureus* and *Bacillus subtilis* bacteria. Ethanol and methanol extracts produced the largest inhibition zones against these bacteria, but there was no inhibition towards *Staphylococcus aureus* under the methanol extract. Hence, methanol extract of lemuni noodle revealed the higher antioxidant and antimicrobial properties compared to distilled water extract.

1. Introduction

Vitex negundo Linn. or lemuni is commonly known as the five-leaved chaste tree in English, *lemuni* in Malay and *nirgundi* in Hindi. It has been used as medicine throughout the greater part of India and is found mostly in warm zones and high altitudes of 1500 m in the outer Western Himalayas (Vishal 2005). In South and Southeast Asia, lemuni is widely used in folk medicine.

The functional food can be either a natural, unmodified food or food with different modifications with a similar purpose, which is to produce food for the benefit of the consumer (Howlett, 2008). Noodles, made with flour, water, and regular or alkaline salt, have been part of the Asian staple diet for thousands of years. At present, noodles take up over 40% of the total flour consumption in East and Southeast Asian countries (Kim *et al*., 2006).

The rationale of this study to be carried is to fully utilise lemuni leaves, which is rich in antioxidant (Rabeta and An Nabil, 2013). The idea of this study came across since there is a study that encourages utilizing this plant for commercial purposes (Brindha *et al.,* 2012). A study on lemuni leaves has provided limited information on its antimicrobial properties (Panda *et al.,* 2009). Hence, the antioxidant and antimicrobial properties of lemuni noodle were mainly focused to encourage people to eat functional and healthy food.

2. Materials and methods

2.1 Plant materials

The leaves of lemuni were collected from Kuala Kurau, Perak, Malaysia. The voucher specimen (USM Herbarium 11461) was deposited in the herbarium of School of Biological Sciences, Universiti Sains Malaysia.

2.2 Preparation of lemuni leaves powder

The following methods were done in Food Processing Laboratory of School of Industrial Technology, Universiti Sains Malaysia. The leaves of lemuni were plucked from the stem and washed thoroughly using tap water. The leaves were kept at -20° C prior to freeze-dried for 3 days at -50°C. The freeze-dried leaves were ground into a fine powder about 250 µm in size using a blender. The powder was kept at -20° C until use.

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2.3 Noodle preparation

The formulation of the noodle was 1 g of lemuni leaf powder, 99 g of wheat flour, 2 g of salt and 50 g of water (Tan *et al*., 2018). First, the salt was diluted with water, and then the lemuni leaf powder was thoroughly mixed with wheat flour. Next, the salt solution was added to the mixture of wheat flour and lemuni leaf powder. The mixture was mixed homogenously. Then, the dough rested for 30 min. By using a noodle machine, the dough was sheeted and slit into noodle shape. The noodles were then cooked using boiling water for 3 mins before being immersed in tap water approximately 1 min.

2.4 Noodle extraction

Extraction of freeze-dried lemuni noodle was performed using the method described by Khairul Ikram *et al.* (2009) with some modifications. Approximately 1 g of freeze-dried lemuni noodle sample was weighed, and 100 mL of methanol (80/20, v/v) was added to the sample. Then, the mixture was homogenized with a Homogenizer IKA® T25 digital ultra-Turrax at 11.0 × 1000 rpm for 1 min. The homogenized sample was poured into a 100 mL conical flask (wrapped with aluminium foil) then transferred to a dry orbital shaker incubator, ALPHA D13100, at 450 rpm and shaken for 12 hours at 29°C. After 12 h, the sample was removed and centrifuged using a Kubota 5100 at 2500 rpm/min for 20 min. The supernatant was collected and maintained in the dark at 20°C until use. The same procedures were repeated using the other solvents: ethanol (70:30, v/v) and water.

2.5 DPPH

The modified DPPH method by Sánchez-Moreno (1998) from the Brand-William *et al.* (1995) method, was followed in this experiment with slight modifications. By using a 1:1 ratio, 2 mL of extract was mixed with 2 mL methanolic solution of DPPH radical (100 µM). The mixture was shaken vigorously and maintained in the dark for 30 mins. The absorbance of the mixture was read at 516 nm using a Shimadzu UV-VIS Spectrophotometer UV-1650PC against a blank of absolute methanol without DPPH.

2.6 FRAP

Ferric Reducing Antioxidant Power (FRAP) was performed according to the method described by Kong *et al*. (2012) with slight modifications. The FRAP reagent was prepared using a mixture of acetate buffer, TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a ratio of 10:1:1. The solution was incubated for 30 minutes at 37°C. A blank was prepared. An amount of 0.2 mL of extracted samples was mixed with 3 mL FRAP reagent in a test tube and then vortex mixed. The absorbance of the mixture was determined using a Shimadzu UV-VIS Spectrophotometer UV-1650PC at 593 nm against the blank. The results were calculated based on a calibration curve plotted using iron sulphate ($FeSO_4$) at 10 mM, 20 mM, 30 mM, 40 mM, and 50 mM concentrations. The value obtained was expressed as µM Fe(II)/g of sample.

2.7 Determination of flavonoid content

Flavonoid content was determined using the calorimetric method using the method described by Wijekoon *et al.* (2011) with some modifications. The sample extract (0.5 mL) was added to the test tube followed by addition of 2.5 mL distilled water and 0.5 mL of 5% (w/v) of $NaNO_2$ into the same test tube. The mixture was maintained for 5 minutes. Then, 0.3 mL (w/v) of 10% $AlCl_3$ was added to the solutions in the test tube and incubates for 6 minutes at room temperature. After incubation, 1 mL of 1 M NaOH was added and the solution was diluted with 0.55 mL distilled water. The mixture was shaken vigorously. Absorbance was measured at 510 nm using a Shimadzu UV-VIS Spectrophotometer UV-1650PC. The total flavonoid content was expressed as µg quercetin equivalent (QE)/g of sample.

2.8 Determination of tannin content

The vanillin-HCl method was used for determination of tannins as proposed by Liu *et al.* (2008). Approximately 0.5 mL of extract was added to 3 mL of 4% vanillin reagent (w/v in methanol). Then, 1.5 mL of concentrated hydrochloric acid was added, and vortex mixed. The solution was maintained in the dark for 15 minutes at room temperature. The absorbance was read at 500 nm using the UV-Vis spectrophotometer (Shimadzu UV-1650PC). The blank was prepared by replacing the 0.5 mL extract with the solvent used in extraction. The tannin content in the sample was expressed as µg catechin equivalents (CE)/g of sample.

2.9 Antimicrobial activity

The bacteria used in this study were as *Escherichia coli, Salmonella* spp*., Staphylococcus aureus* and *Bacillus subtilis.* The bacterial strains were obtained from Microbiology Laboratory, School of Industrial Technology, Universiti Sains Malaysia. For *Salmonella* spp*.,* the pure culture was isolated in xylose lysine deoxycholate (XLD) agar to confirm the species before transferred into nutrient agar. While the other bacterial strains were grown and maintained on nutrient agar in petri dish until used. The bacterial strains in nutrient agar were kept in the incubator at 37°C.

2.9.1 Preparation of nutrient agar

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Approximately 12.6 g of nutrient agar medium was weight and suspended into 450 mL of distilled water in media bottles. The solutions were mixed and dissolved by continuous heating with vigorous stirring (magnetic stirrer) for 15 mins on a hotplate. Once completed, the media bottles were dispensed into a container to sterilize in an autoclave at 121°C for 2 hours. After the autoclave, the nutrient broth will be allowed to cool before being poured into a clean petri dish and solidify before being maintained at room temperature (28°C).

2.9.2 Preparation of nutrient broth

Approximately 8 g of nutrient broth medium were weighed and suspended in 1 L of distilled water in media bottles. The solutions were mixed and dissolved by continuous heating with frequent agitation by a magnetic stirrer on a hotplate. The heating process continued until the solution was completely dissolved. All the media bottles were dispensed into the container to sterilize in an autoclave at 121°C for 15 minutes. After being autoclaved, the nutrient broth was allowed to cool before being poured into a universal bottle and maintained in an incubator at 37°C.

2.9.3 Preparation of the disk

Whatman filter paper No. 1 was used as the disk in this method. The filter paper was punched using a sterile puncher to make it into a disk. The disk was dipped into the extract for later use for the antimicrobial test. The positive control was gentamicin, and the negative control was methanol.

2.9.4 Disc diffusion method

The disc diffusion method described by the National Committee for Clinical Laboratory Standard (NCCLS) (2015) was used for determination of antimicrobial activity of lemuni noodle. The confirmed bacterial strains were transferred into a universal flask containing nutrient broth and incubated approximately 4 to 6 hours before use. A sterilized cotton swab was dipped into the nutrient broth and spread thoroughly on a petri dish of Mueller-Hinton agar. The petri dish was first divided into 5 parts to differentiate the different types of extraction solvents and the positive and negative control. After the spread was dried, the disk containing extract was put on the agar and incubated overnight (18 to 24 h). The result of the inhibition test was determined by measuring the inhibition diameter around the disk that contained the extraction of samples in the disc together with the positive and negative control.

2.10 Data analysis

All the results were reported as mean ± standard deviation (SD) for triplicate determination. Analysis of variance and the significant differences between mean values were determined by using Duncan test at a level of significance of $p<0.05$. Statistical analyses were conducted by using SPSS 20.0 (SPSS for Windows, 2007, SPSS Inc., Chicago, USA).

3. Results and discussion

3.1 DPPH scavenging and FRAP assay activity

In this experiment, the DPPH assay is presented in terms of percentage free radical inhibited by the extract of lemuni noodle. The methanol extract of lemuni noodle produced the highest percentages of inhibition, which was 63.06±0.44% (w/v) followed by ethanol extract 61.72±2.11% (w/v) with no significant difference ($p>0.05$) in inhibition. The lowest percentage of inhibition was from distilled water extract of 29.41±0.92% (w/v) (Table 1).

Table 1. DPPH and FRAP results of *V. negundo* Linn. noodles extracted with various solvents

Solvent	DPPH Inhibition (%)	FRAP (μM Fe(II)/g of sample)
Distilled water	29.41±0.92[a]	12.53±1.79[a]
Methanol (80%)	63.06±0.44[b]	32.37±1.53[b]
Ethanol (70%)	61.72±2.11[b]	29.05±2.19[b]

Values are expressed as mean ± SD (n=3). Letters followed by the same letter in the same column are not significantly different from each other at $p>0.05$.

The highest FRAP activity was from the methanol extract of lemuni noodle with 32.37±1.53 (μM Fe(II)/g), the ethanol extract with 29.05±2.19 (μM Fe(II)/g) and the distilled water extract with 12.53±1.79 (μM Fe(II)/g) (Table 1). The same trends can be seen for both DPPH and FRAP assay, and the methanol extraction of lemuni noodle for both showed the highest antioxidant percentage for DPPH and antioxidant FRAP activity, followed by ethanol solvent.

This result proved that methanol and ethanol are good solvents to extract the compounds that are responsible for antioxidant activity from the sample, lemuni noodle. The addition of water into methanol and ethanol solvent created the moderate polarity of the solvent, resulting in higher extraction of polyphenols (Fatiha *et al.,* 2012) therefore give the highest antioxidant activity. There was also a study by Anokwuru *et al*. (2011) that claimed that methanol was the most suitable solvent for the extraction of polyphenolic compounds because it can inhibit the action of polyphenol oxidase, which may cause oxidation of polyphenols. Since polyphenols are subjected to form complexes with other components after being extracted, methanol can solve this issue (Yao *et al*., 2004). Those

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studies showed that methanol could be a good solvent for extraction, and this experiment also produced similar results for methanol extraction of lemuni noodle.

Paria *et al*. (2012) studied the phytochemical activity of lemuni leaves using different solvent extractions. The results showed the methanolic extract of lemuni leaves produced the highest phytochemical screening as compared to ethanol, chloroform, and petroleum ether solvents. The methanolic extract proved the presence of alkaloids, glycoside, reducing sugar, tannin, phenols, and flavonoids. In the previous study, revealed that the substitution with lemuni leaf powder (1 g (w/w)) in fresh and cooked noodle, tremendously increased the amount of total phenolic compound compared to control noodle (Tan *et al*., 2018). Lako *et al.* (2007) claimed that the preparation, processing, and cooking of the samples did reduce the antioxidant capacity by 15%. Therefore, the antioxidant and antimicrobial results are expected to be low from the literature review and from our previous study, and the extraction solvent might not produce the same result even using the same solvent.

3.2 Flavonoid content

The highest content of total flavonoids in lemuni noodle was obtained from methanol extract (21.31±1.00 µg QE/g). After methanol, ethanol had the highest content of flavonoids (16.46±0.35, µg QE/g) and water produced the lowest extraction of flavonoids (8.05±0.25 µg QE/g) in lemuni noodle (Table 2). The high amounts of flavonoids in lemuni leaf extract was previously reported by Sahayaraj and Ravi (2008). Flavonoids can cope and adapt to the continually changing environment. The latest study of phytochemical and biological evaluation of lemuni by (Ullah *et al*., 2012) also supported the presence of flavonoids in these leaves. Therefore, this result proved the presence of flavonoids in the sample of lemuni noodle. A study by Sanhita *et al*. (2012) showed that the leaf extract of lemuni is rich in antioxidants, phenolic, flavonoids, and many phytochemicals that contribute to antimicrobial, antioxidant, anticancer, hypercholesterolemic, and anti-ulcerogenic activities.

Table 2. Total flavonoids content of *V. negundo* Linn. noodles extracted with various solvents

Solvent	Flavonoids (µg QE/g of sample)[A]
Distilled water	8.05±0.25[a]
Methanol (80%)	21.31±1.00[c]
Ethanol (70%)	16.46±0.35[b]

Values are expressed as mean ± SD (n=3). Letters followed by the same letter in the same column are not significantly different from each other at $p>0.05$.
[A]Quercetin equivalents.

The methanol extract of lemuni noodle showed the highest flavonoid content, DPPH scavenging activity, and FRAP. There was a significant correlation between the scavenging activity of DPPH with flavonoid content (r=0.912; Pearson's correlation analysis) and FRAP activity with flavonoid content (r=0.961). Therefore, the antioxidant activity of lemuni noodle can be said to contribute to the presence of flavonoid compounds in the sample. The same findings were reported on the leaves of lemuni in a study by Zargar *et al*. (2011).

3.3 Tannin content

The distilled water extract of lemuni noodle was 29.61±0.58 (µg CE/g of sample) followed by ethanol and methanol with 4.05±0.29 (µg CE/g of sample) and 3.47±0.23 (µg CE/g of sample), respectively (Table 3). The highest tannin content was from the distilled water extract, which was is significantly different ($p<0.05$) from the ethanol and methanol extract of lemuni noodle.

Table 3. Tannin contents of *V. negundo* Linn. noodles extracted with various solvents

Solvent	Tannins (µg CE/g of sample)[B]
Distilled water	29.61±0.58[b]
Methanol (80%)	3.47±0.23[a]
Ethanol (70%)	4.05±0.29[a]

Values are expressed as mean ± SD (n=3). Letters followed by the same letter in the same column are not significantly different from each other at $p > 0.05$.
[B]Catechin equivalents.

The antioxidant capacity of DPPH and FRAP were negatively correlated with tannin; (DPPH and tannin r = -0.996, FRAP and tannin r = -0.979; Pearson's correlation analysis). The negative correlation occurred mainly because of the opposite result of solvent extraction. Distilled water extract result in highest tannin content, but for DPPH and FRAP, the highest antioxidant activity was observed from the methanol extract of lemuni noodle.

In the Vanillin-HCl assay, the determination of tannin content in the sample was difficult to achieve because another substituted monomeric flavanol might also react instead of tannin itself (Živković *et al*., 2009). Therefore, it can be said that distilled water has extracted compounds other than tannin that affected the estimation of tannin content in distilled water extract of lemuni noodle. The highest tannin content was found with distilled water and can be said to be overestimated. Additionally, tannins have some hydrophobic characteristics due to benzene rings in the structure, so the suitable solvent used to extract it should have hydrophilic characteristics, such as methanol and ethanol, in order to extract the hydrophobic structure of

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Table 4. Diameter of inhibition of *V. negundo* Linn. noodles extract

Bacteria	Diameter of inhibition from various extracts (mm) of samples				
	Distilled water	Methanol	Ethanol	Positive control	Negative control
B. subtilis	-	7.00	6.67	28.67	-
S. aureus	-	-	7.00	22.00	-
Salmonella spp.	-	6.50	6.83	25.33	-
E. coli	-	7.17	6.50	21.67	-

Results are expressed as mean ± SD (n=3). (-) means no zone of inhibition. Disc diameter = 6 mm

tannin (Seabra *et al*., 2017).

3.4 Antimicrobial activity

From Table 4, the extract of distilled water of lemuni noodle showed no inhibition in any bacteria. The methanol extract of the sample showed inhibition of both of gram-negative bacteria, *Salmonella* (6.50 mm) and *E. coli*. (7.17 mm), and one gram-positive bacteria, *B. subtilis* (7.00 mm). The extract of ethanol showed inhibition of all bacteria with the highest inhibition of *S. aureus* (7.00 mm) followed by inhibition of *Salmonella* (6.83 mm), *B. subtilis* (6.67 mm) and *E. coli* (6.50 mm).

The lack of inhibition of *S. aureus* bacteria by the methanol extract of lemuni noodle might be because the different solvents might extract different types and concentrations of compounds extracted (Moghadam *et al.,* 2010). Therefore, due to the inadequate concentration of antimicrobial compounds in the methanol extract of lemuni noodle, it cannot inhibit the growth of *S. aureus,* which makes it survive in this experiment. From the results shown in Table 4, the bacteria were mostly inhibited by ethanol and methanol extracts of lemuni noodle. This finding can be explained by the interference of those solvents because of the lipophilic properties of alcohol. The interference results in ineffective antimicrobial properties because the compounds in the extraction samples together with the solvent inhibited those bacteria (Aleksic and Knezev, 2014).

As for the distilled water extraction, no inhibition was observed against all the tested bacteria. Distilled water is a highly polar compound for use with hydrophilic compounds. A substance that can dissolve in water is known as a hydrophilic substance, whereas one that is immiscible in water is known as a hydrophobic substance. Lipophilic compounds are likely to induce efficient disruption of membrane structure in eukaryotic cells (Perianayagam *et al.,* 2012). Lipophilic compounds are the opposite of hydrophilic compounds. Therefore, compounds extracted by distilled water are mostly polar compounds following the dissolution principle. Therefore, the distilled water extract does not have the ability to disrupt the cell membrane of the bacteria and results in no inhibition against the tested bacteria. Moreover, the inability of distilled water to extract the sterol from the plants also contributes to this result. The reason can be seen from the study by Mbosso *et al.* (2010) in which the sterol-containing compounds were found to promote good antimicrobial properties due to their ability to interfere with membrane structures in bacteria cells as well as in yeasts.

Despite all those reasons, it can still be argued that because the distilled water extraction produced a higher tannin content compared with the methanol and ethanol extracts, all studies on tannin content can claim the challenge of obtaining real estimations of this compound due to the structural, molecular weight and high sensitivity of tannin itself (Alkurd *et al*., 2008; Živković *et al*., 2009). Therefore, the distilled water did extract compounds other than tannin that has overshadowed the antimicrobial ability of tannin to inhibit bacteria. Furthermore, the results from previous studies also proved the impotence of distilled water as an extraction solvent (Sultana *et al*., 2009; Sengul *et al*., 2009; Wijekoon *et al*., 2011; Fatiha *et al.,* 2012; Sujatha and Suresh, 2013). Therefore, there was a reduction in the antimicrobial strength of the distilled water extract.

4. Conclusion

In conclusion, it can be claimed that lemuni noodle did possess antioxidant and antimicrobial properties. This noodle is good to consume due to those properties. Even after being cooked, this noodle still showed antioxidant and antimicrobial properties. From the experimental results, lemuni leaves have been successfully adapted into food that is also carried together with good antioxidant and antimicrobial properties from lemuni noodle. For recommendations, the effect of storage on the antioxidant and antimicrobial properties of this noodle should be investigated to commercialize the lemuni noodle as a functional food.

Conflict of Interest

We declare that we have no conflict of interest.

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