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Cytotoxicity with Brine Shrimp Lethality Test, Antibacterial Activity, and Dipeptidyl Peptidase IV (DPP IV) Inhibitory Activity of Red Betel (*Piper cf. arcuatum* Blume) Stem Extract

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ABSTRACT

Piper cf. arcuatum Blume is a plant that has long been used in traditional medicine in Indonesia. The community has used the leaves as antioxidants, antimicrobials, antidiabetics, anti-inflammatory, and anti-cancer agents. However, the stem part of *Piper cf. arcuatum* Blume has not been widely used. In this study, we evaluated the cytotoxicity, antibacterial, and antidiabetic properties of hexane, ethyl acetate, and methanol extracts of *Piper cf. arcuatum* Blume stem. Cytotoxicity was assessed using the Brine Shrimp Lethality Test (BSLT) with *Artemia salina larvae*. Evaluation of its antibacterial properties against *Escherichia coli* and *Bacillus subtilis* and antidiabetic evaluation via dipeptidyl peptidase IV (DPP IV) inhibition. Based on the results of this research, the three extracts showed very strong cytotoxic effects, with LC_{50} values of 14.65, 17.38, and 33.74 ppm, respectively. In contrast, *E. coli* and *B. subtilis* bacteria did not appear to be significantly affected. The methanol extract possesses the highest potential for inhibiting DPP IV, with a % inhibition value of 49.23±0.1%. These findings indicated that the stem of *Piper cf. arcuatum* Blume could potentially function as an antidiabetic agent and can prevent cancer.

Keywords: *Piper cf. arcuatum* Blume, Cytotoxicity, Brine shrimp, Dipeptidyl peptidase IV.

Introduction

Piper cf. arcuatum Blume (PAB) is a plant that has long been utilised as a traditional remedy in Indonesia.[1] Our previous research has reported that PAB leaves contain alkaloids, flavonoids, phenolics, tannins, saponins, and steroid glycosides. It also has strong antioxidant properties.[1,2] Methanol extract has been previously reported to have high cytotoxicity in the Brine Shrimp Lethality Test (BSLT).[2] Other studies showed that PAB leaf extract contains phenolic compounds, including hydroxychavicol and eugenol, which have antimicrobial, antifungal, antioxidant, anti-inflammatory, and anti-cancer properties *Artemia salina* larvae served as the test organism in a cytotoxicity test conducted using the BSLT protocol. Conducting preliminary tests on leach larvae is normal practice to ascertain a substance's anti-tumor and anti-cancer effectiveness.[4] Numerous studies have discovered evidence supporting the hypothesis that there is a connection between certain types of bacteria and cancer.[5] Antibiotics are the preferred treatment for bacterial infections. Antibiotic resistance develops for various reasons, including antibiotic overuse, inadequate administration, and therapy discontinuation before bacterial eradication.[6] In addition to combating the issue of bacterial resistance, there is a pressing need for the development of new anti-cancer drugs.

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A DPP IV inhibitor (DI) is one of the many medications used to control blood glucose levels in diabetes mellitus.[7] Sitagliptin is one of the DI medications. This drug, however, has adverse effects on the upper respiratory tract, necessitating the search for natural-ingredient-based alternatives.[8] This study evaluated the cytotoxicity, antibacterial, and antidiabetic activity of solvents extract of the stem of *Piper cf. arcuatum* using different protocols.

Materials and Methods

Simplicia Preparation

PAB stems were collected from the author's personal plants in Bogor, West Java, Indonesia, in December 2022, which were identified by Prof. Dr. Eko Baroto Walujo, Head of Botany, Biology Research Center, Indonesian Institute of Sciences, with specimen number 638/IPH.1.02/II .8/V. PAB stem samples were allowed to dry in the open air at room temperature before being ground into a fine powder. Next, 50 g of stem powder was soaked in 500 mL of n-hexane for three days and filtered to produce a crude n-hexane extract. The filtrate obtained was evaporated using Rotary Evaporator type Hei-Vap Expert HL G3, Heidolph, Germany, and the residue from the first soaking was soaked in 500 mL of ethyl acetate for three days, filtered (the result was a crude extract of ethyl acetate), and the filtrate was evaporated. The marc was allowed to soak in 500 mL of methanol for three days to obtain the crude methanol extract.

Cytotoxicity Test of PAB Stem Extract

The BSLT cytotoxicity test refers to the method previously used by Irawan and his team[9]. In a hermetically sealed container containing saline water, 30 mg of brine shrimp eggs derived from *Artemia salina* were introduced. For the eggs to hatch effectively, an air hose was placed at the bottom of the container. After 24 hours, the ova of *A. salina* were hatched and developed into larvae. After that, 10 larvae each were taken and placed into containers containing sample solutions with concentrations of 10, 100, and 1000 µg/mL, respectively. The

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larvae in the sample and control solution were left for 24 hours, then observed and recorded for the number of dead and live larvae. After that, the percentage of live *Artemia salina* larvae was calculated, the probit value was determined, and a linear regression was carried out to determine the LC_{50} values.

Antibacterial Test of PAB Stem Extract

The disc diffusion method was employed to evaluate the antibacterial activity of the extracts against *Escherichia coli* and *Bacillus subtilis*. The diameter of the paper discs used in the study was approximately 6 mm. In this investigation, the antibacterial agents underwent two testing cycles. 200 µL of the test microbial suspension was added to each Petri dish containing agar nutrient media, and then each dish was distributed with a string. Disc paper was prepared to be placed on the agar surface of the petri dish. The discs had previously been soaked in the test solution (concentration 100 mg/L) for 30 minutes in a sterile petri dish. The plates were incubated at 37ºC for 24 hours. Observations were made using a ruler to measure the inhibition zone formed around the paper disc in the form of a clear area.[9,10]

DPP IV Inhibition Activity of PAB Stem Extract

The DI activity test was done on a blank control solution, a positive control (a solution of sitagliptin), and a sample or inhibitor solution made from stem extract. The composition of the solutions and reagents used are presented in Table 1. The mixture was incubated for 30 minutes at 37°C in an incubator. Fluorescence was measured using excitation wavelengths in the 350-360 nm range and emission wavelengths in the 450-465 nm11 range. Wavelength was measured using a Jena Analytical Spectrophotometer, Specord 200 Plus (Germany). The percentage inhibition of DPP-IV activity was calculable using Equation 1. The initial activity is the blank subtracted from the blank control.

$$\%\ Inhibition = \frac{(initial\ activity - inhibitor)}{initial\ activity} x\ 100\% \quad (1)$$

Tukey Test Analysis

The Tukey test, often referred to as the honestly significant difference (HSD) test, was introduced by Tukey in 1953. It compares all pairs of treatment means after an analysis of variance is conducted. Testing with the Tukey test is typically employed when data analysis in a study involves comparing data from two sample groups of equal size, allowing hypothesis comparison. The testing requirement is that the group sizes must all be the same or harmonically averaged. There are two types of testing: through the Sum in groups (T) and the Mean in groups (X). The steps of the Tukey test are as follows:

1. Hypothesis for two-sided test:

H_0: $\mu 1 = \mu 2$

H_1: $\mu 1 \neq \mu 2$

2. Insert the data into the formulas used:

$$Q = \frac{|\overline{Y1} - \overline{Y2}|}{\sqrt{\frac{s^2}{n}}}$$

With $s^2 = \frac{\sum Y_T^2 - \left[\frac{(\sum Y1)^2}{n_1} + \frac{(\sum Y2)^2}{n_2}\right]}{n_T - c.r}$

H_0: a null hypothesis
H_1: alternative hypothesis
µ1: The mean of population1/sample 1
µ2: The mean of population2/sample 2
Q: The value of significance Tukey by comparison with Tukey distribution
S^2: The combined variance
Y_T: Total yield
Y1: Yield population 1/sample 1
Y2: Yield population 2/sample 2
n1: number of sample 1
n2: number of sample 2
n_T: number of total sample
c: number of columns
r: number of rows

Results and Discussion

The results of the cytotoxicity evaluation of PAB stem extract against *A. salina* larvae are presented in Table 1. The table displays the number of *A. salina* larvae and the percentage of larvae death due to differences in extracts and test concentrations. Using this data, probit statistical analysis was performed, resulting in a plot depicting the relationship between probit values and the logarithm of concentration (Figure 1). Probit analysis of hexane, ethyl acetate, and methanol extracts of PAB stem produces a line equation, with y = 2.4867x+1.2; y = 2.3667x+2.065; and y = 2.5175x+2.065, respectively. The LC_{50} values 33.74, 17.38, and 14.65 ppm of the various extracts, hexane, ethyl acetate, and methanol, respectively, were generated from the line equation by extrapolation. This indicates that the extracts were cytotoxic, causing 50% mortality of the test larvae at concentrations less than 1000 ppm.[12] The results further revealed that the three extracts, using the BSLT method, have anti-cancer potential. Several studies have reported a positive correlation between the BSLT method and cytotoxic tests using cancer cell cultures.[13,14] Cytotoxicity is the capacity of a drug to induce the death of cancer cells.[14] The results of the BSLT test show that hexane, ethyl acetate, and methanol extracts from PAB plant stems are toxic. The Tukey Test statistical analysis revealed that the cytotoxicity activity using BSLT for ethyl acetate and methanol extracts was not significantly different. Still, the hexane extract was substantially different from the other two extracts. Based on this information, further *in vitro* and *in vivo* experiments can be conducted on PAB stem extract to determine its potential as an anti-cancer drug.

Table 1: Results of cytotoxicity activity (LC_{50}) and percentage inhibition test

Sample	LC_{50} (mg/L)	% Inhibition
Sitagliptin	-	85.35
Hexane extract	14.65±0.3	9.01
Ethyl acetate extract	17.38±1.8	33.27
Methanol extract	33.74±2.1	49.23

Using the agar diffusion method, the antibacterial activity of PAB stem's hexane, ethyl acetate, and methanol extracts was carried out on gram-negative bacteria, *E. coli*, and gram-positive bacteria, *B. subtilis*. The extract concentration used was 0.11 mg/L. The results are presented in Table 2. The zone of inhibition against *E. coli* produced by hexane, ethyl acetate, and methanol extract has a diameter of 0.65, 0.40, and 0.50 mm, while for *B. subtilis*, it is 0.40, 0.50, and 0.50mm, respectively. Amoxicillin was used as a standard, producing inhibition zones with diameters of 0.70 and 1.10 mm.

The test results showed that the extracts of *Piper cf. arcuatum* did not exhibit antibacterial activity against *E. coli* and *B. subtilis*. The distance between these bacteria's inhibition zones depends on how much their environment influences them.[15]

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Table 2: Results of antibacterial amoxicillin, hexane, ethyl acetate, and methanol extracts

Sample	Bacteria	Average (mm)	The activity of the antimicrobial inhibition zone
Amoxicillin	*E. coli*	0.70	potent
	B. subtilis	1.11	potent
Hexane extract	*E. coli*	0.65	Weak
	B. subtilis	0.40	Weak
Ethyl acetate extract	*E. coli*	0.50	Weak
	B. subtilis	0.50	Weak
Methanol extract	*E. coli*	0.40	Weak
	B. subtilis	0.50	Weak

The zone of inhibition of PAB stem hexane extract against *E. coli* was the largest compared to other extracts but had low inhibitory activity against *B. subtilis* compared to methanol and ethyl acetate extracts. Gram-positive bacteria, such as *B. subtilis*, have an entirely distinct surface morphology compared to gram-negative bacteria, such as *E. coli*. The outermost layer of gram-positive bacteria comprises negatively charged teichoic and teicuronic acids, whereas 90% of the cell wall comprises peptidoglycan. It has been shown that the outer layer of the cell wall of gram-negative bacteria contains between 5 and 20% peptidoglycan.[16]

The DI activity was quantified based on the presence or absence of extract, with sitagliptin as a positive control. The average DI results for PAB stem extract are presented in Table 3. The inhibitory activity of sitagliptin produced in this study was 85.45±2.3, while the hexane, ethyl acetate, and methanol extracts of PAB stems had inhibitory activities of 9.01±1.0, 33.27±1.2, and 49.23±0.1%, respectively. These results indicate that the methanol extract has the highest potential as a DI compared to the other two extracts. The statistical analysis results using the Tukey test revealed that the inhibitory activity of the standard and the other three extracts had significantly different values. This means that the more polar the solvent, the more potent the inhibitory activity, but the inhibitory activity was still lower than the standard. DPP IV has three active sides, namely S1, S2, and S3. The specific active site of S1 comprises a catalytic triad side chain (Ser630, Asn710, and His740) and is involved in strong hydrophobic interactions. The S2 active site of DPP IV is a cavity near residues Glu205, Glu206, and Tyr662. The active site of S3 consists of Ser209, Arg358, and Phe357. The position within the active side of S3 on the DPP IV allows smaller groups to access the side; on the other hand, the outside position of S3 favours larger groups.[17] The DI interacts with the S2 active site in Glu205 and Glu206 by forming a salt bridge. This interaction plays a vital role in inhibiting the enzyme.[18] Alkaloids and polyphenolic compounds, such as flavonoids, from plants, have been reported to have DPP IV inhibitory activity.[19,20,21,22] Based on the results of cytotoxicity, antibacterial, and DPP IV inhibitory activity, the study revealed that PAB stem extract has the potential to be a source of anti-cancer and antidiabetic bioresource.

Table 3: Data on testing the activity of DPP IV inhibition by PAB stem extract

Hole Plate	Buffer Tris-HCl (µL)	DPP-IV (µL)	Solvent (µL)	Inhibitor (µL)	Substrate (µL)
Blank	30	10	10	-	50
Control Blank	40	-	10	-	50
Sitagliptin	30	10	-	10	50
Inhibitor (sample)	30	10	-	10	50

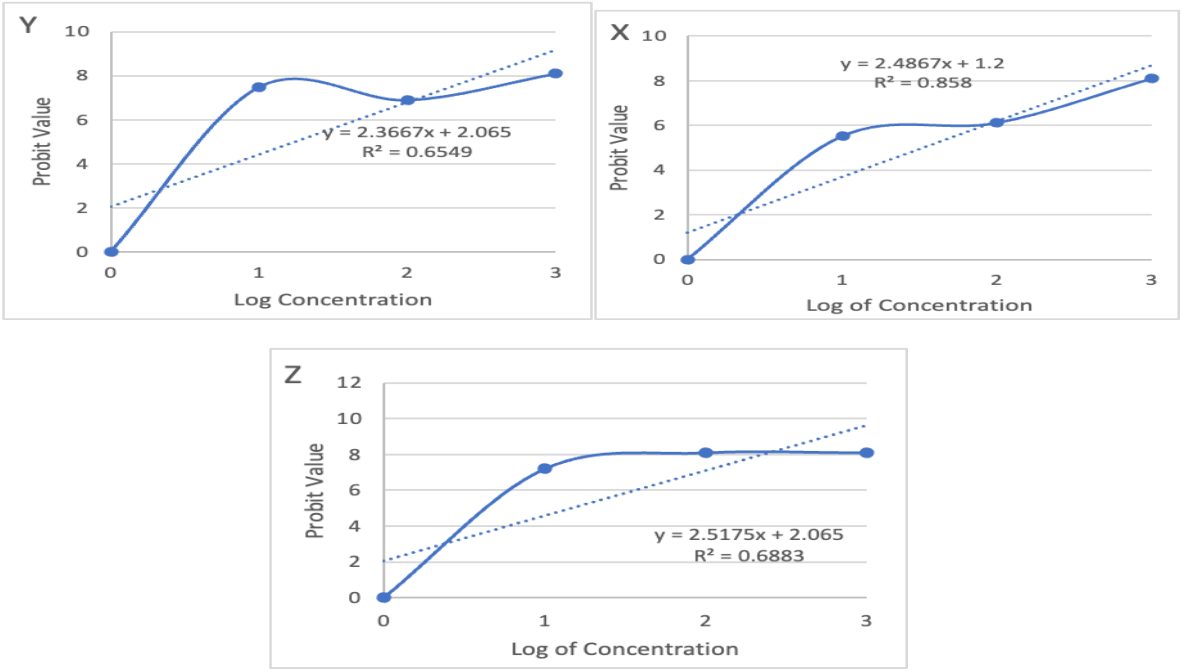

Figure 1: Probit plot for hexane (X), ethyl acetate (Y), and methanol (Z) extracts of PAB stems

Conclusion

The research concludes that the Stem of *Piper cf. arcuatum* Blume exhibits potent cytotoxicity in the BSLT test and DPPIV inhibitory activity, with the highest activity by methanol extract. In contrast, the extracts were not significantly active against *E. Coli* and *B. subtilis* bacteria. However, the stem extracts of *Piper cf. arcuatum* Blume have the potential to be a natural source for treating diabetes and cancer.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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