An examination of medicinal potential of *Pneumatopteris callosa*: phytochemical screening, antibacterial, and antioxidant activity

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Abstract. Ferns have been used as medicinal plants in many tribes and communities all over the world. Nevertheless, some of them are less studied and rarely explored. Herein, we conducted the preliminary phytochemical screening and evaluated the antibacterial and antioxidant activity of methanol extract of *Pneumatopteris callosa*, a fern species used by Balinese community for medicinal purpose. Tannins, alkaloids, triterpernoids, flavonoids, and reducing sugar were present in the extract. Moreover, the extract exhibit antibacterial activity against Gram-negative bacteria *Eschericia* coli and *Pseudomonas sp* and Gram-positive Bacteria *Bacillus cereus* and *Staphylococcus aureus*. The extract also shows the moderate DPPH free radical scavenging activity. These results validate the use of *Pneumatopteris callosa* as a medicinal plant.

1. Introduction

The fern is a significant plant group that has a comprehensive fossil history and remains a noticeable component of land flora [1]. It had passed the series of many adaptive changes of environment. Thus, these plants might could possess the genetic capability of having many useful secondary metabolites. Fern and fern allies have been used since the old past as the source of food, tea, and medicine. It has been well documented as the medicinal plant in the ancient medicinal document such as Ayurveda, as well as in traditional Chinese medicine systems [2]. Most of the ferns are known to possess medicinal properties such as anti-cancer, antimicrobial, antiviral, antidiabetic and wound healing activity [3]. Some species of ferns such as *Pellalea smithii* and *W. magnifica* have high antioxidant potential which relates to its total flavonoid concentration [4]. Moreover, *Lygodium flexuosum* and *Salvinia mollesta* possess antibacterial activities against pathogenic bacteria *Eschericia coli* and *Staphylococcus aureus* [5].

It is evident that ferns have many phytochemical properties such as flavonoids [6], alkaloids, glycosides, saponins, terpenoid, and tannins [7] and it validates the use of fern as traditional medicinal plants for treating human illness. Many tribes and local community all over the world use Pteridophyta for theraupetic purpose. *H. serrata* is commonly used in China for its anti-inflammatory activity [8], Malasar tribes in India use *Hemionitis arifolia* as antidiabetic and antimicrobial agent [9], whereas community of Bali Aga, Indonesia use the decoction of leaves of *Pneumatopteris callosa* for fever and hypertension [10]. Unfortunately, like other less studied fern species, this plant is poorly investigated and has never been evaluated for its pharmaceutical value and phytochemical compounds. This study

aims to conduct the phytochemical screening of *Pneumatopteris callosa*, evaluate its potency as an antibacterial agent against some pathogenic bacteria, and determine its antioxidant activity.

2. Methods

2.1. Materials

The materials needed in this study were methanol, aquadest, dimetilsulfoksida (DMSO), Fe (III) chloride, natrium hidroxyde, kloroform, H_2SO_4, acetic acid, Wagner reagent, Benedict reagent, ascorbic acid, 1.1-Diphenyl-2-picrylhydrazil (DPPH), Mueller Hinton Broth, Mueller Hinton Agar, gentamycin, *S.aureus*, *Eschericia coli*, dan *Pseudomonas sp* bacterial culture from Biology Department of State Islamic University of Maulana Malik Ibrahim Malang, and *B.cereus* INACC B317 for LIPI. The equipment needed in this study were rotary shaker, rotary evaporator vacuum, blender, spectrophotometer UV-Vis, extraction equipment, and glasswares.

2.2. Procurement of plant materials and extraction

The plant specimen was collected from Jatiluwih village, Tabanan, Bali. The aerial parts of the fresh plants were selected and dried at 50°C for 1-2 days, then powdered and filtered. The powder sample (20g) was extracted with methanol (200g) and kept on a rotary shaker (200 pm) within 24 hours. Then, the sample was filtered with Whatman filter paper (No 1) and centrifuged at 5000 rpm (15 minutes). The solvent was evaporated with rotary evaporator and the extract is dissolved in 8% dimethyl sulfoxide (DMSO) to obtain stock solution of 0.63, 0.13, 0.25, and 0.5 g/ml to be used in microbial assay procedure, whereas 0.5 gram extract was diluted with aquadest (50 ml) to be used as stock solution for further analysis of phytochemical screening. Moreover, the plant powder (0.5 g) was added on 20 ml aquadest, boiled, and filtered as another kind of sample to be tested for phytochemical constituents.

2.3. Phytochemical screening

Phytochemical screening was carried out by the following tests:

2.3.1. Test for tannins (Ferric chloride test). To 500 µl of extract, 2 drops of 1 % $FeCl_3$ solution was added. Blue-black or dark green colour appeared indicates the presence of tannins.

2.3.2. Test for flavonoids (Modified Kumar test. 1 ml of extract was treated with 100 µl NaOH. Yellow color indicates that extract contains flavonoids.

2.3.3. Test for saponins (Foam test). 1 ml of extract was added with 1 ml of aquadest and shaken vigorously within one minute. Formation of the consistent froth after 5 minutes of shaking validates the presence of saponins.

2.3.4. Test for triterpenoids (Modified Salkowski test). 1 ml of extract was mixed with 400 µl and 400 µl of sulphuric acid was added carefully to form a layer. A reddish or brown coloration at the interface indicates the positive results for the presence of triterpenoids

2.3.5. Test for polysteroids (Lieberman-Buchard test). 3 drops of acetic acid were added to 500 µl of extract and reacted with 3 drops of concentrated sulphuric acid then allowed to sit for five minutes. The blue or green color indicates the presence of polysteroids

2.3.6. Test for alkaloids (Wagner's test). To 200 µl of extract, a few drop of HCl was added and 500 µl of Wagner's reagent was reacted. A reddish brown flocculent precipitate showed the presence of alkaloids

2.3.7. Tesr for reducing sugar. 1 ml of extract was treated with 1 ml of aquadest and 1 ml of Benedict's reagent and heated slowly in a water bath. Brick red precipitate indicates the presence of reducing sugar.

2.4. Antibacterial activity assay

The methanol extract is tested against three bacterial strains (*S. aureus, Eschericia coli,* and *Pseudomonas sp*) of the culture collection stored in the Department of Biology, State Islamic University of Maulana Malik Ibrahim Malang, Indonesia, and one culture of *B. cereus* (INACC B317) obtained from Research Center for Biology, Indonesian Institut of Sciences. Antibacterial activity of the extract was evaluated by disk diffusion method.

Bacteria inoculation was prepared by selecting one of the well-isolated colonies from a culture agar plate and transferred to an erlenmeyer containing 50 ml of Mueller Hinton Broth. The suspension was incubated at 37°C (18-24 hours) and the absorbance of the suspension was adjusted to the value of OD obtained from its growth curve, approximately 1 x 10^6 CFU/ml. The adjusted inoculum was streaked on the dried surfaces of the petri dishes (90 mm diameter) contain Mueller Hinton Agar (MHA) steril with a sterile swab [11].

The sterile disks (Whatmann No1, 6 mm in diameter) were impregnated in the stock solution extract prepared before and put on a sterile plate under the laminar air flow until it become moist. Then, it put on the agar plate that already inoculated with bacteria. Each plate contains 4 disks with different concentration (0.063, 0.13, 0.25, and 0.5 g/ml) and one disk containing Gentamycin (20 µg / ml) as the positive control. This antibiotic is chosen as the positive control because it is often used as the first line antibiotic in the infection of bacteria [12]. Three replicates were conducted for each bacterial strain. The plates then incubated in 37°C (18-24 hours) and the inhibition zone was determined using a digital caliper.

2.5. Antioxidant activity assay (DPPH free radical scavenging activity)

The measurement of antioxidant activity in this study was compared with ascorbic acid as the standard [12]. Briefly, the extract solution range from 100 - 800 ppm was made by dissolving it with methanol, whereas the ascorbic acid (100 ppm) stock solution was made with the range of 5 - 40 ppm. Then 1 ml of the DPPH solution (0.4 mM) and 1 ml of extract added on test tube that contained 3 ml methanol, thus the final concentration of the extract was 20, 40, 80, 120, 160 ppm respectively, and 1, 2, 4, 6, and 8 ppm for ascorbic acid. Control was made by mixing 1 ml of DPPH and 4 ml of ethanol. Then, the test solutions were mixed with the vortex for 20 seconds and were left in the dark for 30 min. The absorbance of the solutions was measured at 517 nm. Methanol was used as the blank. The measurement was done in triplicates. The inhibition ratio (%) was obtained from the equation 1:

$$\text{Inhibition ratio (\%)} = \left\{\frac{(\text{Ac} - \text{As})}{\text{Ac}}\right\} \text{x } 100 \quad (1)$$

Whereas As was the absorbance at the addition of the sample and Ac was the absorbance of the addition of methanol instead of the sample.The IC_{50} of extract and standard was calculated by plotting the inhibition ratios (y) against the sample concentrations at all five points, and the respective regression line (y = ax + b) was drawn.

2.6. Statistical analysis

The results are expressed as Mean ±SD. The difference between axperimental groups was compared by ANOVA (One Way Analysis of Variance). The significant result of ANOVA followed by Dunnet Multiple comparison test (control × test) using R.

3. Results and Discussion

In the present study, *Pneumatopteris callosa* was screened for the phytochemical constituents. There were seven tests in totals such as test for tannins, flavonoids, saponins, triterpenoids, polysteroids, alkaloids, and reducing sugar performed on two kinds of sample, the methanol extract, and the leaf powder sample. Out of seven test, polysteroid was absent in leaf powder sample, whereas polysteroids and saponins were absent in methanol extract. The result of preliminary phytochemical screening was presented in Table 1.

Plants produce secondary metabolites as their defense systems against other organisms and serve as them survival weapon for them. Besides that, secondary metabolites are useful for human as the unique resources for food additives, pharmaceuticals, and fine chemicals [14]. Preliminary phytochemical screening in this study showed the presence of tannins in both samples. Tannins have potential antiviral, antioxidant, and antibacterial activity. The earlier study also revealed that the fraction of *Blechnum orientale Linn* was highly saturated with condensed tannins and has the ability to treat the external wound, especially diabetic ulcer wounds [15]. Flavonoids as one of the major secondary metabolites also present in both samples. Flavonoids also knew as the fountain of health because their vast biological potential. The initial investigation revealed that *Cheilanthes tenuifolia* owed two flavonoids, rutin, and quercetin that owed the free radical scavenging potential dan anticancer activity.

Table 1. Qualitative phytochemical constituents of methanol extract of *Pneumatopteris callosa* as compared to its leaf powder water infusion.

Sample	Tannins	Flavonoids	Saponins	Triterpenoids	Ploysteroids	Alkaloids	Reducing Sugar
Methanolic extract	+	+	-	+	-	+	+
Leaf water infusion	+	+	+	+	-	+	+

\+ positive
_ negative

Saponins were present in the powder sample diluted by the water. This result might validate the preliminary study conducted by Mir et al. [7]. For 34 species of fern studied, at least the aqueous extracts of 24 species have depicted positive confirmation for saponins. Nowadays, the design of green extraction uses water as the extraction solvent, thus, reduces the use of chemical synthesis [16]. This design might be useful for the extraction of saponins. Beside triterpenoids, alkaloids and reducing sugar also present on both kind of samples. Similar with flavonoids, alkaloids also owe large benefit on human health [17]. Several prior investigations reported the presence of alkaloids in ferns such as *Lycopodiastrum casuarinoides* [18] and *Palhinhaea cernua* [19].

Table 2. Antibacterial activity of methanol extract of *Pneumatopteris callosa*

Extract concentration (g/ml)	Inhibition Zone (mm)			
	Gram (-)ve bacteria (mean		Gram (+) ve bacteria	
	E. coli (mean ± sd)	*Pseudomonas* (mean ± sd)	*S. aureus* (mean ± sd)	*B. cereus* (mean ± sd)
0.063	6.48 ± 0.08*	Nil	Nil	Nil
0.13	7.90 ± 0.17*	Nil	Nil	Nil
0. 25	8.86 ± 0.05	7.7 1± 0.45	7.99 ± 0.08	8.42 ±0.16
0.5	**9.90** ± 0.1*	8.7 ± 0.40	8.6 ± 0.40	**9.04** ± 0.17*
Gentamycin (20 µg/m l)	9.15 ±0.50	9.2 ± 0.88	13.01 ± 4.17	8.3 ± 0

* : P value < 0.05 (Dunnet test)

The evaluation of the antibacterial activity of methanol extract of *Pneumatopteris callosa* is presented in Table 2 and Figure 1. This present study shows that the extract exhibits the inhibitory effect against all tested bacteria. The extract (0.5g/ml) exhibit the maximum 108 % (9.90/9.15) of inhibitory effect against Gram-negative bacteria *Eschericia coli* when compared to that of standard value. The

similar result is shown in the inhibitory effect against Gram-positive Bacteria *Bacillus cereus* which exhibit the maximum 109 % (9.04/8.33) of inhibitory effect as compared to that of the control. It means that the extract is effective against *Bacillus cereus* and *Eschericia coli* than the control measure. Moreover, the extract showed antibacterial activity against *Eschericia coli* in all range of concentration, but for the rest three bacteria, the inhibition zone exhibit from the extract concentration of 0.25 g/ml.

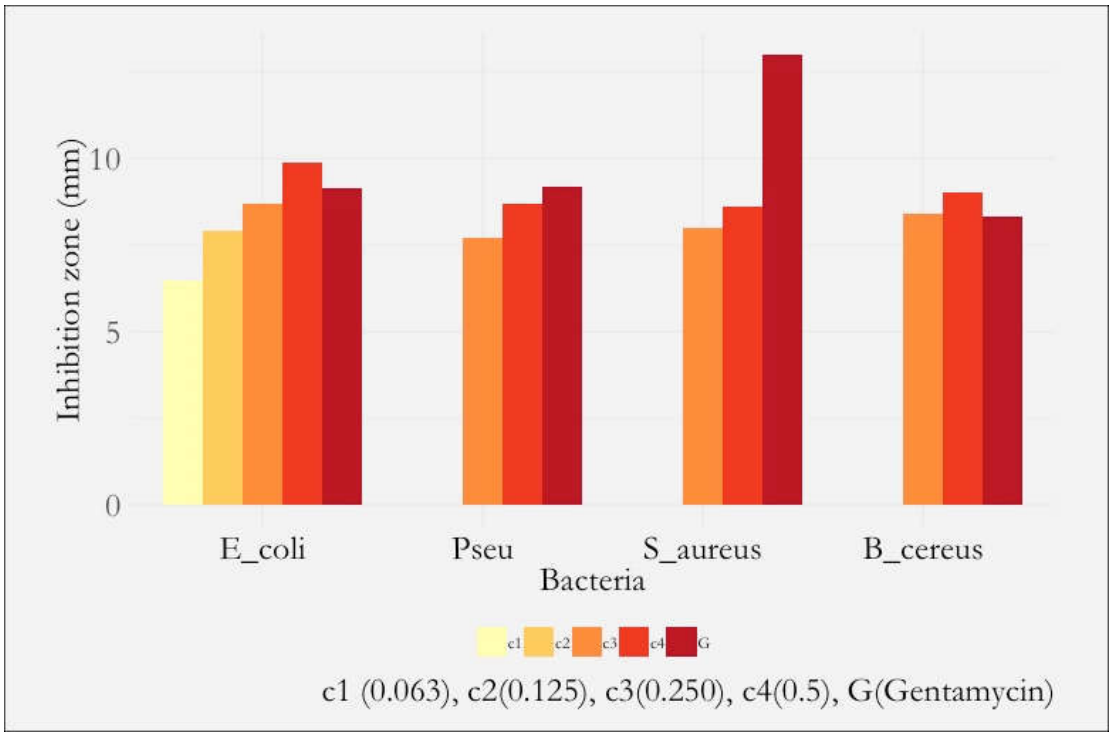

Figure 1. Inhibitory effect of methanol extract in different concentrations against Gram-positive and Gram-negative bacteria.

The ability of the extract to inhibit the bacterial growth might due to the presence of its phytochemical properties, such as flavonoids and triterpenoids, but further investigation is needed to validate it. Flavonoids derived from *C. tenuifolia* (fern) have potent antibacterial activity [20]. Triterpenoid compounds isolated from a fern species Adiantum lunulatum also exhibit the antibacterial activity against *Salmonella typhi* and *Pseudomonas aeruginosa* [21].

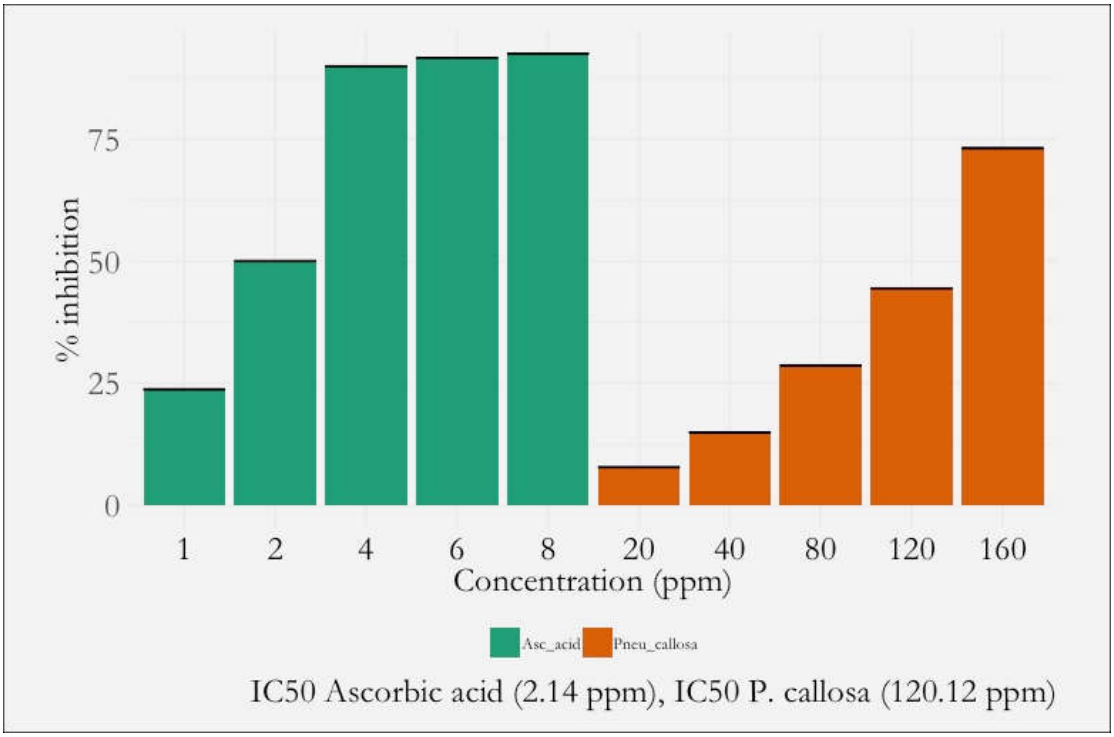

Figure 2. Antioxidant activity of methanolic extract of *Pneumatopteris callosa* as compared to ascorbic acid

This present study shows the moderate antioxidant activity of the methanol extract of *Pneumatopteris callosa* with the IC_{50} values of 120.12 ppm. This value is much lower than the standard. Nevertheless, this result might correspond to the presence of the flavonoids, since several studies found that the antioxidant activity relates with the presence of flavonoids. The DPPH free radical scavenging activity of some ferns including *L. carnosum* and *P. coronans* have a remarkable reciprocal relationship with the total flavonoids contents.

4. Conclusions

From the above results and discussions, it can be concluded that the methanol extract of *Pneumatopteris callosa* possess the phytochemical constituens such as tannins, flavonoids, triterpenoids, alkaloids and reducing sugar. Some of these compounds might responsible to the antibacterial activity of the extract against Gram-negative bacteria *Eschericia coli* and *Pseudomonas sp* and Gram-positive Bacteria *Bacillus cereus* and *Staphylococcus aureus*. Moreover, the extract exhibit moderate DPPH free radical scavenging activity. These foundings at least validate the use of *Pneumatopteris callosa* as one of medicinal plants used by the Balinese community.

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