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Short Communication
Leaf Position Affects Antibacterial Activity of *Spondias pinnata* and its Secondary Metabolite

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Abstract

Background and Objective: Ambarella (*Spondias pinnata* (L.f.) Kurz), Family Anacardiaceae has been traditionally used as an antibacterial in Indonesia. Only the apical leafs of this plant traditionally used for treatment of bacterial infection. The present study aimed to investigate the effect of the leaf position on antibacterial activity and constituent profile of the extracts. **Materials and Methods:** The leaf was collected in three positions (apical, middle and basal leaf) and macerated with 70% ethanol. Antibacterial activity was tested by agar diffusion method using Muller Hinton Agar (MHA) medium against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with amoxicillin as positive control. Characterization of its chemical constituent was performed with chromatography method. **Results:** The results showed that ethanol extract of ambarella leaf contain group compounds of flavonoids, alkaloids, tannins and saponins. The highest antibacterial activity against *S. aureus* and *P. aeruginosa* was observed by the apical position of the leaf with inhibition zones of 14.30 and 14.43 mm, respectively. HPLC chromatogram profile of apical leaf extracts shows different pattern with middle and basal leaf. Fractionation of the active extract given n-hexane and ethyl acetate fractions with inhibition zones of 13.27 and 12.91 mm and ethyl acetate fractions of 12.49 and 12.41 mm against *S. aureus* and *P. aeruginosa*, respectively. While no inhibitory activity was observed on chloroform and water fractions. **Conclusion:** Each position of ambarella leaf has different constituent character and among them, the apical leaves have a stronger inhibitory activity than the other position.

Key words: *Spondias pinnata* (L.f.) Kurz, antibacterial, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, HPLC profile, ambarella leaf, inhibitory activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plant efficacy depends on its chemical constituents. Several secondary metabolites in plants such as tannins, terpenoids, alkaloids, flavonoids, have been reported *in vitro* with antimicrobial activities[1].

One of the promising plants with antibacterial activity is ambarella (*Spondias pinnata*), Family Anacardiaceae. It is known as kedondong in Indonesia and traditionally the apical leaf used for antibacterial. Several phytochemical studies reported that *S. pinnata* leaf extract contains saponins, flavonoids, glycosides, alkaloids, carbohydrates and steroids with an antibacterial activity[2].

The fruit of ambarella contains β-amyrin, oleanolic acid, amino acids, glycine, cystine, serine, alanine, leucine and polysaccharides[3]. While, ethanol extract of *S. pinnata* leaf has been reported contains terpenoids, flavonoids, tannins, alkaloids and saponins[4]. Studies on chemical constituent of aerial parts contains lignoceric acid, 24-methylene cycloartanone, stigmast-4-en-3-one, β-sitosterol and its glucoside, β-amyrin, oleanolic acid, amino acids (alanine and leucine), daucosterol, ellagitannins, galloylgeranin, lignoceric acid and β-carotein[5-7].

The bark extracts *S. pinnata* containing flavonoids and phenolic compounds with a high antioxidant activity[8]. While the leaf methanol and aqueous extract showed antidiarrheal activity[9]. While, the ethanolic extract showed antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[10]. Other studies revealed that ambarella also has biological activity such as hypoglycemic, anthelmintic, anticancer, ulcer protective, cytotoxic, hepatoprotective and thrombolytic activities[11].

Concentrations and types of chemical compounds in plants greatly influence pharmacological effects while the content of chemical compounds was influenced by genetic factors, environment (climate and growth place) and conditions (age and harvesting method) which were closely related to growth components including plant height, number of branches, number of leaf[12,13]. In addition, the position of the leaf also affects the content of the bioactive compounds[14]. In this research, the effect of *S. pinnata* leaf position on antibacterial activity has been investigated including chromatogram profile of secondary metabolite on each extract.

MATERIALS AND METHODS

Study area: The ambarella leaves were collected from Makassar City, South Sulawesi Province, Indonesia at September, 2018 and research was performed until June, 2019.

Materials: Shimadzu LC-20AD UFLC, incubator (Memmert), autoclave (All American), BSC II (Biosafety Cabinet Class II), oven (Memmert), UV 366 and 254 nm lamps, analytical scales (Chiyo JL 200), sonicators, micropipette (Dragonlab), RP-18 silica gel plates, GF 254 silica gel (Merck), 70% ethanol, methanol, chloroform, n-hexane, ethyl acetate, acetonitrile, amoxicillin (Oxoid), DMSO, Nutrient Agar (Merck), Müeller Hinton Agar (Merck), *Staphylococcus aureus* ATCC170718, *Pseudomonas aeruginosa* ATCC170830, ambarella leaf (*Spondias pinnata* (L.f.) Kurz).

Extraction method: Dried ambarella leaf (200 g) and macerated with 1.5 L EtOH. Maceration was carried out for three days and every 24 h the extract was collected and the solvent was replaced with the new solvent. All the extract was filtrated and lyophilized to give the EtOH extract.

Phytochemical screening: Phytochemical screening was carried out to determine the group compounds contained in ambarella extract using chemical reagent[15].

Alkaloid test: The 0.1 g of extract was added with 5 mL of chloroform and 3 drops of ammonia. The chloroform fraction was separated and acidified with 2 drops of H_2SO_4. The fraction was divided into three tubes, each added with Dragendorff, Meyer and Wagner reagents. The alkaloids were characterized by the presence of white precipitate on Meyer reagents, brown in Wagner reagents and red in Dragendorff reagents.

Saponin test: The 0.1 g of extract was added 5 mL of distilled water and heated for 5 min. The extract was then filtered and the filtrate was shaken. Saponins can be seen with the appearance of foam for ±10 min.

Flavonoid test: The 0.1 g of extract was added with methanol then heated. The filtrate was added with H_2SO_4, the formation of red indicates the presence of flavonoids.

Tannin test: The 0.1 g of extract was added with 5 mL of distillated water then boiled for several minutes. The filtrate added with 1% FeCl. Formation of dark blue or green wash black color indicates tannins.

Extract preparation: About 0.1 g extract of ambarella leaf in each leaf position (apical, middle and basal) dissolved with 1 mL of sterile water to give a concentration of 10% (b/v).

Bacterial suspensions: *Staphylococcus aureus* ATCC 170718 and *Pseudomonas aeruginosa* ATCC 170830 were inoculated into agar nutrient (NA) medium, incubated at 37°C for 24 h. The growth of bacteria was taken and inoculated into the medium of Nutrient broth and incubated at 37°C for 24 h. Then a dilution of 10^{-1} to 10^{-3} was carried out and measured using UV-VIS spectrophotometer with OD600 = 1 equivalent to 1×10^{8} cells mL^{-1}. Suspension of *P. aeruginosa* was carried out in the same way as *S. aureus*.

Antibacterial activity: The antibacterial activity of the extract was determined using paper disc diffusion method (Kirby-Bauer) with a layered method[16]. The first layer of the "Base Layer" does not contain the test bacteria. the second layer (seed layer)contain test bacteria mixed into the media and dried for 5 min. Sterile paper disc (6 mm diameter) containing various test extracts (2 mg/disc) was placed on the surface of the agar plate. The plates were then incubated at 37°C for 24 h. Antibacterial activity was determined by measuring the inhibition zone in millimeters. Paper disc which containing amoxicillin (10 µg) was used as a positive control while the paper disc which containing solvent (DMSO) as a control.

HPLC profiling: Ten milligram of extract was dissolved with 10 mL of methanol for HPLC and put into a UFLC auto sampler vial. The extract solution was analyzed with Shimadzu LC-20 AD UFLC with Reverse Phase System, ACN:MeOH: H_2O (40:20:40) using photodiode array (PDA) detectors.

Statistical analysis: Data was analyzed using Ms. Excel 2013.

RESULT AND DISCUSSION

Extraction yield: Extraction of each leaf were obtained 43.7 g of apical leaf, 54.7 g of middle leaf and 40.1 g basal leaf. The extract yield of each part were 21.8, 27.3 and 20.0%, respectively. Based on the result indicated the position of leaf affected the extraction yield with the highest extract was on the middle leaf. Higher value of extraction yield was reported from the leaf of ambarella collected in Western Nepal extracted with 80% ethanol given 32.1%. However, ethanol concentration using in this study (70%) was slightly lower affects in extraction yield[17]. Further phytochemical investigation on extract of ambarella leaf in each position of leaf (apical, middle and basal) using chemical reagent indicated all leaves contain flavonoids, alkaloids, tannins and saponins which were observed by formation of precipitate and color (Table 1). Phytochemical analysis of ethanol extracts of *S. pinnata* leaf has been reported contain flavonoids, tannin, gum and carbohydrate, alkaloid, saponin and terpenoid. While, saponin was not observed in hexane and ethyl acetate extract[4]. This data similar with was observed in our research.

Table 1: Phytochemical ethanol extract of ambarella leaf

Compounds	Apical leaf	Middle leaf	Basal leaf
Flavonoids	+	+	+
Alkaloids	+	+	+
Tannin	+	+	+
Saponin	+	+	+

+: Presence of phytochemical

Chromatogram profile: Characteristic of the chemical component can be observed from the signal and intensity of peak on HPLC chromatogram. The peaks on the chromatogram indicates the presence of chemical compounds while wide and height of the peak indicate the number of compounds in the sample. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants[18,19]. Several research on characterization and quantification of the plant chemical constituent have been reported using HPLC since the resolving power of HPLC is ideally suited to the rapid processing of such multi compounds in extract[20-22].

Based on HPLC chromatogram profile analysis for each extract showed similarity pattern of the middle and basal leaf indicated similarity of the chemical constituents extracted at the middle and basal leaf (Fig. 1b-c). While, presence of two peaks on the apical leaf extract (rt = 11.0 and 11.3 min, Fig. 1a) instead of one sharp peak at retention time of 10.8 min at middle and basal leaf indicates the presence of difference chemical compound. HPLC fingerprint analysis of ethanolic extract *S. pinnata* leaf have not been reported. The other ambarella species known as *S. tuberosa* has been analyzed for its leaf hexane extract showed three main peaks probably corresponded to gallic acid, a flavonoid and hyperoside[20].

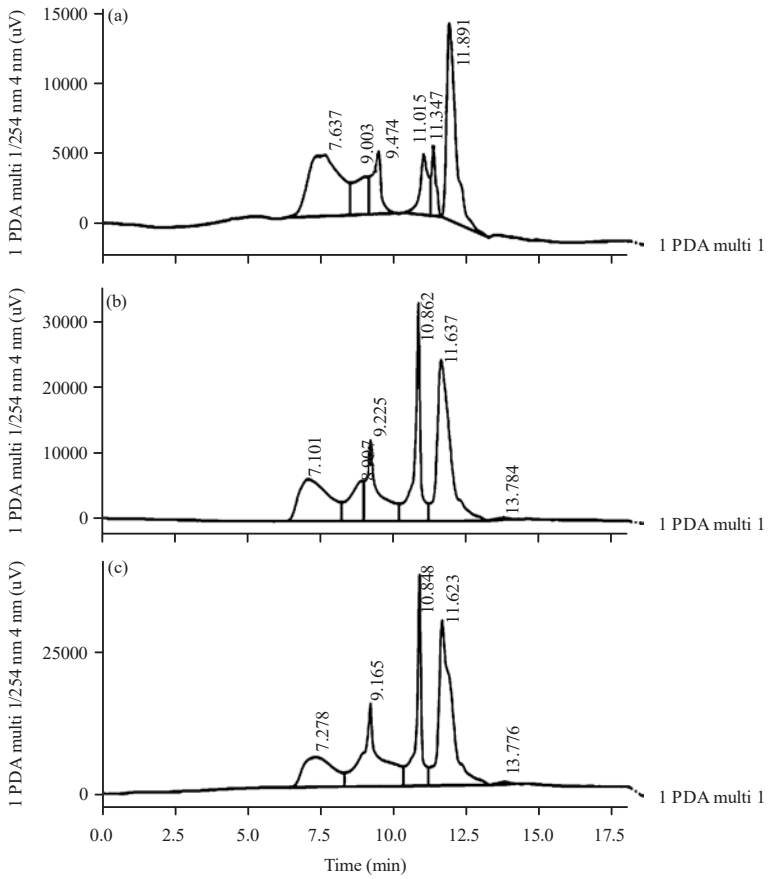

Fig. 1(a-c): HPLC profile of ethanol extract of ambarella leaf at position, (a) Apical, (b) Middle and (c) Basal

Table 2: Inhibition zone of ambarella leaf extract against *S. aureus* at a concentration of 10%

	Inhibition zone diameter (mm)			
Treatments	I	II	III	Average (mm)
Apical leaf	14.00	14.50	14.41	14.30
Middle leaf	13.50	11.00	12.00	12.16
Basal leaf	12.20	10.70	10.00	10.96
Amoxicillin	15.50	12.80	13.83	14.04

Roman numeral indicates replication

Table 3: Inhibition zone of ambarella leaf extract against *P. aeruginosa* at a concentration of 10%

	Inhibition zone diameter (mm)			
Treatment	I	II	III	Average (mm)
Apical leaf	14.40	14.00	14.90	14.43
Middle leaf	11.00	10.00	10.80	10.60
Basal leaf	9.80	11.30	9.00	10.03
Amoxicillin	14.30	14.80	14.10	14.40

Roman numeral indicates replication

Antibacterial activity: Further investigation on antibacterial activity of the leaf extract was carried out by paper disc diffusion method. All the three different position of the leaf showed inhibitory activity against *S. aureus* and *P. aeruginosa* bacteria. The presence of inhibition activity was observed by formation clear zone around the paper disc as a result of the extract (10%) inhibits the bacteria growth. Among the extract, the apical leaf showed the stronger inhibitory activity indicated by the longest clear zone compare to other extract while similar activity was observed with the positive control amoxicillin (10 µg) against *S. aureus* (Table 2) and *P. aeruginosa* (Table 3). The same method for antibacterial assay was reported by Jain *et al.*[10] using ethanol extract of ambarella leaf showed inhibitory activity of 24.6 and 21.1 mm against *S. aureus* and *P. aeruginosa*, respectively. Similar to the methanol extract of *S. tuberosa* showed inhibitory zone of 20.1 mm, while no inhibition was observed by *S. mombin* against *P. aeruginosa*[23]. Inhibitory activity was higher than that we found since the different concentration of solvent using in this research. However, there are no researches on effect of the leaf position on its antibacterial activity. These results indicate that the most effective ambarella leaf used for antibacterial were in the apical leaf. The different peaks on HPLC chromatogram profile of the apical leaf compare than middle and basal leaf indicated the most responsible constituents on its antibacterial activity.

Table 4: Inhibitory activities of ambarella apical leaf fractions against *Staphylococcus aureus*

Treatments	Inhibition zone diameter (mm) I	II	III	Average (mm)
n-hexane	12.83	12.83	14.16	13.27
Chloroform	-	-	-	-
Ethyl acetate	11.33	11.83	14.33	12.49
Water extract	-	-	-	-

Roman numeral indicates replication

Table 5: Inhibitory activities of ambarella apical leaf fractions against *Pseudomonas aeruginosa*

Treatments	Inhibition zone diameter (mm) I	II	III	Average (mm)
n-hexane extract	12.26	12.83	13.66	12.91
Chloroform extract	-	-	-	-
Ethyl acetate extract	10.40	13.33	13.50	12.41
Water extract	-	-	-	-

Roman numeral indicates replication

Liquid-liquid fractionation of the apical leaf extract used four different solvents i.e., n-hexane, chloroform, ethyl acetate and water. The results of partitioning of 10 g of ambarella apical leaf extract, obtained 4.3 g of n-hexane fraction, 0.2 g of chloroform fraction, 1.9 g of ethyl acetate fraction and 3.5 g of water fraction. Antibacterial activity for each fraction was carried out the same as the test in the extract with the same concentration of 10% against *S. aureus* and *P. aeruginosa*. The results showed that n-hexane fraction was the strongest inhibitor while water fraction and chloroform were not show any inhibitory zones (Table 4-5).

Findings from this study suggest that position of *S. pinnata* leaf extracts affects antibacterial activity and can be exploited as a natural drug for antibacterial. These data also give scientific information of the empirical use of the ambarella apical leaf for antibacterial. However, further studies are needed to isolate active compound and evaluate their antibacterial activity.

CONCLUSION

The position of the leaf (apical, middle and basal) influences chemical components seen on HPLC chromatogram profile. Further investigation on their antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed the apical leaf was the stronger compare to middle and basal leaf. These data suggested that the apical leaf of ambarella has antibacterial activity compare to other leaf part.

SIGNIFICANCE STATEMENT

This study discovered that ambarella leaf on apical position have the stronger antibacterial activity compare to the other position that can be beneficial for using as antibacterial. This study will help the researchers to uncover the critical areas of the using herbal medicine and drug discovery from natural medicine. Thus a new theory on the use leaf of ambarella as antibacterial should be the apical leaf position.

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