

Evaluation of Anatomical Structure, Growth, and Antibacterial Activity of *Toxocarpus villosus* Species used as Supplementary Feed for Livestock

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This study was focused on a wild plant named *Toxocarpus villosus* (Blume) Decne which grows naturally in uncultivated lands in the Mekong Delta, Vietnam. The research examined the potential of *T. villosus* to be supplemented in livestock feed through parameters related to the anatomy, growth, and antimicrobial ability of this plant. The plant samples were collected from Vinh Long and Tien Giang provinces, identified based on morphology, examined its anatomical structure and observed the growth rates (the size of somatic organs and yield) by growing under the net-house condition for two months. Moreover, antibacterial activity was assessed using agar diffusion and MIC assays against four tested bacterial species (including both Gram-negative and Gram-positive bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*). The results showed that *T. villosus* was easy to cultivate, have a moderate growth rate (fresh leaf yield at 5.23 tons ha⁻¹), the anatomical structure contains high levels of fiber and its ethanolic leaf extract can resist the examined bacteria.

Keywords: Anatomical structure, antibacterial, growth, *Toxocarpus villosus* (Blume) Decne

INTRODUCTION

Currently, due to the effects of climate change and urbanization, the cultivation area for raw feeds for animal husbandry has been declined (Tran, 2018). Furthermore, antibiotics and chemicals have been restricted or even prohibited in animal feed because of severe consequences including emergence of antibiotic-resistant pathogens and antibiotic residue in meat and milk. Therefore, the plants containing medicinal properties could be supplementary and alternative to antibiotics for animals (Abdallah *et al.*, 2019). *Toxocarpus villosus* (Blume) Decne. is a type of vine, whose Vietnamese name is Dây giáo vàng, a hairy *Toxocarpus* species belonging to the family Asclepiadaceae (Pham, 1999). According to the traditional Vietnamese experience, *T. villosus* has been used to treat rheumatism (bone degeneration), pain, and swelling (water retention in the body, in cells), diarrhea or weak digestive ability and urinary tract (Nguyen, 2003). With these properties, *T. villosus* may contain a lot of compounds that have anti-inflammatory and antibiotic activities (Ghasemian *et al.*, 2016).

In the Mekong Delta, *T. villosus* grows naturally in the bank along canals, and in gardens, which is well adapted and therefore, can give high biomass. Based on the pilot investigation of our group, Tien Giang and Vinh Long provinces have a high density of wild-growing *T. villosus*, and that has been used as forage for livestock (such as goats) and has shown good effects (for high milk and meat content). Thus, *T. villosus* potentially could be exploited as forage for livestock when this source of forage is increasingly scarce (Ghazwani *et al.*, 2023; Hegazy *et al.*, 2022). In this report, the criteria for developing green food sources such as morphological description, anatomical structure, growth, and antibacterial ability of *T. villosus* species were investigated and statistically analyzed.

MATERIALS AND METHODS

Sampling and description of plant characteristics: Sample collection and description of plant characteristics were performed according to the previous study (Nguyen, 2007). Based on the pilot investigation of our group, Tien Giang and



Vinh Long provinces have a high density of wild-growing *T. villosus*. Samples were collected at Tien Giang (10°25'08.7"N 106°22'43.1"E) and Vinh Long provinces (10°03'29.6"N 105°59'22.4"E) with uncultivated roadside habitats. The samples collected in each position were used for identification (samples containing reproductive organs), anatomy (randomly choose 3 mature leaves in each position) and cutting to cultivate in the net house (depending on the growing level of samples). In the case of samples used for antibacterial testing, all plant samples growing on the ground (consisting of leaves and stems) were collected in each mentioned position.

Identification of scientific names: The scientific name was identified based on morphology according to “An Illustrated Flora of Vietnam” (Pham, 1999) and “Flora of Vietnam” (Nguyen, 2003).

Plant anatomy: Microsurgery was thinly cut (by hand) and double stained with carmine aluné - vert d'iod in cross-section across the petiole, midrib, and leaf tip of mature leaves. Microsamples were taken under an optical microscope (Olympus CX23, Olympus Corporation - Japan) with an objective lens of a microscope (E10, E40) and an ocular micrometer (E15) (Mondolot *et al.*, 2001). The size of samples was measured by using Toupview software (ToupTek Photonics, China).

Evaluation of plant growth: The plant growth was evaluated with the criteria of the number of leaves, number of shoots, stem diameter, leaf area, and yield under the net-house condition.

The cuttings were taken in Tien Giang and Vinh Long. The cuttings were selected as mature trees, the segments that were too young or too old were removed, and they were cut into pieces about 15-20 cm long, ensuring each cutting had 2-3 nodes. The cuttings were planted in plastic pots size (40 cm wide – 32 cm height). The soil used for planting was mixed with the formula: 3 kg of soil, 0.5 kg of husk, and 0.5 kg of treated dry goat manure. The cultivating soil was manured with Dau Trau NPK fertilizer 20-20-15 + TE (Binh Dien Fertilizer Joint Stock Company, Vietnam) with the instruction of producer 300-500 kg ha⁻¹ in each manuring time. Water twice daily at 8 am and 4 pm. Cuttings were planted in pots after 2 months (to take care of cuttings and not take the target), then cut off near the base, in each pot, there were only 2 branches, and each branch had 2 nodes (young shoots), take care of the tree for an extra week for the plant to grow well, then start taking targets. The number of pots for the experimental arrangement was 18 pots, the criteria were taken on all pots. The way to measure the indicators was presented in Table 1.

Antibacterial assay: The four bacterial strains used in this study including *Listeria monocytogenes*, *Escherichia coli* (ATCC25922TM), *Staphylococcus aureus* (ATCC25923TM), and *Bacillus cereus* (ATCC14579TM) were provided by the Microbiology laboratory, College of

Aquaculture and Fisheries, Can Tho University. In this study, the antibacterial activity of *T. villosus* extract was evaluated by agar plate diffusion method and determination of MICs - Minimum Inhibitory Concentration (Ngan *et al.*, 2023).

Agar well diffusion: To investigate the ability to inhibit pathogenic bacteria of *T. villosus* extract, we conducted experiments according to the method of Valgas *et al.* (2007), aspirated 100 µL of the suspension of the indicator bacteria strains at the density of 8.5×10⁵ cells mL⁻¹ and injected into the agar plate. Using a sterile cotton swab, spread the bacterial solution evenly over the entire surface of the TSA agar plate. Dilute the extract at concentrations of 0.3, 0.4, and 0.5 mg mL⁻¹ in Dimethyl sulfoxide (DMSO) 15% solution combined with ultrasonication. DMSO 15% solution and Streptomycin 0.5 mg mL⁻¹ were used as negative and positive controls. Add 50 µL of each solution to each well (the diameter of each well is about 6 mm), then incubate at 37°C for 24 h. The diameter of the inhibitory zone (mm) which was transparent, meaning that it did not indicate bacterial growth was measured by ruler to evaluate the inhibitory effect on pathogenic bacteria of the extract.

Table 1. Indicators and measurement of growing data of *Toxocarpus villosus* on a laboratory scale (Illustrated in Figure 1)

Parameters	Measurement
Stem length (cm)	Measure from the ground to the tip of the stem top. Measure from the ground to the top end of the tallest tree in the tree, excluding the tops of the leaves.
Internode length (cm)	Measure the distance between the two nodes of the internode, and choose the 1 st , 3 rd , and 5 th internodes from the top down. Measure the added internodes of the stems 1, 3, 5, and 7 from the top down.
Number of shoots (shoot)	Count the total number of shoots in each pot.
Leaf length (cm)	Choose three types of young, mature, and old leaves of the tree. Measure from petiole to leaf tip
Leaf width (cm)	Choose three types of young, mature, and old leaves of the tree. Measure the width of the widest part of the leaf from the edges of the leaf
Number of leaves (leaves)	Count the total number of leaves in a branch.
Green matter yield (tons ha ⁻¹ per year)	Cut the plant biomass (leave 2 or 3 leaf nodes for the shoots) and weigh all the wires and leaves of each treatment plot.

The green matter yield was determined by measuring all samples when they were collected.

Dry matter yield = Green matter yield x % DM

Dry matter (DM) was defined by drying at the temperature of 105°C for 4 hrs. Total ash (Ash) was determined by heating at 550°C for 3 hrs.

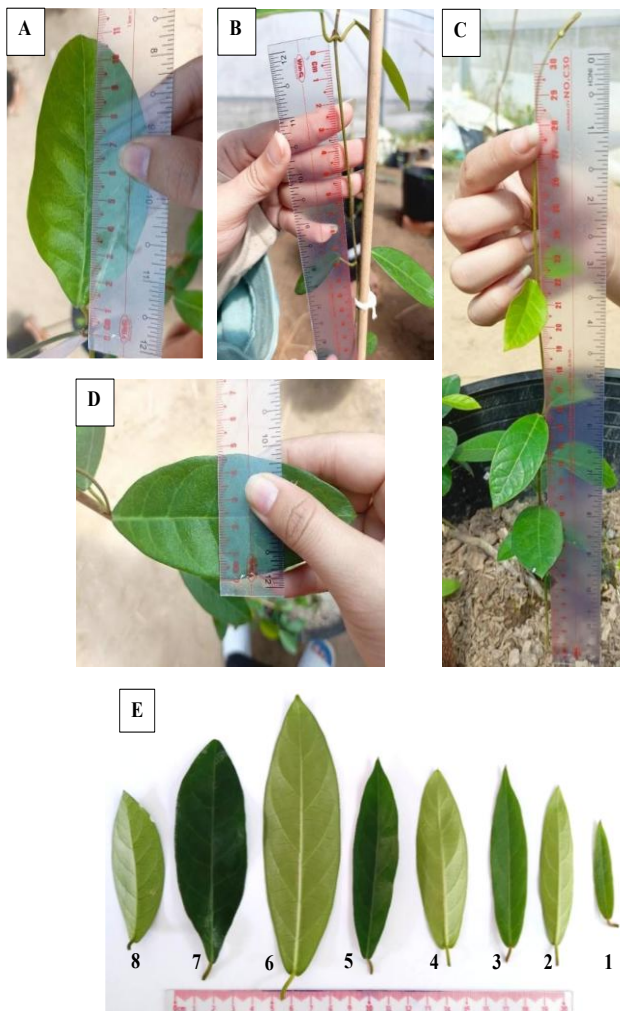


Figure 1. Leaf morphology of *T. villosus* from base to tip (on the same vine). A: measure the length of leaf, B: measure the length of internode, C: measure the height of vines, D: measure the width of leaf, E: types of leaves growing in each vine (1-3: young leaves, 4-6: mature leaves, 7-8: old leaves).

Determination of Minimum Inhibitory Concentration (MICs): Minimum inhibitory concentrations (MICs) were determined by using a 96-well plate dilution method, in which Resazurin (Merck, Germany) played a role as a growth indicator of the test bacteria (Sarker *et al.*, 2007). The Resazurin 10 mg mL⁻¹ solution was prepared in NaCl 0.85% and used for marking bacterial growth. The experiment was performed on a 96-well plate in a sterile medium. Drop 100 µL of NaCl 0.85% solution into each well. A 100 µL of

solutions consisting of 5 mg mL⁻¹ *T. villosus* extract, DMSO 15%, and Streptomycin 0.5 mg mL⁻¹ were injected into the first row of the plate, respectively. Then, dilute the solutions with decreasing concentrations in each well on the plate. The *T. villosus* extract was diluted in DMSO 15% solution (Phengyongsone *et al.*, 2022) with a continuously decreasing concentration range from 5 mg mL⁻¹ to 0.0097 mg mL⁻¹ and repeated four times. Streptomycin 0.5 mg mL⁻¹ solution and DMSO 15% were used as positive and negative controls. Solution F consisting of Isosensitest media (ISO) and Resazurin 10 mg mL⁻¹ was used to check whether the extract was pre-contaminated with microorganisms. In each well, except for the wells where the experiment was performed with solution F, 100 µL of a solution consisting of bacterial suspension (105 CFU mL⁻¹) and Resazurin 10 mg mL⁻¹ was injected into the remaining wells. Then, each well was closely covered with parafilm and incubated for 18 h at 37°C. The color change of the solutions on the plate was observed by naked eyes and compared to the color of the negative control. The color of the negative control solution indicated bacterial growth, and the MICs concentration was determined to be the lowest concentration where no bacterial growth observed.

Statistical analysis: The data indicated in Table 3 and Table 4 were expressed as mean ± standard deviation. A one-way ANOVA test was used to analyze these data and differences in susceptibility of bacteria in various groups including plan extract, negative and positive controls. $P < 0.05$ was considered statistically significant. All statistical tests of variation were performed with Minitab Software Version 18 (Pennsylvania, USA).

RESULTS

Anatomical morphology and growth ability of *T. villosus* grown under net-house condition: *T. villosus* had all the characteristics of a species belonging to the genus *Toxocarpus* with a long vine life form due to long internodes, opposite leaves, white latex stems and leaves, and long stalks. The leaves had 5-6 herringbone veins, the leaf blade was dark green on the upper surface and lighter on the lower surface (Pham, 1999; Tran, 2007). The collected samples showed the diversity of leaf shapes of *T. villosus*. Leaves had oval to oblong blades, shape, size, and color can change during the developmental stages of the species (Fig. 1).

Evaluate the growth parameters of *T. villosus* in the net house after 2 months, the recorded data were shown in Table 2.

The data from Table 2 showed that the growth rate of stem and leaf size increased gradually until 2 months of culture, some parameters were stopped. The data has just illustrated some parameters involving the plant growth which meant that they just had the meaning of growth rates at the time when our group collected the samples as well as did not indicate any relative comparison between each data.

Table 2. The growth parameters of *T. villosus* recorded in each period under the net-house condition

Parameters		1 week	2 weeks	4 weeks	8 weeks
Number of leaves		11.00	17.00	19.00	23.00
Leaf length (cm)	Young	4.80	10.40	11.80	14.10
	Mature	3.30	9.10	11.80	13.00
	Old	4.80	7.70	10.90	10.70
Leaf width (cm)	Young	1.50	7.50	4.60	4.00
	Mature	2.00	5.10	5.30	3.90
	Old	2.14	3.50	5.00	3.10
Number of shoots		5.00	3.00	3.00	4.00
Stem length (cm)		11.90	50.00	86.00	79.40
Internode length (cm)	Internode 1	2.10	6.00	11.40	8.80
	Internode 3	5.20	9.00	10.70	9.90
	Internode 5	5.00	2.30	6.20	8.10
	Internode 7	4.70	3.50	5.80	8.10
Green leaf yield (tons ha ⁻¹)					5.23
Dry matter leaf yield (tons ha ⁻¹)					0.89

Productivity is an important parameter in assessing growth and an essential factor in evaluating the appropriate type of feed for livestock (Nguyen, 2010). The fresh leaf yield of *T. villosus* was reported at 5.23 tons ha⁻¹ and dry matter leaf yield is 0.89 tons ha⁻¹ each year. The yield of potential forage plants varies in each species and crop during a year, ranging from 3.5-4.0 tons ha⁻¹ to 15-17 tons ha⁻¹ (Hamacher *et al.*, 2021). In this study, the yield of *T. villosus* belonged to this range, which could be used as a forage plant.

The anatomical structure helps to determine the crude fiber and some other components of the plant, which is an important criterion for deciding the quality of animal feed (Le *et al.*, 2005). The anatomical structure of the leaves was shown in Fig. 2. Structural features of leaves *T. villosus* had all the common characteristics of dicotyledonous plants with heterotrophic structure, soft tissue (palisade parenchyma and parenchyma) with cellulose structure have advantages.

Antibacterial activity of the leaf extract of *T. villosus*: The ethanol leaf extract of *T. villosus* was used to evaluate the antibacterial activity. The results of agar well diffusion was described in Table 3 and the determination of MIC values in testing bacteria was illustrated in Table 4.

Agar well diffusion: All three tested concentrations of the extract were able to inhibit the indicator bacterial strains. Under the influence of Streptomycin 0.05 mg mL⁻¹, the inhibition against four strains of bacteria *L. monocytogenes*, *B. cereus*, *E. coli*, and *S. aureus* was the highest with the values of inhibitory zone diameter were 20.55, 19.83, 24.33 and 22.00 mm, in turns. In contrast, wells containing DMSO 15% as negative control did not appear the inhibitory zone (Table 3).

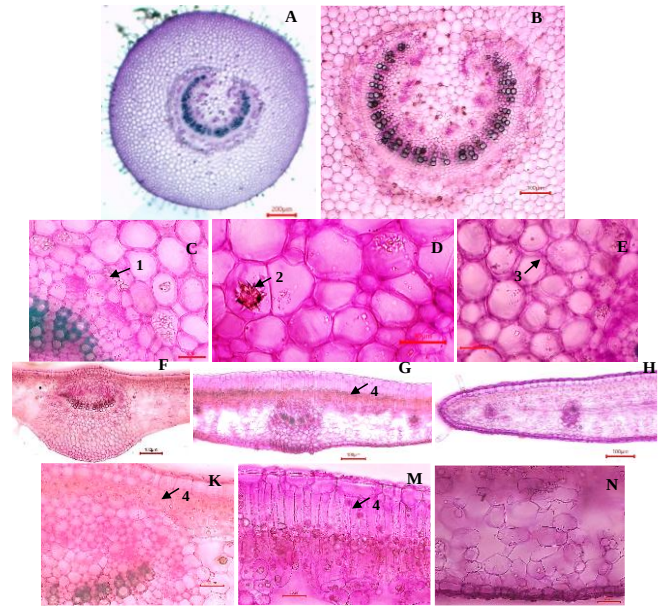


Figure 2. The anatomical structure of the leaves at the mature period. A: petiole with lots of soft tissue, B: Leaf veins in petioles with many small wood vessels, C: parenchymal cells contain starch, D: parenchymal cells contain calcium oxalate crystals, E: parenchymal cell containing oil drop, F: midrib structure of the leaf, G: main vein region of leaf tip, H: structure of leaf blade and leaf margin, K: part of the main vein of the leaf blade with many soft tissues containing chloroplasts and stores, M: structure of leaf blade with many palisades parenchyma containing many chloroplasts, N: structure of spongy parenchyma in leaf blade (1: starch; 2: calcium oxalate crystals; 3: oil drop; 4: palisades parenchyma containing many chloroplasts).

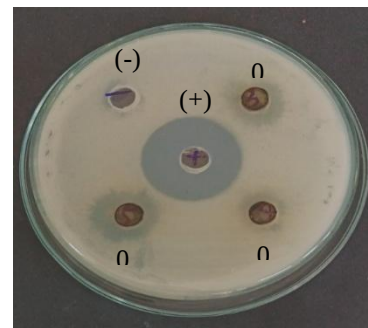


Figure 3. The inhibitory zone of tested bacteria in Petri dish. (+) Control: Streptomycin 0.5 mg mL⁻¹; (-) Control: DMSO 15% and the other wells was the *T. villosus* extract in 0.3, 0.4 and 0.5 mg mL⁻¹.

Table 3. The diameter of the inhibitory zone of *T. villosus* leaves extract recorded in four bacterial species.

Bacteria	Diameter of the inhibitory zone (mm)				
	Streptomycin (0.05 mg/mL)	DMSO (15%)	Concentrations of extract (mg/mL)		
			0.3	0.4	0.5
<i>L. monocytogenes</i>	20.50 ± 0.50 ^b	-	2.33 ± 0.58 ⁱ	4.50 ± 0.50 ^{ghi}	14.67 ± 2.52 ^c
<i>B. cereus</i>	19.83 ± 0.29 ^b	-	3.67 ± 0.58 ^{hi}	5.00 ± 0.00 ^{ghi}	6.83 ± 0.76 ^{fg}
<i>E. coli</i>	24.33 ± 1.16 ^a	-	8.33 ± 0.58 ^{def}	10.00 ± 0.50 ^{de}	10.67 ± 0.58 ^d
<i>S. aureus</i>	22.00 ± 1.00 ^{ab}	-	6.33 ± 0.58 ^{fgh}	7.83 ± 0.29 ^{ef}	9.67 ± 1.16 ^{de}

Note: means of triplicate determination ± standard deviations followed by the same letter are not significantly different according to the Tukey test ($P \geq 0.05$) The positive control was Streptomycin and DMSO 15% was the negative control.

As shown in Table 3, the diameter of the inhibitory zone decreased with the decline of tested concentrations. Letters following the result expressed statistical significance compared to each other in the same column and row. Numbers followed by the same letter were not significantly different according to the Tukey test ($P \geq 0.05$), otherwise, the statistical data would be significant. Specifically, the extracts at concentrations of 0.3 and 0.4 mg mL⁻¹ had the same inhibitory ability on *L. monocytogenes* with diameters of 2.33 and 4.5 mm (the difference was not statistically significant in the same row). The extract at a concentration of 0.5 mg mL⁻¹ had the strongest inhibitory effect on *L. monocytogenes* with an inhibitory diameter of 14.67 mm. For *B. cereus*, the inhibitory ability of the extract at different concentrations did not differ with statistical significance compared to the data belonging to the similar row; the inhibitory diameter was 3.67 and 5.00 mm at the concentrations of 0.3 and 0.4 mg mL⁻¹.

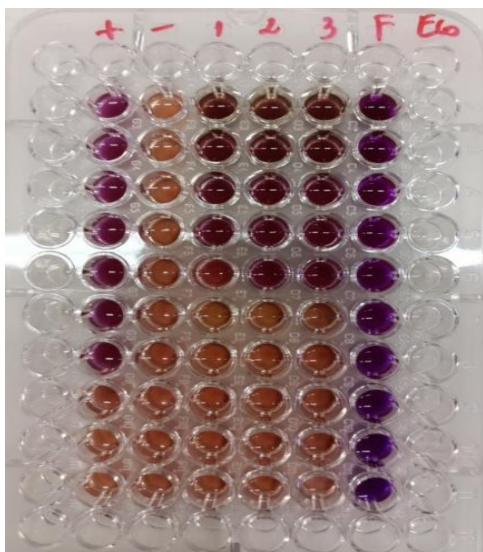
**Determination of MICs**

Figure 4. The color observation based on Resazurin in plate. (+): Streptomycin 0.5 mg mL⁻¹ at first well; (-): DMSO 15% at first well; (1), (2) and (3): three replicates of *T. villosus* leaf extract (5 mg mL⁻¹ at first well); (F): tested solution including ISO and Resazurin 10 mg mL⁻¹

For bacterial strain, *S. aureus*, the inhibitory ability of the extract was highest at the concentration of 0.5 mg mL⁻¹ with the diameter reaching 9.67 mm (the difference was statistically significant for the antimicrobial resistance value at concentrations 0.3 mg mL⁻¹, 6.33 mm). In general, the inhibitory ability of the three bacterial strains above at the high extract concentration of 0.5 mg mL⁻¹ was statistically significant compared with the other two of 0.3 and 0.4 mg mL⁻¹. Therefore, it is possible to choose the appropriate extract concentration for the best antibacterial ability depending on the usage purpose to save the extract and production costs. Finally, the antibacterial effect of the extract against *E. coli* was not statistically significant at the investigated concentrations, 8.33 mm at the lowest concentration (0.3 mg mL⁻¹) and 10.67 mm at the highest concentration (0.5 mg mL⁻¹), respectively.

The solution contained in lane F did not show the change in color meaning that the Resazurin solution used in this study was not contaminated, being a prestigious indicator for antibacterial testing. The color changes between dark violet and orange-pink colors were observed which was able to determine the MICs of examined antibacterial agents in this experiment (Table 4).

Table 4. MICs of *T. villosus* leaves extract recorded in four bacterial species based on Resazurin microtiter assay plate

Bacterial strains	Gram stain	MIC values are in mg mL ⁻¹	
		Streptomycin	Extract
<i>L. monocytogenes</i>	+	3.91×10 ⁻³	3.00×10 ⁻²
<i>B. cereus</i>	+	7.81×10 ⁻³	3.00×10 ⁻²
<i>E. coli</i>	-	6.25×10 ⁻²	8.33×10 ⁻²
<i>S. aureus</i>	-	6.25×10 ⁻²	8.33×10 ⁻²

As shown in Table 4, the *T. villosus* leave extract was able to inhibit the four bacteria in this research via the determined MICs. Specifically, the MICs of plant extract against *L. monocytogenes* and *B. cereus* (Gram-positive bacteria) was 3.00×10⁻² mg mL⁻¹ which was higher than the figure of *E. coli* and *S. aureus* (Gram-negative), was at 8.33×10⁻² mg mL⁻¹. It can be seen that the MICs of testing with Gram-negative bacteria was higher than that of Gram-positive bacteria so that

the antibacterial activity of the plant extract was considered being more efficacious for the group of Gram-positive bacteria. It is elucidated that the Gram-negative bacteria have a strong ability to resist antibiotics and antibacterial agents, which was characterized by its special cellular structure. As the description of Breijyeh *et al.*, (2020), the Gram-negative bacteria cells have a particular structure including three layers. The outer membrane is the first layer, considered a particular characteristic protecting it from attacked factors, making its susceptibility lower. Furthermore, it is examined as the principal reason for resistance to a broad spectrum of antibacterial agents because many hydrophilic components contained in these agents pass via porins and vancomycin are not capable to cross the outer membrane insofar as its structure that curb it from altering any of the passages. Conversely, Gram-positive bacteria do not have this feature, it therefore is more easily influenced by other factors.

DISCUSSION

The growth rate in the number of *T. villosus* leaves over 1 week, 2 weeks, 4 weeks, and 8 weeks increased gradually. However, the size of leaves (ratio of leaves length and width) did not largely change because the shape of *T. villosus* leaves changed during the growth, the initial leaves (1 to 2 weeks old plants) were usually oval. However, the young leaves appearing between 4 and 8 weeks were elongated. The data from Table 2 showed that the leaves reach their maximum size at 4 weeks of age. These results were relevant to plants used for fresh matter harvesting purposes because the growing time would not be too long to be exploited. Although it was harvested for the first time in net house conditions, moderate fresh leaf yield of 5.23 tons ha⁻¹ and dry matter leaf yield of 0.89 tons ha⁻¹ in comparison with some groups of plants with good fresh matter such as *Polygonum multiflorum* Thunb, *Pseuderanthemum palatiferum* (Wall.) Radlk, then could be considered as a potential group that could bring economic returns for farmers (Dieu *et al.*, 2006; Thi *et al.*, 2020). Farmers report that *T. villosus* serves as a valuable forage for ruminants, usually fed as sun-dried and cut-and-carry forage. The anatomical structure through parts of *T. villosus* leaves showed the characteristics of a light-tolerant plant (the upper surface has a two-layer fence pattern) containing a lot of chlorophyll, which helps the plant to photosynthesize well (Fig. 2). In addition to the characteristic of many soft tissues (structure of cellulose). Furthermore, in this parenchyma, there were many secretions such as starch, essential oils, and oxalate calcium crystals. These were necessary reserve substances (protective and hardening crystals) for plants; however, they were also substances that increase the quality of plants for animal feed (Duc *et al.*, 2006). Based on the results related to the antibacterial activity (agar well diffusion) and MICs, it is assumed that the ethanol leaf extract of *T. villosus* can resist bacteria. All three testing

concentrations of the extract can inhibit the indicator bacteria (both Gram-negative and Gram-positive). The extract of *T. villosus* is not currently much studied, the MIC values in this research could demonstrate that the extract has the antibacterial ability to the four bacteria species. Although the results involving antimicrobial activity was lower than that of antibiotics (Table 3), it could be an antibacterial agent, the *T. villosus*, therefore, could help to control the microorganism population living in the digestive system which was suitable to the Vietnamese traditional experiences of using this plant in human. With the antibacterial activity evaluated, the supplement of *T. villosus* in feeding livestock could be a solution for limiting bacterial infection and reducing antibiotic usage, contributing to decreasing the antibiotic resistance in livestock farming.

Conclusions: With good characteristics of growth (could be harvested for 4 weeks with the dry matter yield of 0.89 tons ha⁻¹ per year), leaves had many green leaves with a barrier pattern to help photosynthesis and adapt to the natural conditions of the Mekong Delta. Besides, it was proved that *T. villosus* was easy to cultivate with moderate growth rate (fresh leaf yield at 5.23 tons ha⁻¹). According to the structure, there was a lot of fibrous matter (cell wall is rich in cellulose, a range of starch and secondary secretions in the cell) which would be easy to digest for some groups of animals. Furthermore, the extract had the antibacterial ability in both Gram-negative and Gram-positive bacteria, recorded with MICs value from 3×10^{-2} to 8.33×10^{-2} mg mL⁻¹. The selection of relevant antibacterial concentrations depends on the purposes and needs to be further studied to economize the cost as well as get the high effectiveness in exploring the plant for livestock feeding.

Conflict of Interest: The Authors declare that there is no conflict of interest.

Authors' Contribution Statements: Phung Thi Hang, Lam Ngoc Ngan: Investigation, methodology, design of the experiment, manuscript preparation, editing and formal analysis; Nguyen Khoi Nghia, Ho Thanh Tham, Nguyen Trong Hong Phuc: Conceptualization, investigation, supervision, editing, and finalization.

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