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Total Phenolic, Total Flavonoid, Total Condensed Tannin Contents and Antimicrobial Activity Against Diarrheal Bacteria of the Bark and Fruit of *Terminalia nigrovenulosa* Pierre ex Laness

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ABSTRACT

The bark and fruit of the Thai astringent herb, *Terminalia nigrovenulosa* are some of the most frequently used herbal products for treating diarrhea and dysentery. Unlike other astringent herbs, this plant has never been screened for antimicrobial activity against diarrheal bacteria before. The bark and fruit of this plant were prepared via water extract and ethanol extract to investigate the presence of any antimicrobial activity against various strains of bacterial; this was done by disc diffusion method and broth dilution method. Additionally, total phenolic content, total flavonoid content, and total tannin content was measured. Water and ethanol extracts of the fruit had a total phenolic content of 157.70±2.83 and 371.97±6.13 mg gallic acid equivalence / g extract. Total flavonoid content of the fruit was found to be 332.24±4.59 µg quercetin equivalence/ g extract and 268.90±1.09 µg quercetin equivalence/ g extract for the water and ethanol extracts, respectively. Whereas the total condensed tannin from the bark was 0.89±0.02 µg catechin equivalence/g extract and 0.85±0.03 µg catechin equivalence/g extract for the water and ethanol extracts, respectively. Regarding antimicrobial action against diarrheal bacterial, the ethanol extract of the fruit showed activity against *Shigella sonnei*, *Shigella flexneri*, *Shigella dysenteriae*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtillis*, and *Staphylococcus aureus* with an inhibition diameter of 15, 7, 13, 10, 13, 15, and 15 mm, respectively, which was similar to ciprofloxacin. TheMIC of this plant's fruit was 16.12 mg/ ml compared to ciprofloxacin 0.01 mg/ ml. Overall, this study indicated that *T. nigrovenulosa* fruit has a tendency to inhibit the proliferation of dysentery bacterial and diarrheal bacterial; looking forward, it would be wise to develop further in vitro and in vivo models for future development.

Keywords: Diarrhea and dysentery; *Terminalia nigrovenulosa*; Thai astringent herbs

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1. Introduction

Diarrhea is found in developing countries including Thailand. The incidence of diarrhea is high in young children (under 5 years old) who suffer from health problems due to a lack of basic hygiene in their community. In addition, there is a specific kind of diarrhea, a traveler's diarrhea, which is often caused by eating and drinking contaminated food or beverages [1]. With advances in the understanding of chronic diarrhea, there are several conventional treatments available including antidiarrheals and antispasmodics drugs. These medicines are somewhat expensive when also considering the high relapse rates present in some populations and can cause some adverse effects [2]. It is known that the majority of herbal medicines used as antidiarrheals have properties that result in delaying gastrointestinal processes, suppressing gut motility, stimulating water absorption, and reducing electrolyte secretion which may explain the benefits of using certain herbal medicines for the treatment and management of diarrhea from bacterial infection [3]. According to the concept of Thai traditional medicine, with chronic loose stool, the underlying cause is usually the imbalance and defect of four elements in the body; earth, water, wind, and fire. Normally, the consumption of food with extreme taste such as sour or spicy can disturb the body's elements [4]. Spicy foods can cause an excess of water in the digestive system especially in the stomach and intestine. In Thai traditional medicine theory, diarrhea can cause a severe imbalance in all of four of the elements. Treatment of diarrhea is done by using astringent herbs to alleviate the condition. Astringent herbs are a special type of plant that pulls the body's cells together and also accelerates the contraction of the body's tissues. The active constituents that provide astringent herbs the property of inducing beneficial drying and tightening are known as "tannins". Tannins are responsible for the astringent or bitter taste of several herbs and act to reduce intestinal inflammation. Tannins bind to the protein layer of inflamed mucous membranes and thicken them and slow down the absorption of toxic materials and also inhibit the production of secretions [5].

Terminalia nigrovenulosa Pierre ex Laness. is a Thai medicinal plant in the Combretaceae family that grows wild in deciduous forests in the northeastern part of Thailand and the Southern part of Vietnam. This plant has been used as an anti-diarrheal in the treatment of chronic dysentery in Thailand and Vietnam and has also been used to treat sore throat, laryngitis, and hemorrhoids in Vietnam. According to Thai traditional medicine practice, both the bark and fruit of this plant can be used to treat diarrhea and dysentery with tenesmus. However, in the case of severe diarrhea, only the fruit is recommended for use. Studies have shown that bark extracts of *Terminalia* spp. possess a variety of biological activities. The activities indicated in previous studies include potent antioxidant activity, in vitro anti-cancer activity by inhibiting expression of matrix metalloproteinase enzymes MMP-2 and MMP-9 [6], antifungal activity against *Fusarium solani* [7], and nematicidal activity against *Meloidogyne incognita* [8].

Evidently, there are not sufficient scientific studies that confirm and compare the antimicrobial properties of the bark and fruit of this plant. This study looks into the in vitro antimicrobial activity of these plant parts against 6 pathogenic microorganisms that cause the most common cases of diarrhea and dysentery in the upcountry area of Thailand. The selected solvents for this study were hot water, which corresponded to the menstruum for decoction process, and 95% ethanol as a representative of the solvent used in the laboratory for preparation of the extract of *T. nigrovenulosa* for determination of its activity. In addition to the investigation of antimicrobial activity, this study also evaluated the total phenolic

content, total flavonoid content, and total condensed tannin content of this plant.

2. Materials and Methods

2.1 Plant material

The bark and fruit of *T. nigrovenulosa* used for this study were collected during October 2018 from multiple zones in the north eastern area of Thailand (Fig. 1). The samples were classified taxonomically by the division of pharmacognosy and toxicology; voucher specimens were made for all accessions and conserved at the herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. The voucher specimen number is TNKKU2018-45.

Fig. 1. *T. nigrovenulosa* Pierre ex Laness. (Fruit and Bark).

2.2 Preparation of plant extracts

The bark and fruit of this plant were washed, dried with sunlight, cut to small pieces, and dried again in a hot air oven at 40ºC until completely dry. The dried plants were ground to a coarse powder by grinder; the powders were then kept in zip-locked bags and stored in a dry area [9]. Sixty g of powder from each plant was macerated with 1000 mL of ethanol 95%, at 25ºC for 7 days. The maceration with solvent was conducted 3 times. The extracting solvents were filtered and evaporated by rotary evaporator (BUCHI Labortechnik AG, Switzerland) until the solvents were completely removed. For the water extraction, 60 g of each plant powder was boiled in 2 liters of water to imitate the actual preparation (conducted only 1 time with the temperature around 85-90ºC) for 30 minutes. The extracting solvents were then filtered and the water was removed using a freeze dryer (SCANVAC cool safe 110-4 Pro, Denmark), all crude extracts were stored at -10°C until the antioxidant assay experiments were performed [10].

2.3 Total phenolic content

The total phenolic content of the plant extracts was determined using Folin-Ciocalteu reagent (Merck KGaA, Germany) following a slightly modified method [11], [12]. Gallic acid (Fluka, Switzerland) was used as a reference standard. Prepared by serial dilutions with distilled water, various concentrations of gallic acid ranging from 12.5 to 100 µg/mL were prepared from its stock dilution. The plant extracts were prepared at the concentration of 500 µg/mL. For sample measurement, 100 µL of Folin-Ciocalteau's reagent and 80 µL of 7% sodium carbonate were added to the 20 µL of sample in 96 well plates. The solutions were mixed and incubated at room temperature for 30 minutes. Absorbance at 765 nm was measured. Data presented are average values of 3 measurements for each sample. The total phenolic contents were expressed as mg of gallic acid equivalent (GAE)/g extract.

2.4 Total flavonoid content

The determination of total flavonoid content referred to [13]. 0.5 mL of the extract was mixed with 4.5 mL of methanol. To the mixture, 0.1 mL of 10% aluminum chloride (Fischer Scientific, UK) and 0.1 mL of 1 mol/L sodium acetate (Fischer Scientific, UK) was added. The mixture was kept at room temperature for 30 min and the absorbance was measured at 415 nm using UV/visible spectrophotometer (Perkin Elmer, USA). The total flavonoid content of the extracts was calculated using a standard graph of quercetin (Aldrich, Germay). Stock quercetin solution was prepared by dissolving 5.0 mg quercetin in 1.0 mL methanol, then the standard solutions of quercetin were prepared by serial dilution using methanol (100-1000 µg/mL). The

results were expressed as quercetin equivalent (mg/g).

2.5 Total condensed tannin contents

The determination of condensed tannin content referred to [14-15] , using catechin (Aldrich Germany) as a reference compound. A volume of 400µL of extract was added to 3 mL of a solution of vanillin (4% in methanol) and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation, the absorbance was read at 500 nm. The condensed tannin content was expressed as milligrams of catechin per gram of dry weight (mg CE/g DW).

2.6 Determination of antimicrobial activity (CLSI. Methods) [16]

2.6.1 Microorganisms used

The test organisms *Shigella sonnei* ATCC 1160, *Shigella flexneri* ATCC 29903, *Shigella dysenteriae* ATCC 13313, *Salmonella typhimurium* ATCC 13311, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 25923 were obtained from the microbiology laboratory, Department of Medical Sciences, Nonthaburi, Thailand. The Department of Medical Sciences provided various cultures from their ATCC bacteriology collection with the specific restriction to Universities only, for research purposes only.

2.6.2 Culture media

The medium used for the activation of the microorganisms was soybean casein broth (SBCB). The following selective agar media were used for the antimicrobial test: Baird- Parker (*S. Aureus*), McConkey (*E. coli*) , and Xylose Lysine Deoxycholate agar (*Salmonella* and *Shigella*). All the culture media were prepared and treated according to manufacturer guidelines (Becton Dickinson® M.D. USA). Ciprofloxacin was used as positive control.

2.6.3 Inoculum

The microorganisms were inoculated into SBCB and incubated at 35±2°C for 4 h. The turbidity of the resulting suspensions was diluted with SBCB to obtain a transmittance of 25.0% at 580 nm. After that, the percentage was found spectrophotometrically comparable to 1 McFarland turbidity standard. Agar diffusion assay.

The antimicrobial activity test was performed by employing the pour plate method as well as disc diffusion methods.

The freeze-dried extracts of the plant were dissolved in water and solutions of concentrations ranging from 10 to 100 mg/ ml were prepared. Then, 25-30 µl of each sample was applied to a hole- punched plate. All bacterial strains were grown in their respective media overnight at 37°C. The suspension of microorganisms was adjusted to 105 - 107CFU/ ml in broth media. In this procedure, 100 µl of bacterial suspension was prepared in the nutrient broth and was inoculated in the nutrient agar in petri dishes at room temperature in sterile condition and mixed thoroughly to ensure uniform growth. All the seeded petri dishes were incubated at 27 °C for 24 hours. The assessment of antimicrobial activity was based on measurements of the inhibition zones formed around the discs. The appearance of a bacterial free zone around the disc is known as an inhibitory zone, and is considered as a positive response [17-18].

A minimal inhibitory concentration (MIC) evaluation was then conducted; this method was done on plant extracts that showed antimicrobial activity. This test was performed at 4 concentrations for each extract (6.3, 12.5, 25, 50 µg/ml) employing the same modified agar well diffusion method. All values are expressed as mean ± standard deviation. The MIC data for each microorganism were analyzed using one-way analysis of variance (ANOVA) and the differences among group means were analyzed using the Dunnett's multiple

comparisons test. A p-value < 0.05 was considered as significant. The software MINITAB® was employed for the statistical analysis.

3. Results

Water extraction with *T. nigrovenulosa* bark led to the highest yield (16.17%) followed by the water extract of fruit (14.43%), methanol extract of bark (11.87%), and lastly methanol extract of fruit (7.27%). The total phenolic content, total flavonoid content, and total tannin content results are presented in Table 1. TPC in the different herbs was determined by Folin-Ciocalteu assay, expressed as gallic acid equivalents by reference to a standard curve ($y=0.0051X-0.0034$, $R^2=0.9976$). The TPC varied from 89.17±1.43 to 371.97±6.13 mg GAE/g extract. The results show that ethanol extract of the fruit produces the highest phenolic concentration (371.97 ± 6.13 mg GAE/g extract), followed by water extract of the fruit (157.70±2.83 mg GAE/g extract). Ethanol extracts of the fruit and the bark exhibited a higher phenolic content than those of the water extracts. Water extract of bark presented the lowest level of TPC (89.17±1.43 mg GAE/g extract).

Total flavonoid content of the plant extracts was determined in comparison with standard quercetin and the results are expressed in terms of mg quercetin/ g (Standard curve $y=0.00014X-0.0908$, $R^2=0.9961$). Similar to total phenolic content, the ethanol extract of fruit contains the highest amount of flavonoid (332.24±4.59 mg/g) followed by water extract of fruit (268.90±1.09 mg/g). The lowest amount was presented in water extract of bark (239.48±1.30 mg/g).

Contrary to TPC and TFC results, the highest total tannin content was found in the bark extract with 0.89±0.02 and 0.85±0.03 µg CE/g extract for water and ethanol extract respectively.

Table 1. Total phenolic content, total flavonoid content and total condensed tannin content of *T. nigrovenulosa* ethanol and water extracts (fruit and bark).

Plant	Total phenolic content (mg GAE/g extract)	Total flavonoid content (µg QUE/g extract)	Total condensed tannin content (µg CE/g extract)
T. nigrovenulosa (fruit)			
Ethanol	371.97±6.13	332.24±4.59	0.66±0.01
Water	157.70±2.83	268.90±1.09	0.65±0.01
T. nigrovenulosa (bark)			
Ethanol	143.72±2.27	246.41±0.75	0.85±0.03
Water	89.17±1.43	239.48±1.30	0.89±0.02

Results are mean ± SD from three sets of independent experiments, each set-in triplicate.
GAE=gallic acid equivalence, QUE=quercetin equivalence, CE=cathechin equivalence.

In the present study (Table 2), ethanolic extract and water extract from fruit and bark of this plant were investigated for activity against various diarrheal bacteria (*S. sonnei*, *S. flexneri*, *S. dysenteriae*, *S. typhimurium*, *E. coli*, *B. Subtilis*, and *S. aureus*). Some extracts had clearly visible zones of inhibition, while some had moderate to small, or even no zones of inhibition. According to the agar disc diffusion study, ethanol and water extracts from the fruit exhibited antimicrobial activity against 7 strains of diarrheal bacterial including *S. sonnei*, *S. flexneri*, *S. dysenteriae*, *S. typhimurium*, *E. coli*, *B. subtillis* ATCC6633, and *S. aureus*. Inhibition zones of bark and fruit extract applications against *S. sonnei*, *S. dysenteriae*, *E. coli*, and *S. aureus* ranged from 13-15 mm, whereas those of ciprofloxacin ranged from 15-17 mm. In addition, water extract from the fruit exhibited an inhibition zone of 19 mm compared to the 16 mm inhibition zone of ciprofloxacin. The strongest MIC of this plant was found in ethanol extract from the fruit with a value of 16.12 mg/ml against *S. sonnei* compared to ciprofloxacin (0.01 mg/ml). The MIC concentrations of bark extract ranged from 31.25 to 125 mg/ml and were mostly very high, ranging from 0.008 to 256mg/ml. The ethanol extracts exhibited stronger antimicrobial activity and a much broader spectrum of action than those of the aqueous extracts.

Table 2. Disc Diffusion Assay of *T. nigrovenulosa* ethanol and water extracts (fruit and bark).

Extracts	Average Inhibition Zone (mm) (n=3, mean±SD)						
	SS	SF	SD	ST	EC	BS	SA
Water extract							
(Fruit)	15±0.12	7±0.08	13±0.10	10±0.07	13±0.09	15±0.11	15±0.10
(Bark)	11±0.08	7±0.05	17±0.12	0	0	10±0.10	0
Ethanol extract							
(Fruit)	19±0.15	8±0.03	10±.05	11±0.07	8±0.09	14±0.10	13±0.11
(Bark)	1.5±0.02	0	0	0	0	0	0
Ciprofloxacin**	16±0.05	25±0.09	17±0.05	22±0.08	15±0.09	25±0.08	16±0.08

*SS=*Shigella sonnei* ATCC1160, SF=*Shigella flexneri*, SD=*Shigella dysenteriae*, ST=*Salmonella typhimurium* ATCC13311, EC=*Escherichia coli* ATCC25922, BS=*Bacillus subtillis* ATCC6633, SA=*Staphylococcus aureus* ATCC25923;
**Ciprofloxacin (12.5µg/ml) used for positive control of bacteria *T.nigrovenulosa* extract concentration (62.5µg/ml)

Table 3. Minimum Inhibitory Concentration of *T. nigrovenulosa* ethanol and water extracts (fruit and bark).

Extracts	Minimum Inhibitory Concentration (mg/ml)						
	SS	SF	SD	ST	EC	BS	SA
Water extract							
(Fruit)	62.5	31.25	N/A	31.25	N/A	62.5	62.5
(Bark)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ethanol extract							
(Fruit)	16.12	N/A	31.25	N/A	N/A	31.25	125
(Bark)	125	N/A	N/A	31.25	N/A	N/A	125
Ciprofloxacin**	<0.01	<0.02	<0.01	0.01	<0.01	0.02	<0.1

*SS=*Shigella sonnei* ATCC1160 , SF=*Shigella flexneri*, SD=*Shigella dysenteriae*, ST=*Salmonella typhimurium* ATCC13311, EC=*Escherichia coli* ATCC25922, BS=*Bacillus subtillis* ATCC6633, SA=*Staphylococcus aureus* ATCC25923 ;
**Ciprofloxacin (12.5µg/ml) used for positive control of bacteria, Na = Non-applicable : result value was higher than 500 µg/ml

4. Conclusion

All extracts from *T. nigrovenulosa* exhibited high total phenolic content, total flavonoid content, and total condensed tannin content. The high level of flavonoid content of bark extract is consistent with the results of a previous study [5] which isolated luteolin and catechin from the bark of this plant. The present study is the first to use the fruit extract for chemical analysis and antimicrobial activity testing. The fruit extract exhibited a higher total phenolic and total flavonoid content than the bark extract, however, the bark extract showed a slightly higher total condensed tannin content than the fruit.

All of the extracts showed varying degrees of antimicrobial activity on the microorganisms tested. Some of these plants were more effective than ciprofloxacin to combat the diarrheal microorganisms. Total phenolic content of *T. nigrovenulosa* exhibited a similar pattern to total flavonoid content but was inconsistent with total condensed tannin content. Several MIC values (Table 3) did not correlate with inhibition zones. Possible factors potentially leading to apparently contradictory results between MIC determination and diffusion assay results could be the solubility and molecular size of active compounds, which effect the diffusion of the compounds in the extract through agar, and therefore the resulting zone size [19].

The chance to find antimicrobial activity of *T. nigrovenulosa* was higher in ethanol extracts compared to water extracts. In addition, bark extract presented the lowest MIC compared to fruit extract. All extracts exhibited a high MIC compared to ciprofloxacin. Further work is needed to isolate secondary metabolites from the extracts studied in order to test specific antimicrobial activity. According to a Thai traditional medicine concept, herbs with a high tannin content are considered as

astringent herbs with cold taste. The cold taste can counter the action of excess fire element in diarrhea patients. The excess fire element is the origin of the illness in diarrhea patients that further causes the imbalance of water element (watery stool) and earth element (gastrointestinal motility disorders). Overall, this study indicated that *T. nigrovenulosa* fruit has a tendency to inhibit dysentery bacteria and diarrheal bacterial; further *in vitro* and *in vivo* models should be studied for the future development of this plant's medical use.

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