Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 2337-2342
Received: 20-07-2017
Accepted: 21-08-2017

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Phytochemical screening and antibacterial activity of selected medicinal plants against laboratory diarrheal bacteria strains

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Abstract
Diarrheal diseases are major causes of mobility and mortality especially among children in developing countries. This study aimed at investigating the antibacterial activity of organic and aqueous medicinal plant extracts against diarrhea causing bacteria strains. The plant extracts were screened for the presence of phytochemical compounds by thin layer chromatography. *In-vitro* antibacterial activity was determined against *E. coli, Shigella bodyii, Staphylococcus aureus, Salmonella typhi* and *Listeria monocytogenes* by disc diffusion method on Mueller- Hinton agar plates; the Minimum inhibitory concentration was determined using Nutrient broth. Phytochemical screening revealed the presence of triterpernoids, saponins coumarins and alkaloids in all organic extracts and higher presence of tannins and saponins in aqueous extracts. The organic and aqueous extracts of *T. sericea* showed broad spectrum antibacterial activity against different diarrheal bacteria. *S. linnaeanum* showed the highest antimicrobial activity against *Listeria monocytogenes* with a concentration dependent effect. *T. sericea* and *C. apiculatum* portrayed the lowest Minimum inhibitory concentration of 62.5 µg/ml against *S. aureus* and *E. coli*.

Keywords: Diarrhea, Antibacterial, Medicinal plants, Phytochemical screening

Introduction
Diarrheal diseases are a major cause of mobility and mortality especially among children in developing countries [1]. Statistics have shown that 5.9 million children around the world die before reaching their fifth birthday, 9% were due to diarrhea which was the second highest after pneumonia which caused 16% of all deaths [2].
In Namibia, diarrheal diseases are among the top 10 causes of death [3], 518 cases of cholera were recorded in the northern part of Namibia (Kunene, Oshana and Omusati regions) with a total of 17 deaths in 2014 [5].
Various strategies have been developed to combat diarrhea such as immunization for rotavirus, cholera and measles immunization, Oral rehydration solution, zinc supplements, tetracycline, ampicillin and fixed ratio combination treatment of antibiotics [2, 6]. However, despite the available medicine and vaccines, diarrhea remains one of the top causes of preventable mortality and morbidity especially in developing countries[6] because of poor sanitation and lack of access to treatment options for diarrhea [7, 8]. People in rural settlements live far from hospitals hence, it takes several hours or days to reach the health facilities and in many cases they have no access to the antibacterial pharmaceuticals [9]. Another factor for the continuing morbidity and mortality is that some pathogens such as *Shigella, Campylobacter, Nontyphoidal Salmonella,* and *enterotoxigenic Escherichia coli* have become resistant to most of the available antibiotics [10]. Hence, there is a need for a viable alternative treatment to treat diarrheal diseases.
Traditional medicine is currently the fastest growing medical field with herbal therapies becoming increasingly popular [11]. Statistics showed that 80 % of the people from developing countries depend on medicinal plants as means of primary health care [12], whereby 70 % of the people that rely on herbal medicine are from African countries [13]. In Namibia, The traditional medical practice is well documented and about 53 medicinal plant species are in demand for medicinal trade in Windhoek [14]. This is due to a long tradition of using medicinal plants for the treatment of infectious disease in Namibia [15]; Different medicinal plants are used traditionally to relieve ailments cause by microbial agents such as malaria, cough, diarrhea [15], fever and sore throat [16]. Although, there is sufficient information on the traditional medicinal use in Namibia, There is limited scientific evidence on their phytochemical compositions and efficacy.

Hence, this study was conducted to determine the phytochemical screening and antibacterial activity of selected Namibian medicinal plants against laboratory strains that cause reported cases of diarrhea.

Materials and Methods

Sample Collection

A plant collection permit was obtained from the Ministry of Environment and Tourism of Namibia (MET) to license collection of plant materials used in this research. Plant materials (bark, roots and leaves) of *B. albitrunca Z. mucronata C. apiculatum, S. linnaeanum* and *T. sericea* were collected from Kunene region in Namibia. Plants collected were authenticated by the National Herbarium at the National Botanical Research Institute.

Preparation of extracts

The collected plants materials were air dried for 4 weeks at ambient temperature, and grounded. Organic and aqueous plant extracts were prepared using 90% ethanol and distilled water respectively by submerging powdered plant material in 90% respective solvents and incubated at room temperature for 24 hours. What man no 1 filter papers were used for filtration and the filtrate were dried using rotary evaporation and freeze drying [17].

Phytochemical screening

Aqueous and organic extracts were tested for the presence of flavonoids, alkaloids, tannins; steroids and coumarin by Thin layer Chromatography (TLC) using 0. 05 ml of prepared plant extracts. The analysis was performed using solvent systems, chromogenic reagents and positive controls as depicted in table 1; based on a methods from [18].

Table 1. The solvent systems used for phytochemical screening [18].

Plant compound	Solvent system	Chromogenic reagents	Positive controls
Alkaloids	Chloroform: Ethanol (9:1)	Dragendorff reagent	Quinine
Flavonoids	Butanol: acetic acid: Water	Antimony chloride in chloroform	Quercetin
Saponins	Chloroform, Methanol	Vanilin, Ethanol, sulfuric acid	Saponin
Steroids	Chloroform, acetone	A: Phosphoric acid, water B: Methanol, phosphoric acid	β-sitosterol
Tannins	1% ferric chloride in 50% aqueous methanol	1 % Potassium hydroxide in Methanol	β-sitosterol
Coumarins	Chloroform	Copper sulphate, sodium citrate, anhydrous sodium carbonate	Coumarins
Triterpenoids	Hexane: Ethyl acetate (17:3)	Liebermann reagent: cool 5 ml acetic acid and 5 ml of Sulfuric acid mixed with 50 ml ethanol	β-sitosterol

Bacterial Cultures

Standard pathogenic cultures of *E. coli* ATCC25922, *L. monocytogenes* ATCC13932, *S. boydii* ATCC9207, *S. typhi* ATCC9216 and *S.aureus* were used for the present study. The bacteria strains were cultured in Nutrient broth at 37°C for 9 hours and stocked at 4°C on Muller Hinton agar plates. The inoculum size of the bacteria cultures were standardized according to the committee for Clinical Laboratory Standards with the final inoculum size of 10^5 CFU/mL [20]. Diarrheal pathogens were inoculated in Nutrient broth for 9 hours and the turbidity was compared to 0.5 McFarland standard.

Preparation of discs for antibacterial activity

Dry powder of different aqueous and organic extracts were used to prepare different concentrations of plant materials (250, 500 and 1000µg/ml) using the formula $C_1V_1=C_2V_2$, where c= concentration and v= volume as described by (Khanam *et al.* 2014) [19]. Antibacterial of organic and aqueous plant extracts was determined on Muller Hinton agar plates at 250, 500 and 1000 µg/ml concentrations by disc diffusion method. Inoculums were spread over the Muller Hinton agar plates using sterile cotton swabs. Sterile paper discs were then soaked in respective extract concentrations and placed on agar plates. Tetracycline (35µg/ml) was used as a positive control for *E. coli*, vancomycin (10µg/ml) for *L. monocytogenes*, gentamycin (30µg/ml) for *S. aureus* and ampicillin for *S. boydii and S. typhi.* Distilled water and 90% ethanol were used as negative controls. All the plates were incubated at 37 °C for 24 hours [20]. Antibacterial activity was evaluated and the diameter of the zone of inhibition was recorded. Antibacterial activity was classified as strong (≥15 mm), moderate (≥ 7 mm) and inactive (≤6 mm).

Determination of Minimum Inhibitory Concentration

The MIC test was performed in Nutrient broth by broth dilution method at 9 different concentrations (1000 to 3.91µg/ml) as described by Sule & Agbabiaka (2008) [21]. Double fold serial dilution method was used to prepare different extracts concentrations. Tubes of different concentrations of plant extracts were inoculated with. 0.1 ml of the standardized bacterial cell suspensions and incubated at 37° Celsius for 24 hours. The growth of the inoculum in the broth was indicated by cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organism was taken as the MIC. Controls were set up as follows: Nutrient broth only; Nutrient broth and plant extract; and finally Nutrient broth, and a test organism and stored in the refrigerator after preparation until further use. After incubation, a loop full from each tube was sub cultured on Nutrient agar to determine if bacterial growth was inhibited by plant extracts (Minimum Bactericidal Activity). The MIC was defined as the lowest concentration of an antibacterial that inhibited the visible growth of a bacterium after overnight incubation with zero CFU/mL after sub culture on Nutrient broth [21].

Results and Discussion

The Thin layer chromatography chemical profiles for the plant extracts investigated are portrayed in table 2 for aqueous extracts and table 3 for organic extracts. All organic plant extracts showed the presence of triterpenoids, saponins, coumarins and alkaloids. On the contrary, all aqueous extracts with an exception of *S. linnaeanum* only showed the presence of tannins and saponins. The organic extracts of *B. albitrunca* showed the presence of all the phytochemical compounds screened for; whilst its aqueous extract only showed the presence of saponins and tannins. Of all aqueous extracts, only *Z. mucronata* and *S. linnaeanum* and showed the presence of flavonoids; this finding contradicts Tiwari *et al*. (2011) [22], since according to Tiwari, water is only considered best in extracting sugars, tannins and saponins. The higher

presence of phytochemical compounds in organic plant extracts than aqueous plant extracts could be because more bioactive compounds such as flavonoids, steroids and alkaloids are more soluble in organic ethanol that was used in preparing organic extracts [13], while water can only extract sugar, tannins and saponins [22].

Table 2: Phytochemical screening of aqueous plant extracts using TLC

Plant name	Triterpernoids	Saponins	Flavonoids	Steroids	Coumarins	Tannins	Alkaloids
B. albitrunca	-	+	-	-	-	++	-
Z. mucronata	-	++	+	-	-	+	+
C. apiculatum	-	+	-	-	-	++	-
S. linnaeanum	+	-	+	-	-	-	+
T. sericea	-	+++	-	-	-	+++	-

Key: +++ High presence, ++ moderate presence, + low presence, - absent

Table 3: Phytochemical screening of organic plant extracts using TLCZ

Plant name	Triterpernoids	Saponins	Flavonoids	Steroids	Coumarins	Tannins	Alkaloids
B. albitrunca	++	+	+	+	+++	+	++
Z. mucronata	++	++	++	+	+	-	+++
C. apiculatum	++	+	-	-	++	-	+
S. linnaeanum	+	+++	+++	-	+++	-	+++
T. sericea	+++	++	+++	+++	+++	-	++

Key: +++ High presence, ++ moderate presence, + low presence, - absent

Only aqueous extracts of *T. sericea* showed a broad spectrum antibacterial activity against *E. coli, S. typhi, S. boydii, L. monocytogenes* and *S. aureus* as depicted in figure 1. Aqueous extracts of *T. sericea* showed Moderate antibacterial activity against *S. boydii* and *E. coli* (9 mm± 0.57). There is a concentration dependent effect in antibacterial activity of aqueous extracts against different diarrheal bacteria. There was no antibacterial activity against any diarrheal bacteria reported for aqueous extracts at the lowest concentration of 250 µg/ml. Vuuren *et al.* (2015) [23] also reported higher antibacterial activity in *T. sericia* organic and aqueous extracts against *S. aureus, E. coli, S. typhi* and *S. flexneri* in which organic extracts showed more potent antimicrobial activities than aqueous extracts (concentrations ≤ 1. 0 mg/ ml). In this study, organic *T. sericia* extracts also showed higher activity whilst aqueous extracts exhibited lower activity similar to the findings of Vuuren *et al* (2015) [23]. *T. sericea* is used traditionally to treat diarrhea, coughs and infected wounds[24]. It has been reported that organic extract had more phytochemicals compounds than aqueous extracts [22].

Studies such as Tiwari *et al.*, (2011) [22], Kumar *et al.* (2010) [25] and Murugan *et al.* (2013) [26], have linked the antibacterial activity of plant extracts to the phytochemical compounds in the plant extracts. Secondary metabolites inhibit microbial activities through different mechanisms such as by forming complexes with bacteria cell walls, membrane disruption, and protein lysis and by enhancing membrane absorption of sodium and water [22]. Hence, the presence of saponins in both aqueous and organic extracts of *T. sericia* may have contributed to the antibacterial activity. Kannabiran *et al.*, (2009) [27] isolated saponin fractions that exerted antimicrobial activity higher than the aqueous, organic extracts and standard antibiotics. The presence of phytochemical compounds may be responsible for antimicrobial activity portrayed by the different plant extracts in this study. Each plant extract included in this study inhibited the growth of at least one diarrheal pathogen, with a concentration dependent effect. The findings of Bhalodia and Shukla (2011) [28] also showed antibacterial activities of medicinal plants with a concentration dependent effect against different pathogenic bacteria.

Although Kirsoy, (2006) [29] and Gürbüz *et al.* (2015) [30] reported on the toxicity of *S. linnaeanum* and the presence of solamargine and solasonine glycoalkaloids with medicinal properties in *S. linnaeanum.* There is no other study to date that reported on the presence of other phytochemical compounds in the plants. Hence, the findings of the presence of phytochemical compounds such as saponins, triterpenoids, coumarins and flavanoids with antibacterial activity in *S. linnaenum* obtained in this study are new and have not been reported in other studies. Furthermore, the antibacterial activity of *S. linnaenum* have also not been documented.

Among the laboratory bacterial strains, *L. monocytogenes* was most sensitive to the plant extracts since all five plant extracts showed activity against *L. monocytogenes* at least at one extract concentration used. *S. linnnaeanum* showed the highest antibacterial activity (15 mm ± 0.57) followed by *T. sericea* (7.7 mm ±0.57). *Z. mucronata* showed moderate antibacterial activity against *L. monocytogenes* (7.3 mm ± 0.58) at 250 µg/ml as depicted in figure 4. Positive control vancomycin showed moderate antibacterial activity with the average inhibition of 11 mm ± 0.57. These findings are significant since to date, there are no commercially available vaccines to protect against infection by *Listeria* which is acquired by consuming raw (unpasteurized) milk or food that contain unpasteurized milk [31]. This makes these medicinal plants potential sources of anti- listeria agents that can be used in treatment against listeriotic diarrhea.

Salmonella typhi on the other hand was less sensitive to the aqueous plant extracts, since none of the aqueous extracts inhibited its growth. On the contraly, organic extracts of *S. linnaeanum* showed strong antibacterial activity against *Salmonella typhi* (18 mm ± 1.15) at 1000 µg/ml. The positive control ampicillin also showed strong antibacterial activity of 19.7 mm ± 0.56 against *S. typhi* as depicted in figure 6.

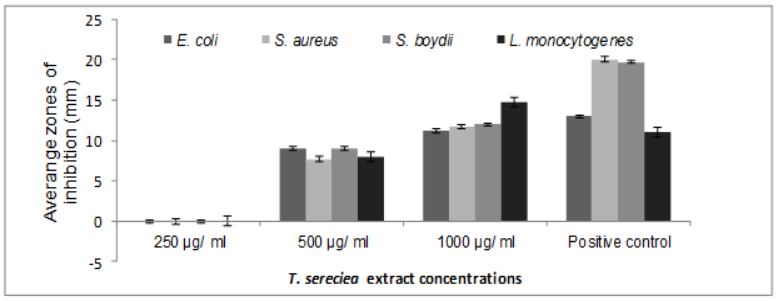

Fig 1: Antibacterial activity of aqueous extracts of *T. sericea* after 24 hours incubation against different diarrheal strains.

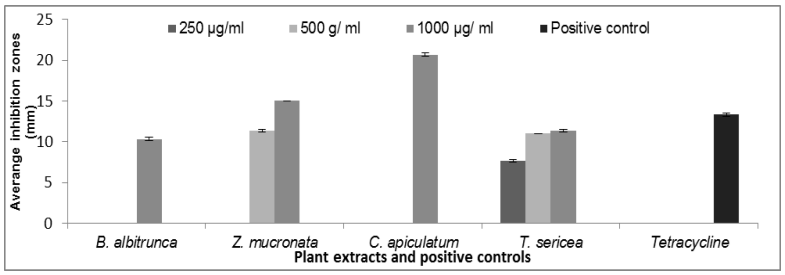

Fig 2: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum* and *T. sericea* against laboratory *E. coli* after 24 hours incubation

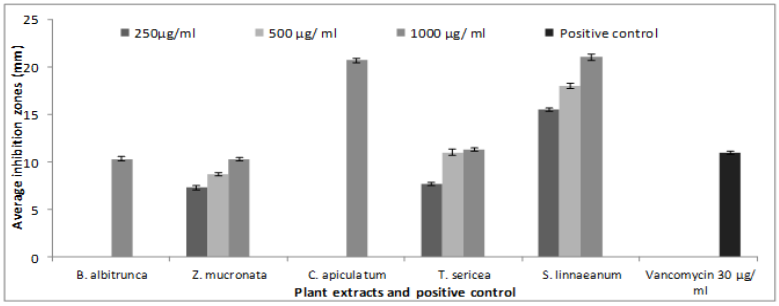

Fig 4: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *T. sericea* and *S. linnaeanum* against laboratory *L. monocytogenes* after 24 hours

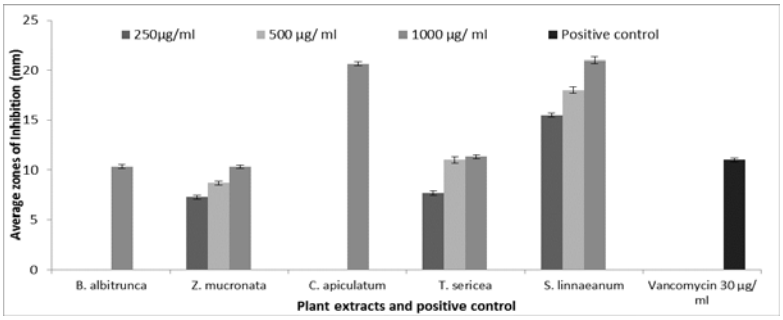

Fig 4: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *T. sericea* and *S. linnaeanum* against laboratory *L. monocytogenes* after 24 hours

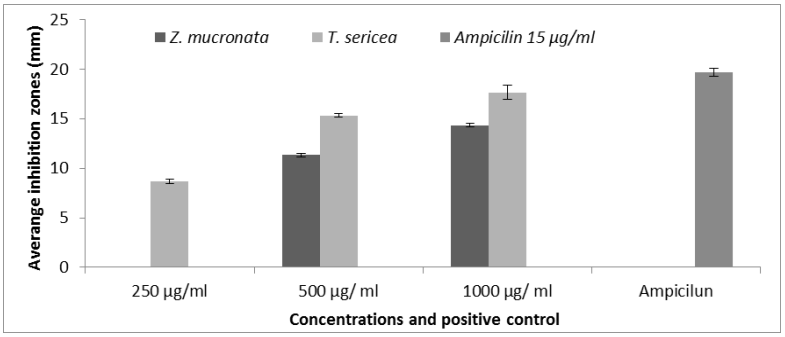

Fig 5: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. muronata*, *C. apiculatum*, *T. sereciea* and *S. linnaeanum* against laboratory *S. boydii* after 24 hours incubation.

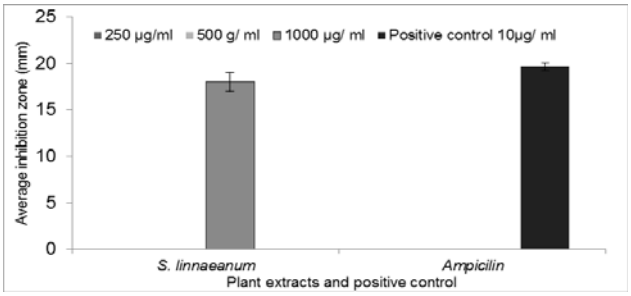

Fig 6: Antibacterial activity of organic extracts of *S. linnaeanum* against laboratory *S. typhi* after 24 hours incubation and ampicillin was used as a positive control.

Over all, Organic extracts showed potent antibacterial activity than aqueous extracts and all plant extracts showed antibacterial activity with a concentration dependent effect.

Findings on Minimum inhibitory concentrations are shown in table 4 for aqueous extracts and table 5 for organic extracts. *T. sericea* aqueous extracts showed an MIC of 500 µg/ml against *S. aureus, S. boydii, E. coli* and *L. monocytogenes*. The findings of this study also showed a difference in MIC of organic plant extracts against different laboratory diarrheal strains. *T. sericea* and *C. apiculatum* portrayed the lowest Minimum inhibitory concentration of 62.5 µg/ml against *S. aureus* and *E. coli*.

Table 4: Minimum inhibitory concentrations of *T. sericea* aqueous extracts against different selected diarrhea causing bacteria

Aqueous plant extracts	Test organisms			
	***E. coli* ATCC25922 (µg/ml)**	***S. aureus* ATCC25923 (µg/ml)**	***L. monocytogenes* ATCC13932 (µg/ml)**	***S. boydii* ATCC9207 (µg/ml)**
T. sericea	500	500	500	500

Table 5: Minimum inhibitory concentrations for organic plant extracts against selected diarrhea causing bacteria

Organic plant extracts	Test organisms				
	***E. coli* ATCC25922 (µg/ml)**	***S. aureus* ATCC25923 (µg/ml)**	***L. monocytogene* ATCC13932 (µg/ml)**	***S. typhi* ATCC9216 (µg/ml)**	***S. boydii* ATCC9207 (µg/ml)**
B. albitrunca	125	125	1000	>1000	> 1000
Z. mucronata	250	250	500	>1000	500
C. apiculatum	62.5	250	500	>1000	> 1000
T. sericea	500	62.5	250	>1000	125
S. liannaeanum	>1000	>1000	250	1000	>1000

The ability of these plant extracts in inhibiting different diarrheal pathogens add value to the existing knowledge on ethno medicinal use of these plants for treating diarrhoea in Namibia. Hence, these findings strengthen and support the potential of future antidiarrheal drug development from these plants.

Conclusion

The presence of at least one phytochemical compound screened in each plant extract attributed to the observed antibacterial properties of the plants against different diarrheal pathogen. The ability of *T. sericea* to portray antibacterial activity in aqueous and organic extracts prove why it is widely used in different counties to treat diarrheal diseases. The medicinal plants investigated are used in the traditional setting to treat diarrhea in Namibia and the results of the antibacterial activity and phytochemical composition of this study validates the use of these plants. Although these extracts have proven to portray antibacterial properties against diarrheal pathogens investigated, further studies such as cytotoxicity assays, GC/MS and *in vivo* antibacterial assays have to be performed before these extracts can be recommended as alternative treatment for diarrhea.

Acknowledgements

DAAD for funding the project

University of Namibia, Biological department and Multi-disciplinary research center, for providing research laboratories

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