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Original Article

Antibacterial Activity and Cytotoxicity against MCF-7 Breast Cancer Cell Lines of *Tabernaemontana pandacaqui* Lam. (Apocynaceae)

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ABSTRACT: *Tabernaemontana pandacaqui* Lam. is a widely distributed plant species in the Philippines. It is used as an herbal medicine and an ornamental plant. Limited studies had been done on the potential use of *T. pandacaqui* as alternative medicine compared to other *Tabernaemontana* species. In this study, antibacterial activity and cytotoxicity of *T. pandacaqui* were determined using disc diffusion and MTT assays using ethanolic extracts from wild type and dwarf cultivar vegetative parts. Disc diffusion assay was done against *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*. The stem of the wild-type obtained the highest antibacterial activity (33.33 mm) against *B. cereus* at 100 mg/mL. Roots of wild-type and dwarf cultivar exhibited consistent antibacterial activity against all bacterial strains used. MTT assay was done using HTB-22 MCF-7 human breast adenocarcinoma cells. The root of dwarf cultivar extract showed highest cytotoxicity at 5 and 50 µg/mL concentrations. The observed bioactivities may be due to alkaloids present in the plant samples which are abundantly found in the cortex, pericycle and near epidermal tissues. Saponins tested positive only in old roots of dwarf cultivar of *T. pandacaqui* prominently observed at or near the pericycle and the branching root.

KEY WORDS: *Tabernaemontana pandacaqui*, antibacterial, cytotoxicity, phytochemical screening

INTRODUCTION

In recent years, one of the major threats to public health particularly in developing countries is the increase of infectious diseases (Bradacs *et al.*, 2010). Resistance of the pathogenic microorganisms is due to the indiscriminate use of commercially available antimicrobial drugs (Rahman *et al.*, 2011a). Aside from the infectious diseases, one of the most prevalent causes of death is cancer. According to Parkin *et al.* (2001), over ten million new cases of cancer worldwide had been observed in 2000. There had been a 22% increase of cancer incidence and mortality since 1990. According to the Global Burden of Cancer Study (GLOBOCAN) in 2012, breast cancer is the second most common cancer in the world next to lung cancer, especially among women in both developed and developing countries.

The alarming increase of the emergence and spread of drug resistance against pathogens along with the increase of occurrence of cancer brought forth the development of new alternative drugs from potentially antibacterial and anticancer agents of plant

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origin (Bradacs *et al.*, 2010). In the Philippines, infectious diseases and cancer are among the top ten causes of death in the country according to the Department of Health (2013).

A plethora of medicinal plants are available for various research for drug discovery. Members of family Apocynaceae are exploited for the presence of secondary metabolites, particularly; alkaloids. The genus Tabernaemontana and its species are well-known for their antileishmanial (Munoz *et al.*, 2014), antiviral (Boligon *et al.*, 2015), antimicrobial (Bijeshmon and Shibu, 2014), antibacterial (Marathe *et al.*, 2013), antimycobacterial (Boligon *et al.*, 2015), antidiabetic (Rahman *et al.*, 2011b), antitumoral (Rizo *et al.*, 2013), antiprotozoal (Bradacs *et al.*, 2010), anti-inflammatory (Taesotikul *et al.*, 2003), antioxidant (Pereira *et al.*, 2005), antinociceptive (Shaker *et al.*, 2011), antipyretic (Taesotikul *et al.*, 2003), and neuropharmacological activities (Taesotikul *et al.*, 1998). The presence of triterpenoids, steroids, and several indole alkaloids are known to be responsible for their therapeutic activities are previously described in the studies previously conducted (Cruz *et al.*, 2015; Pallant *et al.*, 2012; Pereira *et al.*, 2005; Taesotikul *et al.*, 1998, 2003; Van Beek *et al.*, 1984).

A widely distributed species of this genus in the Philippines, *T. pandacaqui*, locally known as kampupot or pandakaki-puti, had been used therapeutically and as a landscape plant. However, previous studies done on the potential of *T. pandacaqui* as an alternative source of medicine is limited. Unlike *T. divaricata* which has its pharmaceutical properties studied from roots to flowers, *T. pandacaqui*'s bioactive constituents have not been explored. There are differences in the concentration and identity of bioactive components among plants at the genus level, as well as in the cultivated types and localization within the plant organ (Nwokocha *et al.*, 2011). Hence, these differences should be studied to effectively choose the plant materials to be used for drug discovery.

This study was done to evaluate the bioactivity of *T. pandacaqui* as a potential source of phytopharmaceuticals, specifically to test the bioactivity of *T. pandacaqui* extracts antibacterial agent; to assess the cytotoxicity of *T. pandacaqui* extracts against MCF-7 breast cancer cell lines; to locate selected bioactive compounds present in the different vegetative parts of *T. pandacaqui* through histochemistry; and to compare wild-type and dwarf cultivar *T. pandacaqui* in terms of bioactivity and histochemical profile.

MATERIALS AND METHODS

A. Selection of Plant Material Sample Collection and Preparation

Vegetative parts, leaves, stems, and roots from both wild-type and dwarf cultivars of *T. pandacaqui* were collected from March to April 2017 near the Social Forestry and Forestry Governance building, University of the Philippines Los Baños and Bay, Laguna, respectively. Plant samples were certified as *T. pandacaqui* by the Museum of Natural History. Fresh samples were used for morpho-analysis and histochemistry. Other set of samples were oven-dried for 48 hours at 40°C. After drying, plant samples were ground and used for crude extraction, antibacterial and cytotoxicity analyses.

Sample Extraction for Antibacterial and Cytotoxicity Assays

Ten grams of each powdered sample was extracted with 150 mL ethanol in 500 mL Erlenmeyer flask for 12 h with constant shaking. The addition of 150 mL ethanol was

done after 5, 7, and 12 h with constant shaking. Extracts were filtered after extraction period using a Buchner funnel and Whatmann® no. 1 filter paper. Filtrate was concentrated using a Bl chi® Rotary An evaporator was attached to a circulating chiller with built-in vacuum (Paralaqua®). The resulting concentrate was collected and stored in an amber bottle. Extraction was repeated two more times to maximize the extractable phytochemicals present from the plant material. Final samples were collected in dried powdered form, placed in Eppendorf's tubes and were labeled accordingly for antibacterial and cytotoxicity assays. Ethanol was used as an extraction solvent since it can dissolve many polar and non-polar plant secondary metabolic compounds.

B. Antibacterial Analysis

Analysis of the antibacterial activity of T. pandacaqui extracts was done using disc diffusion method described by Rahman et al. (2011a) and Nascimento *et al.* (2000).

Two Gram-Positive, *Staphylococcus aureus* and *Bacillus cereus*, and one Gram-Negative, *Escherichia coli* were used as test organisms. These were obtained from the Microbiology Division, University of the Philippines Los Baños. Organisms were cultured on Nutrient agar at 30°C for 24 h and the stock culture was maintained at 4°C.

Spread plating was done to introduce the test organisms. Sterilized paper discs were applied with the treatments: water for negative control; streptomycin for positive control; low dose 50 mg/mL and; high dose 100 mg/mL. After drying, the discs were placed in four separate corners. The plates were kept in a storage area (37°C) to allow the growth of the bacteria. The antibacterial activity of the test agent was determined by measuring the diameter of the zone of inhibition in terms of millimeters. Three replicates for each concentration were done. Susceptibility of extracts was determined when zone of inhibition is greater than 7 mm as described by Nascimento *et al.* (2000). Statistical analysis was performed.

C. Cytotoxicity Assay

Cytotoxic activity of the plant extract against HTB-22 MCF-7 breast adenocarcinoma was evaluated using MTT Cell Proliferation Assay as described in the American Type Culture Collection (2011) and commissioned to Marine Science Institute, University of the Philippines Diliman.

Cell Line and Culture Medium

HTB-22 MCF-7 breast adenocarcinoma cell line obtained from Marine Science Institute, University of the Philippines Diliman, Quezon City was used in this study. Cells were cultured in Minimal Essential Medium with 10% Fetal Bovine Serum (FBS) and maintained under an atmosphere of 5% CO_2 and 95% air at 37°C.

In Vivo Assay for Cytotoxic Activity

Ten thousand cells were introduced in each well of the microtiter plate with total volume of 100 µL and incubated at 37°C for 24 h. The cells were treated with dried ethanolic extract dissolved in 0.1% DMSO, specifically with 50 µg/ml and 5 µg/ml for high and low dose treatments, respectively. Three replicates were done for the assay. The positive control was Doxorubicin with 4 µM and 0.4 µM for high and low dose treatments, respectively. The treated cells with extracts were then incubated for 72 hours. The media was removed and was added with 15 µL of MTT and incubated for 3 hours. Then, the formazan product was solubilized with 100 µL DMSO. Absorbance was measured at

570 nm. Cell viability was calculated using the formula below. Statistical analysis was performed.



D. Statistical Analysis

Experimental results were expressed as mean ± SD. All measurements were replicated three times. The data were analyzed by a two-way analysis of variance ($P < 0.05$) using GraphPad Prism 7.03.

E. Phytochemical Screening

Powdered vegetative plant parts of wild-type and dwarf cultivar *T. pandacaqui* were extracted in different solvents: ethanol, methanol, chloroform, and water. Extracts were analyzed individually for presence of secondary metabolites using the methods described by Sivagnanam and Kumar (2014), Shaker *et al.* (2011) and Goyal *et al.* (2010) with slight modifications in extract preparation.

Two hundred milligrams of each of the powdered plant parts of both wild and dwarf cultivars of *T. pandacaqui* were extracted using water, chloroform, ethanol and methanol. These were extracted with constant shaking for an hour. Extracts were filtered using Whatmann® no. 1 filter paper in Buchner funnel. Filtrates were applied with specific reagents for the different tests.

Tannins. The filtrate was added with 1 mL ferric chloride ($FeCl_3$). A blue-black or greenish-black precipitate indicated the presence of tannins.

Saponins. Frothing test was done for the screening of saponins. Five (5) mL of distilled water was added to the filtrate in a test tube and was shaken for 30 seconds. Persistent frothing confirmed the presence of saponins.

Flavonoids. Shinoda's test was done to analyze the presence of flavonoids. The filtrate was added with magnesium ribbon and concentrated HCl. A pink or red color indicated the presence of flavonoids.

Steroids. Liebermann-Burchard's test was used for the screening of steroids. The filtrate was added with 1 mL acetic anhydride and 0.50 mL concentrated sulfuric acid (H_2SO_4). A blue-green ring observed confirmed the presence of steroids.

Triterpenoids. The filtrate was added with 3 mL of conc. H_2SO_4 to form a mono-layer of reddish-brown coloration of the interface as positive result for the triterpenoids.

Cardiac glycosides. The filtrate was added with 1 mL of glacial acetic acid containing 1 drop of $FeCl_3$ solution, under-layered with 1 mL of conc. H_2SO_4. A brown ring obtained at the interface indicated the presence of a deoxysugar characteristic of cardioids.

Anthraquinones. Bornträger's test was done for the test of anthraquinones. The filtrate was added with 2 mL of 10% ammonia solution. A pink-violet or red color in the ammoniacal layer indicated the presence of anthraquinones. Alkaloids. The filtrate was added with 2 or 3 drops of Dragendorff's reagent. Formation of reddish-brown precipitates indicated the presence of alkaloids.

F. Histochemical Analysis

Localization of specific secondary metabolites was done through histochemical testing described by Dhale (2011) and De Padua (1975). Two tests were done for each of the three desired secondary metabolites: alkaloids, tannins and saponins (glucoside) with confirmatory tests.

Alkaloids. A drop of 20% hydrochloric acid was added on the tissue placed on a slide. This was covered by a cover glass and a few drops of Dragendorff's test composed of bismuth-potassium iodide were added under the glass. Formation of reddish-brown spheres indicated the presence of alkaloids.

Confirmatory test was done using Wagner's Reagent composed of iodine potassium iodide. Two drops of this was added on the tissue and a chocolate brown precipitate indicated the presence of alkaloids.

Tannins. Ten percent of ferric chloride plus a little sodium carbonate was added to the sections. A blue-green color indicated the presence of tannins.

Confirmatory test was done using HCl. A few drops of 20% hydrochloric acid were added on several fresh thick tissue sections. This was heated gently until boiling. Formation of a red insoluble substance indicated the presence of tannins.

Glucoside: Saponins. Detection of the metabolite was done using Prussian blue reaction. A thick section was placed in 5% alcoholic potassium hydroxide for about 1 minute. This was transferred to an aqueous solution containing 2.5% ferrous sulfate and 1% ferric chloride and kept at 70°C for 10 minutes. This was transferred to 20% hydrochloric acid. A bright blue color formation indicated the presence of glucosides.

Confirmatory test was done using Sulfuric acid test. Fresh sections were placed in a drop of concentrated sulfuric acid on a slide and a series of color reactions were observed. The following sequence occurred: immediately a yellow color formed, changing to red in 20 minutes, and finally became violet or blue-green in a few instances. This indicated the presence of saponins.

RESULTS

A. Antibacterial Analysis

Table 1 shows the zone of inhibition against the test organisms. Root of dwarf cultivar exhibited the highest zone of inhibition of 25 mm against *S. aureus* at 100 mg/mL concentration followed by the stem of wild-type with 22.33 mm zone of inhibition. At 50 mg/mL, root of dwarf cultivar showed highest zone of inhibition (22 mm), followed by the leaf of the wild-type (20.67 mm). Both stem and leaf of the dwarf cultivar showed no antibacterial activity against *S. aureus* at low dose. At high concentration, both extracts showed activity with zones of inhibition of 8.67 and 7 mm, respectively.

As observed in Table 1, the stem of the wild-type obtained the highest zone of inhibition with 33.33 mm against *B. cereus* at 100 mg/mL concentration. The leaf of dwarf cultivar showed the lowest antibacterial activity at high concentration among the other extracts against *B. cereus* with a 21 mm zone of inhibition. At 50 mg/mL, the stem of dwarf cultivar showed the lowest antibacterial activity (5.33 mm) compared to the other extracts, while the root of dwarf cultivar showed the highest (16.67 mm) zone of inhibition.

The stem of the wild-type showed the highest antibacterial activity against *E. coli* at a high concentration with a 18.67 mm zone of inhibition. The leaf of dwarf cultivar showed no antibacterial activity at both concentrations, obtaining only a 3.33 mm zone of inhibition.

Data obtained indicate that test organisms react differently to extracts at low and high concentrations. At high concentration, test organisms became more sensitive to

Table 1. Zone of inhibition of wild-type and dwarf cultivar *T. pandacaqui* extracts against *S. aureus*, *B. cereus*, and *E. coli* at 50 mg/mL and 100 mg/mL concentrations.

PLANT SAMPLE		ZONE OF INHIBITION (mm)					
		S. aureus		*B. cereus*		*E. coli*	
		50 mg/mL	100 mg/mL	50 mg/mL	100 mg/mL	50 mg/mL	100 mg/mL
Wild-type	Root	10.33±3.21	17.00±1.73	15.67±1.53	31.33±3.51	11.33±4.16	18.33±2.08
	Stem	10.33±0.58	22.33±4.04	9.67±8.74	33.33±4.51	9.00±0	18.67±0.58
	Leaf	20.67±7.23	18.67±2.31	13.67±1.53	31.00±2	9.67±1.15	15.67±0.58
Dwarf cultivar	Root	22.00±2.65	25.00±1	16.67±1.53	25.00±5.20	12.33±2.08	16.33±2.31
	Stem	0	8.67±0.58	5.33±4.73	23.33±4.62	13.00±1.73	10.00±2.65
	Leaf	0	7.00±12.12	9.00±9	21.00±5.57	3.33±5.77	3.33±5.77

Antibacterial activity (inhibition Zone ≥ 7 mm)

T. pandacaqui extracts obtaining higher zones of inhibition. Both roots of wild-type and dwarf cultivar exhibited constant antibacterial activity even at low concentration with the zone of inhibitions greater than 10 mm. The leaf of dwarf cultivar showed lower zones of inhibition, even at high concentration against *S. aureus* and *E. coli.*

A. Cytotoxicity Assay

Cytotoxicity of ethanolic extracts of *T. pandacaqui* against HTB-22 MCF-7 human breast adenocarcinoma cells was determined using MTT assay. The summarized results of the cytotoxicity screening are shown in Table 2. The crude extract of *T. pandacaqui* can be considered active using MTT assay if there is a significant decrease in percent viability of the HTB-22 MCF-7 human breast adenocarcinoma cells. The root of the dwarf cultivar at high concentration (50 µg/ml) obtained a negative percent cell viability indicating the complete absence/death of cells in the well.

All extracts displayed cytotoxic activities on the cell line due to a decrease in the cell's viability. The roots of both wild-type and dwarf cultivar of *T. pandacaqui* extracts showed highest potent cytotoxicity against the MCF-7 cell lines even at low concentration (5 µg/mL). Of all the extracts, root of dwarf cultivar exhibited most potent cytotoxicity with 23.76 percent cell viability even at low concentration. Root of wild extracts showed closely similar values as that with Doxorubicin obtaining percent survival of cells 1.71 and 1.41, respectively, at a high concentration.

C. Phytochemical Screening

Table 3 shows the phytochemicals obtained using water, chloroform, ethanol and methanol as solvents. Chloroform extracted more of secondary metabolites that include steroids, cardiac glycosides, triterpenoids, and saponins. Saponins and alkaloids were detected in the extracts when water was used as an extraction solvent. Tannins and alkaloids were detected in ethanol and methanol.

D. Histochemical Analysis

Tests for the presence of alkaloids in the vegetative plant parts of wild-type and dwarf cultivar of *T. pandacaqui* consistently showed

Table 2. Percent cell viability of MCF-7 breast cancer cell lines exposed to wild-type and dwarf cultivar *T. pandacaqui* extracts at 5 µg/mL and 50 µg/mL.

PLANT SAMPLE		% CELL VIABILITY			
		5 µg/mL	50 µg/mL	0.4 µM	4 µM
Wild-type	Root	47.15±8.83	1.71±0.56		
	Stem	85.18±9.74	29.82±5.52		
	Leaf	78.08±4.62	7.9±6.12		
Dwarf cultivar	Root	23.76±1.08	-0.12±0.11		
	Stem	94.55±3.75	63.23±0.13		
	Leaf	82.36±1.77	18.92±2.24		
Doxorubicin (Positive Control)				24.83±1.17	1.41±0.18

positive results unlike the other tests that showed false positive results. The presence of saponins in older roots of dwarf cultivar was detected during the experiment. Figure 1–2 show the positive result obtained when detecting alkaloids in wild-type *T. pandacaqui* using Dragendorff's and Wagner's reagents.

Saponins were also localized but positive result only showed in old roots of dwarf cultivar of *T. pandacaqui* prominently observed at or near the pericycle and the branching root. Figure 5 shows the cross section of old roots of dwarf cultivar *T. pandacaqui* showing the positive result for saponins.

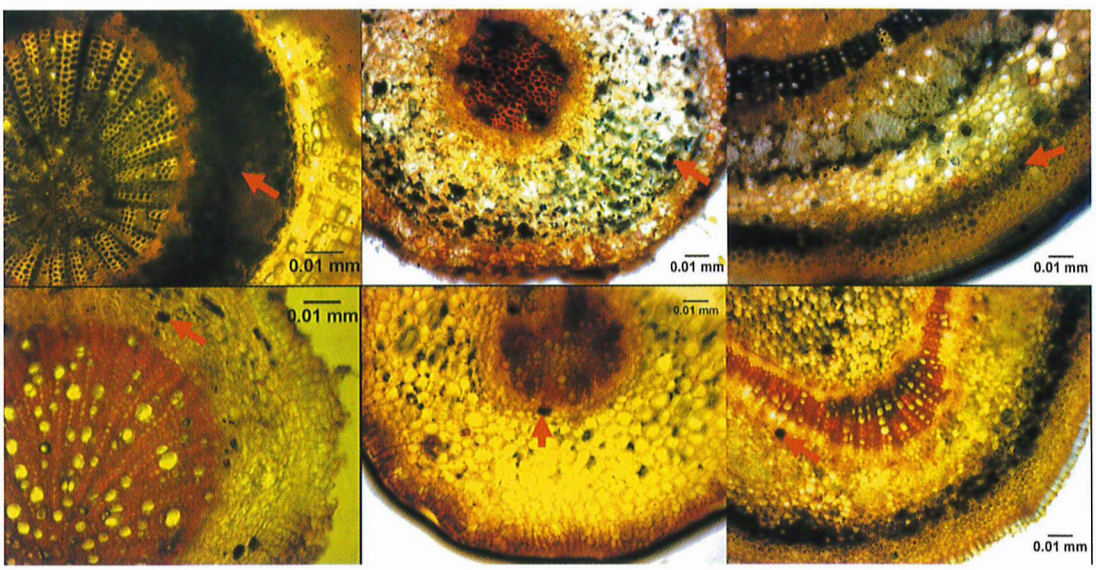


Figure 1. Cross-sections of wild-type (A) and dwarf cultivar (B) *T. pandacaqui* old root (I), young root (II) and stem (III) viewed under the microscope (100X) showing reddish brown spheres (arrows) indicating the presence of alkaloids detected through Dragendorff's test.

Table 3. Phytochemical screening of wild-type and dwarf cultivar *T. pandacaqui* using water, chloroform, ethanol and methanol as solvents.

		Water									Ethanol									Chloroform									Methanol								
		Tannins	Saponins	Flavonoids	Steroids	Tri-terpenoids	Cardiac glycosides	Anthraquinones	Alkaloids		Tannins	Saponins	Flavonoids	Steroids	Tri-terpenoids	Cardiac glycosides	Anthraquinone	Alkaloids		Tannins	Saponins	Flavonoids	Steroids	Tri-terpenoids	Cardiac glycosides	Anthraquinone	Alkaloids		Tannins	Saponins	Flavonoids	Steroids	Tri-terpenoids	Cardiac glycosides	Anthraquinone	Alkaloids
Wild-type	Root	-	++	-	-	-	-	-	++		+	-	-	-	-	-	-	++		-	++	-	-	+	-	-	-		+	-	-	-	-	-	-	+
																		+																			
	Stem	-	+	-	-	-	-	-	++		+++	-	-	-	-	-	-	++		-	+	-	-	++	+	-	-		+	-	-	-	-	-	-	++
	Leaf	-	+++	-	-	-	-	-	+		+++	-	-	-	-	-	-	+		-	++	-	-	-	-	-	-		+	-	-	-	-	-	-	++
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Dwarf cultivar	Root	-	+++	-	-	-	-	-	++		+	-	-	-	-	-	-	++		-	++	-	+	++	++	-	-		-	-	-	-	-	-	-	++
									+									+			+		+													+
	Stem	-	+	-	-	-	-	-	++		+	-	-	-	-	-	-	++		-	+	-	+	++	+	-	-		-	-	-	-	-	-	-	+
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	Leaf	-	++	-	-	-	-	-	++		+	-	-	-	-	-	-	+		-	++	-	-	++	++	-	-		-	-	-	-	-	-	-	+
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Legend: (-) negative; (+) slight; (++) moderate; (+++) abundant

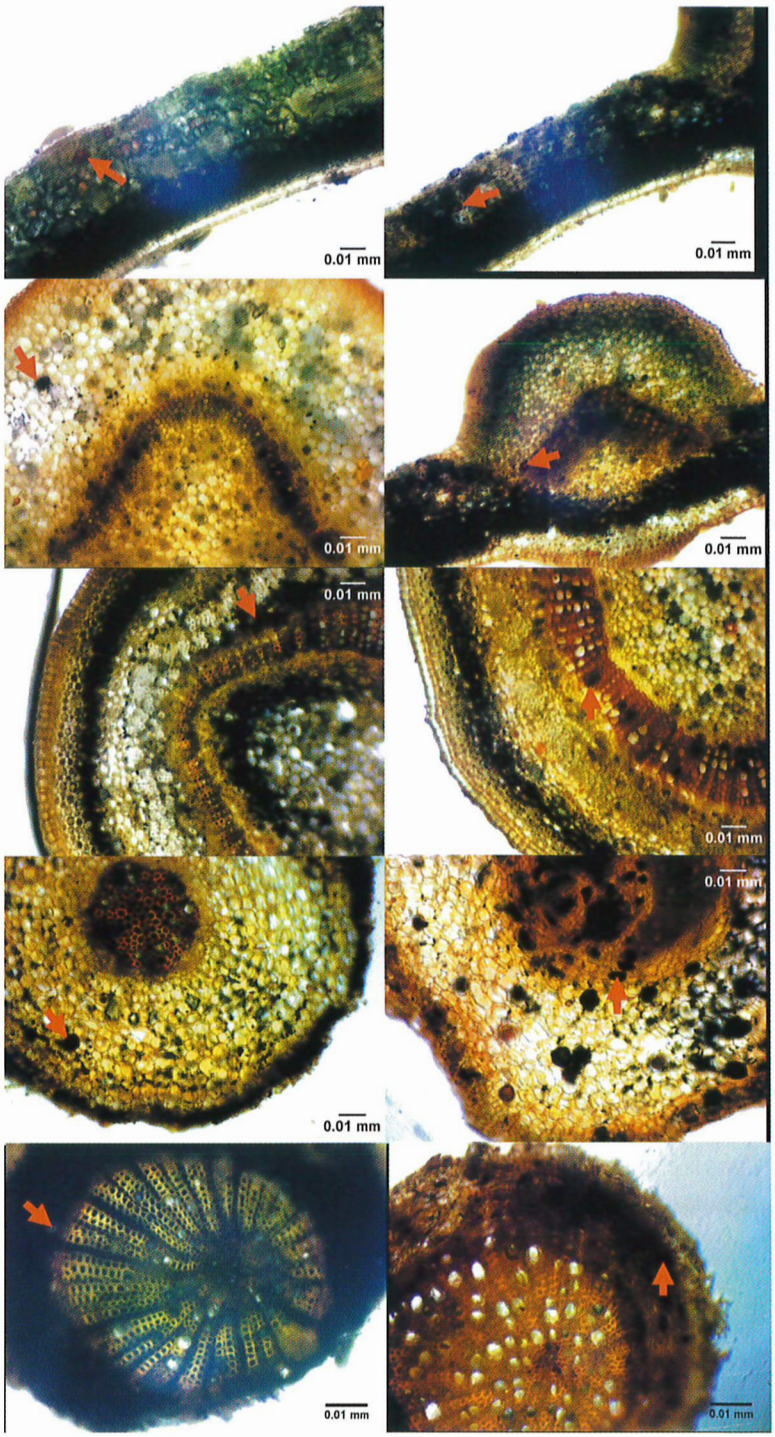


Figure 2. Cross-sections of wild-type (A) and dwarf cultivar (B) *T. pandacaqui* leaf blade (I), midrib (II), stem (III), young root (IV) and old root (V) viewed under the microscope (100X) showing chocolate brown precipitate (arrows) indicating the presence of alkaloids detected through Wagner's test.

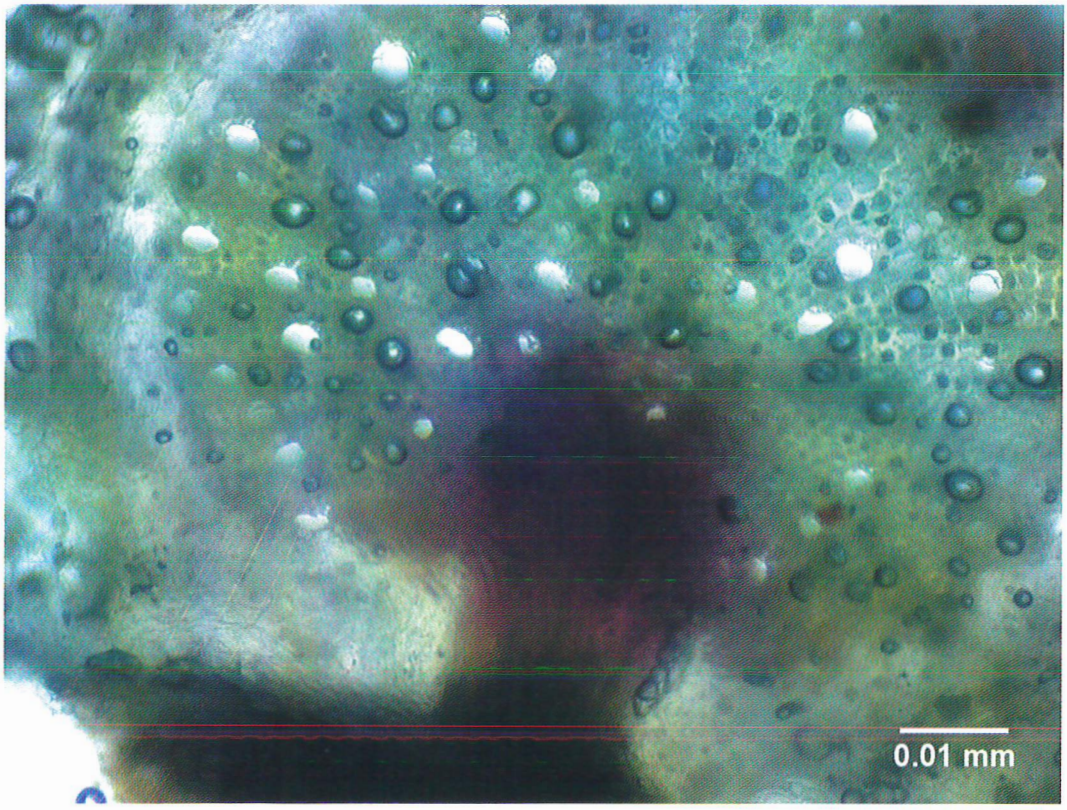


Figure 3. Cross-section of fresh *T. pandacaqui* dwarf old root viewed under the microscope (100X) showing violet color indicating the presence of saponins detected through H_2SO_4 test.

DISCUSSION

A. Antibacterial Assay

Susceptibility of the test organisms, *S. aureus*, *B. cereus* and *E. coli* to the different extracts of wild-type and dwarf cultivar *T. pandacaqui* parallels the results of previous studies conducted by Marathe *et al.* (2013), Sathishkumar *et al.* (2012), Rahman *et al.* (2011), Suffredini *et al.* (2002) and Van Beeket *et al.* (1985; 1984) in other *Tabernaemontana* species. Antibacterial activity against pathogenic organisms had been observed in other *Tabernaemontana* species using different extracts from different plant organs. Stem bark and leaves are two of the commonly studied for the antibacterial activity of *Tabernaemontana* species (Marathe *et al.*, 2013; Sathishkumar *et al.* 2012; Rahman *et al.*, 2011; Suffredini *et al.*, 2002).

It was observed that test organisms have different sensitivities to each extract. Wild-type extracts obtained higher zones of inhibition than the dwarf extracts with 30 mm and above zones of inhibition against *B. cereus* where stem of wild-type had the highest. Zones of inhibition of wild-type extracts against *B. cereus* are closely like the zones of inhibition of the positive control, Streptomycin.

The antibacterial activity of the medicinal plants studied in recent years have been reported due to the presence of phenols, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, tannins, and phytosterols.

Some of the simplest bioactive phytochemicals are composed of a single substituted phenolic ring (Sathishkumar *et al.*, 2012). According to Cowan (1999), the hydroxylated phenols (catechol and pyrogallol) are shown to be toxic to microorganism. It was stated that the position and number of hydroxyl groups in the phenol group are responsible for their relative toxicity to the microorganisms where evidence that the increase in hydroxylation leads to increased toxicity.

It was also found that the more highly oxidized the phenols are, the more they are inhibitory. The proposed mechanisms responsible for phenolic toxicity to microorganisms are: enzyme inhibition by the oxidized compounds, which might be due to the reaction with the sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999).

B. Cytotoxicity Assay

The potent cytotoxicity of root of *T. pandacaqui* dwarf cultivar may be due to the increase amounts of secondary metabolites present in the plant part. Similar results in which dwarf cultivars obtained higher amounts of phytochemicals when screened was shown in the study conducted by Li *et al.* (2016). Propagated dwarf *Campotheca* species were observed to possess higher amounts of anti-cancer campothecin (CPT) in leaves than in other species.

Leaves of wild-type *T. pandacaqui* showed the next highest decrease in the cells' viability next to the roots of both plant samples with a mean value of percent survival of 7.9 at high concentration. This is evidenced by the study of Bradacs *et al.* (2010) where *T. pandacaqui* leaves showed cytotoxic activity against renal and melanoma cancer cell lines. Only the extract of stem of dwarf cultivar did not exhibit cytotoxic activity against HTB-22 MCF-7 human breast adenocarcinoma cells with percent cell survival ranging from 60 to 90 at high and low concentrations, respectively.

Lack of cytotoxicity activity in the crude extract of dwarf stem could have been due several factors which influence the concentration of alkaloids in the plant such as precursor availability, microbial attack or other environmental stressors (Pallant *et al.* 2012).

Cytotoxic activity of *T. pandacaqui* extracts was parallel in the studies done by Rumzhum *et al.* (2012), Boligon and Athayde (2012) and Van Beek *et al.* (1984) where different *Tabernaemontana* species using various plant parts, particularly stem and leaves, were evaluated for their cytotoxicity. Isolated indole alkaloids such as voacangine showed cytotoxicity as described in the study of Van Beek *et al.* (1984).

The cytotoxicity assay done in the study was only to show if wild-type and dwarf cultivar *T. pandacaqui* influence the cell viability of the HTB-22 MCF-7 human breast adenocarcinoma cells. The mechanism of action of the cytotoxic property was not elucidated. Morphological tests should be coupled in cytotoxicity assays to see whether cell death was induced by apoptosis or whether there was only growth inhibition and cell adhesion that occurred. Further studies could be done to determine the mode of action of the cytotoxic activity of *T. pandacaqui* extracts.

The data obtained a statistically significant difference as shown in Figure 2 in the effects of concentration and plant part extract used to the cell viability of HTB-22 MCF-7 human breast adenocarcinoma cells. Varying cytotoxicity effects of extracts to HTB-22 MCF-7 human breast adenocarcinoma cells

were observed. As dosage was increased, percent cell viability of HTB-22 MCF-7 human breast adenocarcinoma cells decreased.

The results in both antibacterial and cytotoxicity assays showed that each extract has various effects on the test organisms and that their concentrations affect the sensitivity of these organisms. This may be attributed to the number of phytochemicals present in a particular plant part. The difference in secondary metabolites in each species under one genus or various cultivars might affect the antibacterial or cytotoxic activity of the extracts due to the varying concentration of phytochemicals extracted from each plant part or plant material (Raya *et al.*, 2015). According to Nwokocha *et al.* (2011) in their comparative study of phytochemical screening of Jatropha L. species, each secondary metabolite varies among the plant parts and in species due to their difference in adaptation and response to stress. Environmental factors might also play a role in this difference (Ramakrishna and Ravishankar, 2011). The bioactivity of the crude extract may have been due to the synergistic effects of the phytochemicals present in each plant cultivar and their plant parts.

C. Phytochemical Screening

The different extraction solvents with varying polarities were used in the phytochemical screening to determine which solvents can better extract the metabolic compounds. Chloroform tested negative for the presence of alkaloids. It could be due to the interaction of certain secondary metabolites or extraction time was not enough to fully extract the alkaloids present. Chloroform is commonly used as a solvent in alkaloid extraction as used in the study of Piana *et al.* (2014). There were different types of alkaloids with different chemical structures which would render different solubility.

Ethanol was also used as an extraction solvent for the different *Tabernaemontana* species as observed in the studies of Sivagnanam and Kumar (2014) and Shaker *et al.* (2011). Results obtained for phytochemical screening showed only alkaloids and tannins when ethanol was used as an extraction solvent.

Studies of Cruz *et al.* (2015) and Ruttoh *et al.* (2009) showed similar results obtained in the experiment when methanol was used as an extraction solvent in which alkaloids and tannins. Methanol can be used for a wide range of phytochemical extraction.

Based on Table 3, dwarf extracts obtained the higher number of secondary metabolites extracted in various solvents compared to the wild extracts. Within the dwarf cultivar, root of dwarf cultivar obtained the most abundant precipitate when specific chemical tests were done. This could be attributed to the increase of production of secondary metabolites when the plant undergone pruning or under environmental stress as described by Li *et al.* (2016).

D. Histochemical Analysis

Alkaloidal components are seen to be localized in cortical tissues and near the epidermal tissue. In root sections, alkaloids are found in the pericycle, since it is biosynthesized here (Choshi, 2008). Findings in the studied are verified by the data obtained by De Padua (1975) that alkaloids are abundantly found in epidermis, cortex, phloem, and in some isolated cells of pith.

Formation of reddish-brown spheres and chocolate brown precipitate, the positive results, in the roots of the dwarf cultivar were higher than in the roots of the wild-type. This

could be attributed to the induced endocides, metabolites that can poison or inhibit the parent via induced biosynthesis of secondary metabolites in propagated plants such as dwarf cultivar *T. pandacaqui* as described by Li *et al.* (2016).

Saponins were also localized, but positive results only showed in old roots of dwarf cultivar of *T. pandacaqui* prominently observed at or near the pericycle and the branching root. Figure 3 shows the cross section of old roots of dwarf cultivar *T. pandacaqui* showing the positive result for saponins.

Presence of saponins in dwarf cultivar old root may contribute to its high bioactivity as antibacterial and cytotoxic agents. No previous studies on *Tabernaemontana* species showed the accumulation of saponins in the roots. The test for saponins were negative for the young and old roots of wild-type and young roots of dwarf cultivar. The study of Wang *et al.* (2011) on saponin accumulation in the seedling root of Panax notoginseng (Araliaceae) showed a time-dependent increase after the germination of *P. notoginseng.*

The roots of dwarf cultivar of *T. pandacaqui* had the highest content of secondary metabolites based on phytochemical and histochemical screenings. It also exhibited potent bioactivity on the assays done together with roots of the wild-type T. pandacaqui. Between the two, the root of dwarf cultivar had the higher potency of bioactivity for both assays. This could be attributed on the synergistic effects of the phytochemicals present in the roots of the dwarf cultivar as shown in the phytochemical screening. This could also be due to the endocidal regulation of secondary metabolites mentioned by Li *et al.* (2016).

According to Li *et al.* (2016), an increase in the production of secondary metabolites in the parent organism induces dwarfism or even death due to autotoxicity. The results in the phytochemical screening showed that the dwarf cultivar obtained high amounts of secondary metabolites. These possibly contributed to its high bioactivity due to the synergy of phytochemicals since the crude extract was used for the two assays.

The results from the phytochemical screening and histochemical analysis supports the bioactivity of *T. pandacaqui* extracts as an anti-bacterial and cytotoxic agent against HTB-22 MCF-7 human breast adenocarcinoma cells.

CONCLUSION

The two assays and histochemical analysis used to determine the potential use of *T. pandacaqui* as alternative source of medicine showed promising results. Dwarf roots exhibited consistent and higher bioactivity as antibacterial agent against the tested organisms and cytotoxicity against HTB-22 MCF-7 human breast adenocarcinoma cells. Histochemical analysis showed that the old root of dwarf cultivar was the only one that tested positive for the presence of saponins. Presence of these phytochemicals in the roots of the dwarf cultivar may have contributed for its high bioactivity in the two assays done among the other plant parts. Higher amounts of phytochemicals in the root of dwarf cultivar could be due to the response the plant exhibits when exposed to various stress.

Information gathered in this study can be used for the quantification of active components present in wild and dwarf cultivars. Isolation of the active constituents could determine the specific compound responsible for the bioactivity observed when the crude extract

was used. Varying the concentration of extracts to determine the IC_{50} value for antibacterial and cytotoxicity assays can provide the efficacy of the extracts as source of alternative medicine. Determination of the appropriate age of plant parts with highest phytochemicals present can improve the extraction yield of the desired phytochemicals.

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