

Determination of Antimicrobial Properties of Crude Aqueous Leaves Extracts of Selected Medicinal Plants Using Resazurin-Based Microtiter Broth Dilution Method

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The Philippines has a wide range of medicinal plants species that need to be tested for safety and efficacy for the possibility of serving as basis in drug development in the future. Five medicinal plants commonly used in the province of Cavite, Philippines were selected for crude aqueous leaf extraction. These were *Annona muricata* L., *Cymbopogon citratus* DC., *Graptophyllum pictum* (L.) Griff, *Jatropha curcas* L. and *Piper betle* L. Extracts were tested for antimicrobial activities using resazurin-based microtiter broth dilution method. *P. betle* showed the maximum antimicrobial activity with minimum inhibitory concentrations (MIC) of 4.69 mg/ml against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and 37.50 mg/ml against *Candida albicans*. This then validated the efficiency of *P. betle* in treating skin diseases and other infections as reported in the locality.

Keywords: antimicrobial, microtiter broth dilution, minimum inhibitory concentration, resazurin

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1. Introduction:

Traditional medical therapy makes use of plants (WHO, IUCN and WWF, 1993), and these may contain secondary metabolites and essential oils that can be used to treat infections (Morilla *et al.*, 2014). It is therefore necessary to investigate the antimicrobial properties of these ethnomedicinal plants which are commonly not pharmacologically studied and clinically tested (Gutierrez *et al.*, 2013).

In the Philippines, its plant communities are so diverse that countless extracts from local medicinal plants have the potential to be used as agents against microbial pathogens (Manuel *et al.*, 2012). About two decades ago, only a small portion of this plant diversity had undergone pharmaceutical screening (Roberson, 2008).

During this recent time, advancement of these studies could serve as a promising

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way to discover new antibiotic compounds, new anti-viral drugs, anti-cancer drugs (Oladele *et al.*, 2011; Roberson, 2008), and other treatments against a number of existing, emerging, and re-emerging diseases (Gutierrez *et al.*, 2013).

Locally, only 10 plants are recommended by the Department of Health (DOH) and Philippine Institute for Traditional and Alternative Health Care (PITAHC) for medicinal use since there is a lack of pharmacological studies concerning other local plants (Principe and Jose, 2002). Hence, there are more local medicinal plants to be tested for efficacy. In this study, five plants of medicinal importance to locals of Cavite, Philippines were used namely, *Annona muricata* L., *Cymbopogon citratus* DC., *Graptophyllum pictum* (L.) Griff., *Jatropha curcas* L. and *Piper betle* L. Antimicrobial activities of these plants were determined using resazurin-based microtiter broth dilution assay.

2. Materials and methods:

2.1. Medicinal plants under study

Five medicinal plant species commonly used in Cavite, Philippines were selected for crude aqueous leaf extraction namely, *Annona muricata* L. (vernacular name: *guyabano*), *Cymbopogon citratus* DC. (*tanglad*, *salay*), *Graptophyllum pictum* (L.) Griff. (*morado*), *Jatropha curcas* L. (*tuba-tuba*) and *Piper betle* L. (*litlit*, *ikmo*) (Figures 1-5). All these plants are usually found abundant in the area and have leaves recorded as one of the plant parts that are being used in practicing traditional health care. These are also plants that do not belong to the 10 medicinal plants recommended by DOH-PITAHC for the primary purpose of focusing more on lesser scientifically investigated local medicinal plants. Fresh samples of these plants were submitted to The National Museum of the Philippines for verification of identity.

2.2. Collection of leaf samples

Leaves were collected into labeled zip lock bags and were stored in an ice cooler until transported to the drying site (Biswas *et al.*, 2013). Leaves were first washed with tap water to remove unwanted particles. These were then air dried under shade (average temperature = 26.59°C; relative humidity = 81.52%), ground and pulverized using electric osterizer.

2.3. Crude aqueous leaf extraction

Since water is the most common solvent used in preparing the medicinal plants for use in Cavite, aqueous extraction was done. Following Gakunga *et al.* (2014), for every 50 g of powdered dried leaves, it was extracted with 250-ml distilled water for 48 h using a mechanical shaker. It was then heated and allowed to boil for 20 mins before filtering using Whatmann filter paper No. 1 (Penduka *et al.*, 2011). Filtrates were transferred into clear, wide-mouthed glass vials of known weight, and oven-dried at 50°C until dried crude extract was left (Igbokwe *et al.*, 2010). Weights of resulting extracts were calculated using the following formula: $W_{extract} = W_f - W_i$, where W_f is the final weight of the vial that contained the dried crude extract and W_i is the weight of the vial before receiving the filtrate. Calculated volumes of 10% DMSO (dimethyl sulfoxide) were then added into each vial to give a final crude extract concentration of 300 mg/ml. Crude extracts were finally sterilized by autoclaving (Hashemi *et al.*, 2008) and refrigerated at 4°C prior to use (Selvamohan *et al.*, 2012). Sterility of extracts was ensured by plating them on nutrient agar every before any experiment was performed.

2.4. Determination of antimicrobial activity of ethnomedicinal plants

2.4.1. Test organisms

Plant extracts were tested for their activity against microorganisms of medical importance to humans. Microbial cultures were purchased from the University of Santo Tomas-Collection of Microbial Strains (USTCMS), Manila. As presented in Table 1, four pathogenic species were used; namely, bacteria: *Staphylococcus aureus* USTCMS 1090, *Pseudomonas aeruginosa* USTCMS 1080, and *Escherichia coli* USTCMS 1030; and fungus: *Candida albicans* USTCMS 1235. Bacterial and fungal cultures were maintained on nutrient agar (NA) and potato dextrose agar (PDA), respectively.

For use in the determination of minimum inhibitory concentration (MIC), microbial cultures were first allowed to grow in nutrient and potato dextrose broths for 24 h at 37°C and 25°C for bacterial and fungal isolates, respectively. Resulting bacterial broth cultures were diluted in physiological saline solution and its absorbance was compared spectrophotometrically to 0.5 McFarland turbidity standards to give an approximate cell density of 1 to 5×10^6 cells per ml at 530 nm. The same comparison was made for *C. albicans* (Lee and Chee, 2010; Arevalo *et al.*, 2003) but was further diluted to prepare a final suspension of 1 to 5×10^3 cfu/ml.

2.4.2. Preparation of resazurin solution

Resazurin dye is a redox indicator that was used to indicate microbial growth following the procedures of [Gahlaut & Chhillar \(2013\)](#). The solution was prepared by dissolving 300 mg of resazurin dye in 40-ml sterile water and was sterilized prior to use. Growth inhibition was indicated by a change of dye color from purple to pink due to the reduction of resazurin to resofurin, which in turn could further be reduced to uncolored hydroresofurin.

2.4.3. Determination of minimum inhibitory concentration (MIC) by microtiter broth dilution method

All plant extracts proceeded to determining MIC. This followed the microtiter dilution broth method described by [Mariita *et al.* \(2011\)](#) with modification on the solvent used for the reconstitution of the extract.

Each well of the 96-well microplates was initially filled with 75- μ L NB and PDB for bacterial and fungal test isolates, respectively. Seventy-five microliters of the previously prepared crude extract (300 mg/ml) were dispensed into the first well of the microplate from where a two-fold serial dilution began and terminated at the 6th well. Seventy-five microliters of each test organism were then transferred in each well. This gave the following plant extract concentrations (mg/ml) per well: 75.00, 37.50, 18.75, 9.38, 4.69 and 2.35. The remaining two wells in each row of the microplate were then allotted for negative (*i.e.* extract replaced with 50 μ L of 10% DMSO) and positive (*i.e.*, extract replaced with 50 μ L of antibiotics: 600- μ g/ml chloramphenicol for *S. aureus*, 200- μ g/ml streptomycin for *E. coli* and *P. aeruginosa*, and 500- μ g/ml fluconazole for *C. albicans*) controls, respectively. Lastly, 10 μ L of resazurin solution was added into each well. All tests were performed in triplicates and were incubated for 24 h at 37°C and 25°C for bacterial and fungal isolates, correspondingly. MIC values were then recorded as the least amount of plant extract capable of preventing visible microbial growth organism ([Andrews, 2001](#)) or a no change in resazurin dye color.

2.4.4. Determination of minimum bactericidal/fungicidal concentration (MBC/MFC)

Minimum bactericidal or fungicidal concentration (MBC/MFC) is the lowest concentration of the extract capable of eliminating 99.9% of the test organism ([Andrews, 2001](#)). To determine MBC/MFC, 100 μ L of the content of each well that did not show microbial growth was subcultured on NA or PDA and was incubated for 24 or 72 h, correspondingly. MBC/MFC was recorded as the lowest concentration of extract that did not show any formed colony ([Mariita *et al.*, 2011](#)).

3. Results:

3.1. Percentage yield

Percentage yield of crude aqueous leaves extracts that resulted from the medicinal plants used is presented in Table 2. This shows the crude extract recovery for every 50 g of powdered dried leaf samples that was then prepared for experimental use. *G. pictum* had the highest percentage yield (3.61 %), followed by *A. muricata* (2.20 %), *P. betle* (1.65 %), *C. citratus* (1.61%) and *J. curcas* (1.44%).

3.2. Minimum inhibitory concentration (MIC) and minimum bactericidal/ fungicidal concentration (MBC/MFC) of crude aqueous leaves extracts

Crude aqueous leaves extracts of the five medicinal plants were screened for their antimicrobial potential by determining their minimum inhibitory concentrations (MICs) using microtiter dilution broth method. MICs were obtained for the activity of these water extracts against four microorganisms capable of causing some ailments reported in the study. These were represented by three bacterial species namely *Escherichia coli* USTCMS 1030, *Pseudomonas aeruginosa* USTCMS 1080 and *Staphylococcus aureus* USTCMS 1090 and a fungal species that was *Candida albicans* USTCMS 1235.

As shown in Table 3, *P. betle* showed a promising activity as it inhibited the growth of three test cultures, namely *P. aeruginosa* (MIC = 4.69 mg/ml), *S. aureus* (4.69 mg/ml) and *C. albicans* (37.50 mg/ml) at a very low concentration. These were also identified as the maximum activities against these cultures. Next to this plant, *G. pictum* also showed a high antimicrobial activity but not as high as the aforementioned observations for *P. betle*. However, unlike *P. betle*, *G. pictum* was recorded to inhibit the growth of all test organisms used, with high activity (37.50 mg/ml) against all bacterial test pathogens. Comparatively, *J. curcas* and *C. citratus* had lower inhibitory activities as it was observed at highest concentrations only. However, the activity was observed against all the microbial cultures, except for the non-inhibitory activity of *C. citratus* against *P. aeruginosa*. Furthermore, *A. muricata* was recorded as the least active in inhibiting microbial growth as this activity was only observed against two microbial cultures—*S. aureus* (75.00 mg/ml) and *C. albicans* (75.00 mg/ml).

Minimum bactericidal/fungicidal concentrations of crude extracts that showed inhibitory activities were also determined. Investigations revealed that no extracts had killed the test cultures completely. This means that these extracts were more of microbiostatic rather than microbicidal. This then suggests that higher concentrations of these extracts could be responsible for such bactericidal and fungicidal activities.

4. Discussion:

4.1. Antimicrobial activities of crude aqueous leaves extracts

Determining antimicrobial properties of plant extracts can be of great importance in therapeutic cure (Jyoti *et al.*, 2015). Previous studies over the years also reported the antimicrobial activities of the five medicinal plants used in this study. *P. betle* showed the maximum activities among all plant extracts tested. The same inhibitory activity against the bacterial species used was also observed in other investigations (Pradhan *et al.*, 2013; Subashkumar *et al.*, 2013; Agarwal *et al.*, 2012; Datta *et al.*, 2011; Chakraborty and Shah, 2011; Shukla *et al.*, 2009). Likewise, anti-candidal activity was reported to be exhibited by its aqueous leaf extract (Nanayakkra *et al.*, 2014; Rekha *et al.*, 2014). The result showing resistance of *E. coli* to the aqueous extract was then found to be in contrast with the result of other studies (Subashkumar *et al.*, 2013; Ghanwate and Thakare, 2012; Chakraborty and Shah, 2011). This could be attributed to the *E. coli* strain used and the application of heat in extraction that could have altered the structure of phytochemicals responsible to specifically inhibit *E. coli* growth.

Though there were only few resources available for *G. pictum*, its activity against the growth of *E. coli*, *S. aureus* (Jiangseubchatveera *et al.*, 2015) and *C. albicans* (Wahyuningtyas, 2008) was still presented in reports.

C. citratus was also found to be antibacterial and antifungal showing inhibitory activity against *E. coli*, *S. aureus* (Jyoti *et al.*, 2015; Hindumathy, 2011; Ravinder *et al.*, 2010; Fagbemi *et al.*, 2009; Oloyede, 2009; Osaniye *et al.*, 2007) and *C. albicans* (Silva *et al.*, 2008; Ravinder *et al.*, 2010). Similarly, non-susceptibility of *P. aeruginosa* to aqueous *C. citratus* extract was also observed (Fagbemi *et al.*, 2009). However, in contrary to the findings of this study, *E. coli* and *S. aureus* were reported to show resistance to the extract (Asaolu *et al.*, 2009). This difference in result could be attributed to the application of heat in extraction in this study which could have helped in releasing the active compounds responsible for exhibiting inhibitory activity.

Several studies also supported the activity of aqueous extracts of *J. curcas* against *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans* (Dada *et al.*, 2014; Adamu *et al.*, 2013; Ekundayo and Ekekwe, 2013; Omoregie and Folashade, 2013; Adamu *et al.*, 2013; Namuli *et al.*, 2011).

Further, *A. muricata* leaves extracts, similar to the result of this study, demonstrated its antimicrobial activity against *S. aureus* (Kumar *et al.*, 2014; Kedari and Khan, 2014; Wisdom *et al.*, 2014; Abubacker and Deepalakshmi, 2013; Pathak *et al.*, 2010; Vieira *et al.*, 2010) and *C. albicans* (Ragasa *et al.*, 2014; Torres *et al.*, 2011). It also revealed the non-inhibitory activity of the extract against *P. aeruginosa* and *E. coli* that was similarly observed in the study of Abah & Egwari (2011). Conversely, there was a report that aqueous extract of *A. muricata* was inhibitory against *E. coli* (Wisdom *et al.*, 2014). This could be attributed to the researchers' non-use of heat, thus preserving the chemical

structure of extracted active compounds, in the extraction of plant material. Likewise, *E. coli* and *P. aeruginosa* were found to be susceptible to ethanolic extracts of *A. muricata* (Haro *et al.*, 2014; Vijayameena *et al.*, 2013). This could be due to water-insoluble phytochemicals that can only be obtained using ethanol as extracting solvent.

These activities of plant extracts in inhibiting microbial growth are attributable to water-soluble active compounds they contain. Investigations carried out by various authors on the phytochemistry of aqueous leaves extracts of these plant extracts revealed the presence alkaloids, anthraquinones, flavonoids, glycosides, phenolics, tannins, terpenes, saponins and steroids (Jyoti *et al.*, 2015; Dada *et al.*, 2014; Dwivedi and Tripathi, 2014; Manvitha and Bidya, 2014; Adamu *et al.*, 2013; Vijayameena *et al.*, 2013; Ravinder *et al.*, 2010; Igbinosa *et al.*, 2009). These compounds are identified to be biologically potent and thus support the antimicrobial activities of *A. muricata*, *C. citratus*, *G. pictum*, *J. curcas* and *P. betle*.

4.2. Efficacy of medicinal plants in traditionally treating ailments

Considering the ailments for which each plant was reported to be used (Balinado & Chan, 2017), the results obtained suggest that the crude aqueous leaves extracts of the five plants used are efficacious in treating microbial-caused ailments (Table 4). Crude aqueous extracts of *A. muricata* were effective against abdominal pain and diabetes, ailments that could be attributed to *S. aureus* infection (Tortora *et al.*, 2010; Rich and Lee, 2005). It was also shown that *C. citratus* extracts were inhibitory against *S. aureus* and *E. coli*, thus supporting the effective use of the plant in dealing with abdominal pain and urinary tract problems, respectively (Tortora *et al.*, 2010). Moreover, the use of *G. pictum* and *J. curcas* in curing swollen muscles could be attributed to the antibacterial effect of the extracts against *P. aeruginosa*, a pathogen known to play role in soft tissue infections (Friedrich, 2015). Furthermore, the inhibitory activities exhibited by *P. betle* extracts against *P. aeruginosa* and *S. aureus* supported its role in treating bacterial infections in general, while its activities against *S. aureus* and *C. albicans* explained its use in dealing with skin infections (Tortora *et al.*, 2010).

Conclusions:

Crude aqueous leaves extracts of *A. muricata*, *C. citratus*, *G. pictum*, *J. curcas* and *Piper betle* L. revealed that *P. betle* had the maximum antimicrobial activity with minimum inhibitory concentrations (MIC) of 9.38 mg/ml against *P. aeruginosa* and *S. aureus* and 75.00 mg/ml against *C. albicans*. Likewise, *G. pictum* was inhibitory against all bacterial cultures including *P. aeruginosa* at a 75.00-mg/ml MIC. These results then suggest the efficacy of these plants in treating ailments caused by these pathogens.

Table 1. List of test microorganisms and their medical importance to humans (Tortora *et al.*, 2010) that were used to determine the antimicrobial potential of selected crude aqueous leaves extracts.

TEST ORGANISMS	DISEASES CAUSED
Bacteria (Gram-positive)	
<i>Staphylococcus aureus</i>	Gastroenteritis, endocarditis, abscesses, mastitis, impetigo, scalded skin, pneumonia
Bacteria (Gram-negative)	
<i>Pseudomonas aeruginosa</i>	Gastrointestinal infection, eye ulcers, septicemia, pneumonia
<i>Escherichia coli</i>	Urinary tract infection, septicemia, gastroenteritis
Fungi	
<i>Candida albicans</i>	Esophagitis, gastritis, septicemia, endocarditis, cutaneous infection

Table 2. Percentage yield of crude aqueous leaves extracts of *A. muricata*, *C. citratus*, *G. pictum*, *J. curcas* and *P. betle*.

PLANT NAME	WEIGHT OF LEAF SAMPLE (in g)	WEIGHT OF CRUDE EXTRACT (in g)	PERCENTAGE YIELD (in %)
<i>A. muricata</i>	50.00	1.10	2.20
<i>C. citratus</i>	50.00	0.80	1.61
<i>G. pictum</i>	50.00	1.81	3.61
<i>J. curcas</i>	50.00	0.72	1.44
<i>P. betle</i>	50.00	0.83	1.65

Table 3. Minimum inhibitory concentrations (MIC, in mg/ml) of crude aqueous leaves extracts of five selected ethnomedicinal plants against *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans*.

PLANT NAME	MIC (in mg/ml)			
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>C. albicans</i>
<i>A. muricata</i>	0.00	0.00	75.00	75.00
<i>C. citratus</i>	75.00	0.00	75.00	75.00
<i>G. pictum</i>	37.50	37.50	37.50	75.00
<i>J. curcas</i>	75.00	75.00	75.00	75.00
<i>P. betle</i>	0.00	4.69	4.69	37.50

Table 4. Medicinal efficacy of crude aqueous leaves extracts of the five selected medicinal plants in treating several ailments in Cavite known to be caused by the test pathogens used in the antimicrobial assay.

PLANT NAME	AILMENTS TREATED	ASSOCIATED PATHOGEN
<i>A. muricata</i>	Abdominal pain; diabetes	<i>S. aureus</i>
<i>C. citratus</i>	Abdominal pain	<i>S. aureus</i>
	Urinary tract problem	<i>E. coli</i>
<i>G. pictum</i>	Swollen muscles	<i>P. aeruginosa</i>
<i>J. curcas</i>	Swollen muscles	<i>P. aeruginosa</i>
<i>P. betle</i>	Bacterial infection	<i>S. aureus, P. aeruginosa</i>
	Skin infection	<i>S. aureus, C. albicans</i>



Figure 1. *Annona muricata* L. plant, its leaves and fruit.



Figure 2. *Cymbopogon citratus* DC. plant.



Figure 3. *Graptophyllum pictum* (L.) Griff plant, its leaves and flower.



Figure 4. *Jatropha curcas* L. plant and its leaves.



Figure 5. *Piper betle* L. plant and its leaves.

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Competing interests:

The authors have no conflicts of interest relevant to the ideas and/or contents of the manuscript.

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