Antibacterial properties of *Dioscorea alata* (purple yam) peel extracts against selected gram-negative and gram-positive bacteria

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Abstract

Rapid emergence of resistant bacteria is endangering the efficiency of antibiotics which previously saved millions of lives [1]. The antibacterial agent in plants is a notable source of new antibiotics alternatives [2]. In the Philippines, *Dioscorea alata* is a main export banner crop since the country is the only supplier of the tuber in the world market [3]. *D. alata* tubers have bioactive compounds including alkaloids, tannins, flavonoids, and saponins [4]. In this study, the antibacterial properties of *Dioscorea alata* peel extracts were evaluated against four selected human pathogens including gram-negative bacteria, *Escherichia coli* and *Enterobacter aerogenes* and gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis.* Extracts were obtained through maceration and rotary evaporation. Activities of different concentrations (25%, 50%, 75%, 100%) of the peel extracts were evaluated using agar-well diffusion method on Mueller-Hinton agar. Inhibitory properties were identified only against selected gram-positive bacteria strains, *S. aureus* (50%, 75%, 100%) and *B. subtilis* (75% and 100%). For more results, *D. alata* extracts are recommended to be tested against other bacteria strains by different solvents with varying polarity.

Keywords: *antibacterial, agar well-diffusion method, Dioscorea alata, purple yam, peels*

Introduction. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century [2]. The use of plant extract for medical treatments have been well-known since the 1990s [5]. Technological and research advances have been established through the years. Some of these folklore medicines are tested in order to develop medicines to prove that these plants can treat target diseases. About 80% of the world population is wholly or partially dependent on drugs derived from plants. Therefore, medicinal plants are significant remedial aid for various ailments [2].

Compounds in plants are notable agents against bacterial infections, but in the last three decades, rapid increases in antibiotic resistance has been threatening human health, especially the immune suppressed patients [2]. Bacteria are prone to mutations due to their prokaryotic nature which enables them to develop antibiotic resistance [1,6]. This continuous discovery of antibiotic-resistant bacteria through surveillance have urged scientists to study other sources to address these problems. Therefore, antibacterial studies using different sources and solvents have been recurrent in research and drug development. Plants have been one of the most prominent sources for antibacterial agents.

Studies have shown that yam extracts exhibit antidiabetic, antimicrobial and antioxidative activity. Some yams are used as medicine in oriental countries to prevent diarrhea and diabetes [7]. It was also determined that *Dioscorea esculenta* exhibited antibacterial activity against selected human pathogens [8]. The ethanolic extracts of the *D. bulbifera* peels were also reported to be more effective in terms of antibacterial properties against selected human pathogens than its bulbils [9].

Scientific papers also focus on the compounds present in plants that are attributed to their medicinal properties called phytochemicals. Plants are excellent sources of phytochemical agents in the field of clinical microbiology. There are many kinds of phytochemicals that have different functions including antibacterial properties. Phytochemical drugs are used to aid drug resistance due to misuse of market antibiotics [5]. Yam species have great potential as alternatives due to their observed high phenolic and bioactive compounds content.

Yam species contain various bioactive components such as polyphenols, diosgenin, and carotenoids which are shown in different studies testing hypoglycemic, antimicrobial, and antioxidant activities [2]. Phenolic compounds are potential agents with antimicrobial activity and are found in yam varieties, *Dioscorea hirtiflora* and *Dioscorea dumetorum* [2]. Phytochemical analysis of root extracts of *Dioscorea alata* revealed the presence of phytochemicals; results include alkaloids, tannins, flavonoids, phenols, glycosides, saponins, anthraquinones, resins, carbohydrates, and terpenoids [4].

D. alata, commonly known as “ube” in the Philippines, is a large genus in the family Dioscoreaceae. It is also one of the staple foods in many tropical countries. Ube is one of the main root crops commercially produced in the Philippines since it is the only supplier in the world market. Thus, the crop is considered one of the important export banner crops [3].

In this study, antibacterial activities of ethanolic extract of *Dioscorea alata* (purple yam) peels of Tinta variety against two gram-negative bacteria,

Escherichia coli and *Enterobacter aerogenes* and two gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis* were determined using agar well-diffusion method at different concentrations: 25%, 50%, 75%, and 100%. It specifically aims to:

(i) Determine the zones of inhibition (mm) exhibited by the ethanolic extracts of the peels of *Dioscorea alata* at concentrations (25%, 50%, 75%, and 100%) against *E. coli*, *E. aerogenes*, *S. aureus*, and *B. subtilis*.

(ii) Determine if there is a significant difference between the mean zones of inhibition exhibited by co-trimoxazole, distilled water, and the ethanolic extracts of *D. alata* peels against *E. coli*, *E. aerogenes*, *S. aureus*, and *B. subtilis* at each of the following concentrations: 25%, 50%, 75%, 100%.

(iii) Determine if there is a significant difference between the mean zones of inhibition exhibited by co-trimoxazole, distilled water, and the different concentrations of ethanolic extracts (25%, 50%, 75%, 100%) of the peels of *Dioscorea alata* against each of the following bacteria: *E. coli*, *S. aureus*, *E. aerogenes*, and *B. subtilis*.

(iv) Identify which bacteria is most susceptible to the peels of *D. alata* ethanolic extracts at the following concentrations: 25%, 50%, 75%, and 100%.

(v) Identify which concentration exhibit the greatest zones of inhibition (mm) for each of the following species of bacteria: *E. coli*, *S. aureus*, *E. aerogenes*, and *B. subtilis*.

Methods. Purchased *Dioscorea alata* tubers were thoroughly washed and peeled. Peels were oven dried and homogenized. Powdered plant material was then subjected to extraction by maceration in 95% ethanol and filtered using Whatman no.1 filter paper. Collected filtrate had undergone rotary evaporation, and treatment concentrations were then prepared and used for agar-well diffusion method. Results were analyzed using one-way analysis of variance with 95% level of confidence and independent sample t-test.

Plant Material. Purple yam tubers were purchased in the province of Aklan, Philippines within November to December 2017. Variety of the plant species (Tinta) purchased was verified by the Department of Agriculture, Makato, Aklan.

Controls. Co-trimoxazole used for the assay was the suspension form of the antibiotic with the concentration of 400mg/80mg per 5ml suspension. Distilled water was used as the negative control.

Bacteria. Freeze dried cultures of *Enterobacter aerogenes* Hormaeche and Edwards BIOTECH 1146, *Escherichia coli* (Migula) Castellani and Chalmers BIOTECH 1203, and *Bacillus subtilis* subsp. *subtilis* (Ehrenberg) Cohn BIOTECH 1573 were purchased from the Philippine National Collection of Microorganisms, National Institute of Molecular Biology & Biotechnology at the University of the Philippines Los Baños, Laguna. Freeze dried culture of *Staphylococcus aureus* Rosenbach BIOTECH 1582 was obtained from the University of San Agustin, Gen. Luna St., Iloilo City.

Glasswares and Equipment. Glasswares and equipment, such as the autoclave (Delixi LS-B35L), the oven (Binder ED53), and the incubator (Binder BD115), were available in Philippine Science High School-Western Visayas Campus, Brgy. Bito-on, Jaro, Iloilo City. The rotary evaporator (Eyela Autojack NAJ-100) of Department of Science and Technology Region VI, Magsaysay Village, Lapaz, Iloilo City was used during the extraction process.

Preparation of Plant Samples for Extraction. Purple yam tubers were thoroughly washed under running water and brushed for removal of unnecessary residues. Plant samples were then peeled using a sterile peeler and acquired peels were oven dried at 35°C for a week until peels were easily crushed. Oven dried samples were powdered using a homogenizer (Spice & Herb Grinder IC-04A).

Extraction. The powdered samples were soaked in 95% ethanol for 48 hours [10]. The mixture was filtered with Whatman no. 1 filter paper. Filtered plant solution was set up for rotary evaporation (Eyela Autojack NAJ-100) at 40°C with 100rpm. Acquired crude extracts were stored in the refrigerator at until use to prevent degradation.

Treatment Preparation. To prepare the different concentrations, pure extracts were mixed with distilled water to obtain the intended concentrations using the following extract to water ratio: 1:3, 2:2, 3:1 and 4:0, measured in milliliters (ml)

Media. Nutrient agar and nutrient broth were used for the revival process of the freeze-dried bacteria culture. Nutrient broth was used for the observation of bacterial growth. The bacterial colony was inoculated into nutrient agar plates by quadrant streaking. Nutrient agar enabled the bacteria to grow accordingly until it reached the log phase. The medium used for the antibacterial assay was Mueller-Hinton Agar (MHA), a culture media commonly used in antibacterial testing. Prepared MHA plates were stored in the refrigerator at about 2- 8°C until use.

Standard Inoculum. The 0.5 McFarland Turbidity Standard was used for adjusting the concentration of bacterial colonies suspended in the inoculum in preparation for inoculation on MHA plates. For the preparation of bacterial strains, the turbidity of each strain suspension was compared visually with the McFarland turbidity standard tubes using Wickerham card [11].

Antibacterial Assay. Six setups per bacteria strain were generated composing of one plate of positive control, and another plate of negative control. Other remaining setups were the four treatment concentrations (25%, 50%, 75% and 100%) which were used for the antibacterial analysis, agar-well diffusion method. The cultures were placed in the incubator at 37°C. Plates were initially checked for any zone of inhibition at the 18th hour of incubation, but data was only gathered after 24 hours of incubation.

Handling and Disposal of Solvent. Ethanol is extremely flammable and should be kept away from fire ignition prone materials or location. Container was kept tightly closed and sealed until ready for use in a cool, well-ventilated area. Excess solvents were disposed in a chemical waste container in accordance with state and local environment control regulations [12] and its corresponding Material Safety Data Sheets.

Handling and Disposal of Bacteria. Appropriate protective lab attire was worn until the process was done. Used media plates were soaked with 10% solution of bleach and were left in a safe area for 30 mins. Agar residues were removed using a stirring rod to avoid contact. Culture was also handled and disposed following the Pathogen Safety Data Sheets of each strain.

Standard Inhibition Zone. Standard zone of inhibition used was the mean zone of inhibition of the positive control, co-trimoxazole. The average inhibition zones of bacteria strains in each treatment concentrations were compared with that of the co-trimoxazole. The zones of inhibition on each plate were measured using a Vernier caliper for more precise measurements in millimeters.

Data Analysis. To determine if there was a significant difference in the antibacterial activity of *D. alata* ethanolic extracts against the different bacteria strains, the mean zone of inhibition for each plate was compared using One-way Analysis of Variance (ANOVA) with a 95% level of confidence. Only the selected gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, showed significant difference between the mean zones of inhibition. Therefore, an Independent Samples t-test was done in order to determine if there was a significant difference at the susceptibility of the gram-positive bacteria strains at the specified treatment concentration.

Results and Discussion. Table 2.1 presents the mean zones of inhibition displayed by *D. alata* ethanolic extracts at 25%, 50%, 75%, and 100% treatment concentrations and the control treatments, distilled water (negative control) and co-trimoxazole (positive control) against selected bacteria. *D. alata* ethanolic extract at treatment concentrations of 50%, 75%, and 100% showed zones of inhibition against *S. aureus* while only the extracts at 75% and 100% treatment concentrations showed inhibitory properties against *B. subtilis*. There was no zone of inhibition observed on the selected gram-negative bacteria, *E. aerogenes* and *E. coli*, for all treatment concentrations of *D. alata* ethanolic extracts.

According to the sensitivity pattern range [13], *B. subtilis* at concentrations 75% and 100% and *S. aureus* at concentrations 50%, 75%, and 100% shows intermediate sensitivity pattern. *E. coli* and *E. aerogenes* are resistant at all concentrations (25%, 50%, 75%, and 100%). For the plant extract inhibition activity ranges [14], treatment concentration of 75% was partially active against *B. subtilis* while 100% treatment concentration was active against *B. subtilis*. For the *S. aureus*, it showed that the *D. alata* peel extracts were partially active against *S. aureus* at 50% and 100% treatment concentrations, and active at 75% treatment concentration. Treatments are inactive against the gram-negative bacteria, *E. coli* and *E. aerogenes*. The summary of data can be seen in Table 2.2 and Table 2.3.

The selected gram-positive bacteria, *S. aureus*, showed a significantly wider zone of inhibition compared to *B. subtilis* at the 75% treatment concentration with a confidence level of 95%. *B. subtilis* exhibited a relatively bigger zone of inhibition than *S. aureus* at 100% treatment concentration; however, the difference was not significant ($t_{crit} > 0.05$). At 75% treatment concentration, the zones of inhibition were not significantly different from the results at 100%. *S. aureus* was relatively more susceptible to the positive control than *B. subtilis*, but the difference is also not significant. The t-test for comparison of *S. aureus* and *B. subtilis* at each treatment can be found in Table 2.4.

Gram-negative bacteria showed resistance to the *D. alata* peel extracts which may be caused by the gram-negative bacteria having a protective membrane present around the cell wall. This membrane increases resistance to antibiotics and other microbial agents and is absent in gram-positive bacteria. Gram-negative bacteria also have porin channels which are absent in gram-positive bacteria. Porin channels are water-filled channels that facilitate transport of low molecular weight substances including antimicrobial agents into the cell. They can prevent the entry of harmful chemicals, antibiotics, and other antibacterial agents and can also expel out antibiotics causing the treatment to be much more difficult to inhibit. Another difference between the two gram stains is their possession of exotoxins and

Table 2.1. Zones of inhibition (mm) of different concentrations of Dioscorea alata peels ethanolic extracts against selected gram-negative and selected gram- positive bacteria.
Mean values of the same bacteria strain with varying superscripts have a significant difference at $p<0.05$
Mean values of the same bacteria strain with the same superscripts have no significant difference at $p<0.05$
Each agar-well measures 7mm in diameter

	Treatment concentrations of *Dioscorea alata* peel extracts					
	25%	50%	75%	100%	-	+
B. subtilis	7.00	7.00	11.50 ± 0.50^{a}	13.50 ± 1.00^{b}	7.00	19.50 ± 1.32^{c}
S. aureus	7.00	10.17 ± 0.29^{a}	13.50 ± 0.50^{b}	12.00 ± 1.50^{ab}	7.00	22.83 ± 2.26^{c}
E. aerogenes	7.00	7.00	7.00	7.00	7.00	18.67 ± 0.58
E. coli	7.00	7.00	7.00	7.00	7.00	16.00 ± 2.00

Table 2.2. Bacteria sensitivity pattern of selected bacteria strains against *D. alata* peel extracts
Note: >10mm = Resistant, 10-15mm = Intermediate, 15< = Susceptible

	Treatment concentrations of *Dioscorea alata* peel extracts					
	25%	50%	75%	100%	-	+
B. subtilis	R	R	I	I	R	S
S. aureus	R	I	I	I	R	S
E. aerogenes	R	R	R	R	R	S
E. coli	R	R	R	R	R	S

Table 2.3. *Dioscorea alata* peel extracts concentrations (treatment) inhibition activity against selected bacteria
Note: >10mm = inactive, 10-13mm = partially active, 14-19mm = active, 19< = very active

	Selected bacteria			
	B. subtilis	*S. aureus*	*E. aerogenes*	*E. coli*
25%	I	I	I	I
50%	I	PA	I	I
75%	PA	A	I	I
100%	A	PA	I	I

Table 2.4. Independent samples T-test for comparison of *Staphylococcus aureus* and *Bacillus subtilis* at each treatment. Mean difference marked with '*' have a significant difference at p<0.05

Treatment	Mean Difference	p- value
75%	2.00*	0.008
100%	-1.50	0.223
Co-trimoxazole	3.33	0.092

endotoxins [15]. Gram-negative bacteria possess both exotoxins and endotoxins while gram-positive bacteria only have exotoxins [15].

Since gram-negative bacteria has endotoxins which are lacking in gram-positive bacteria, they might have caused the resistance of the selected gram-negative bacterial strains (*Escherichia coli* and *Enterobacter aerogenes*) to *D. alata* peel ethanolic extracts. Endotoxins are bacterial toxins consisting of lipids that are located within a cell while exotoxins are toxic substances secreted by bacteria and released outside the cell. Major component of endotoxins are lipopolysaccharides (LPS) which contribute to the ability of the bacteria to cause diseases [2]. Endotoxins are essential to the function of the outer membrane. The outer membrane acts as a protective permeability barrier which is impermeable to large molecules and hydrophobic compounds in the environment and is permeable only to low molecular weight, hydrophilic molecules [16]. These bacterial toxins may enhance or inhibit the pathogenicity of infection, depending upon the infecting microorganism, dose and route of injection of endotoxin, and the interval between administration of endotoxin and initiation of infection [17]. Variations in LPS structure provide for the existence of different antigenic strains of a pathogen that may be able to bypass a previous immunological response to a related strain [16]. Considering all the aforementioned factors, gram-negative bacteria, naturally, are more resistant to antibiotics or antibacterial agents compared to gram-positive bacteria. This was affirmed in the results of the present study which shows that *E. coli* and *E. aerogenes* exhibited no susceptibility to *D. alata* peels ethanolic extract.

The antibacterial activity of an extract depends on the concentration and potency of the bioactive compounds that contribute to its antibacterial effect. The obtained crude extracts from rotary evaporation may have contained various bioactive compounds, but not all these compounds exhibit antibacterial properties. In order to obtain the bioactive compounds present in *D. alata* tubers, 95% ethanol was used as the solvent since it is mostly used in the extraction of phenolic compounds in plants. Ethanol is a highly polar substance; however, it also has a nonpolar portion, the ethyl part. Therefore, ethanol can dissolve both polar and nonpolar compounds. *D. alata* tubers had been discovered to have the following bioactive compounds: alkaloids, tannins, flavonoids, phenols, glycosides, saponins, anthraquinones, resins, carbohydrates, and terpenoids [4]. Some of these bioactive chemicals have polar and nonpolar parts in their composition. In the case of the results of this study, the concentrations and the yield of the bioactive compounds obtained from the extraction were not evaluated to determine the specific substance that affected the zones of inhibition measured. One inference that can be formed was that bioactive compounds present in the crude extracts obtained may not be effective antibacterial agents.

Phenolic compounds are expected to be extracted from the peels of *D. alata*. These phenolic compounds are also referred to as secondary metabolites and are reported to possess antibacterial properties which is attributed to their redox properties. These includes tannins, flavonoids, flavonols, alkaloids, saponins, and the likes. According to Eleazu et al. [18], the ethanolic extracts obtained from *Dioscorea alata* peels theoretically would contain 1.85 ± 0.14% flavonoids, 1.33 ± 0.04% tannins, 0.60 ± 0.00% alkaloids, and 5.36 ± 0.01% saponins.

Pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents because of their analgesic, antispasmodic and antibacterial properties [18]. Alkaloids mode of action against pathogens have been identified to be by disruption of virulence gene regulation, inhibition of sortase and disruption of fimbriae and adhesins, inhibition of bacterial defences against host immune system, inhibition of secretion systems, inhibition of exotoxin-mediated effects, inhibition of destructive enzyme- mediated effects, and inhibition of biofilm formation [19]. Antibacterial mechanisms of flavonoids are by inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, alteration of the membrane permeability, and attenuation of the pathogenicity [20]. The mode of action of saponins are maybe by the disruption of membrane, and/or induction of fungal apoptosis [21]. Phenolic compounds like tannins found in plant cells are potent inhibitors of hydrolytic enzymes used by plant pathogens [22].

Diverse phenolic compounds are generally believed to principally perform their antibacterial

mechanism against the cytoplasmic membrane of bacterial cells, which is mainly attributed to the presence of hydroxyl groups. Due to the difference in outer cell membrane composition, the mechanism in destroying the protective layer of the bacteria have different effectivities. The phenolic compounds present in the crude extracts were more effective in disrupting the gram-positive bacteria membrane than the gram-negative bacterial membrane. The accumulation of hydrophobic phenolic groups in the lipid bilayer may disrupt lipid–protein interaction and increase membrane permeability, further causing alterations in membrane structure and accelerating the extensive leakage of intracellular constituents, finally destroying membrane integrity to facilitate the entry of more antibacterial agents [23].

In the case of 75% and 100% treatment concentration, the zone of inhibition values seems to suggest that the 75% treatment was more effective than the 100% treatment. This is an unexpected result knowing that 100% treatment concentration meant that the treatment was pure. In this case, statistical analysis states that the two treatment concentrations, 75% and 100%, were statistically equal, in which the two treatments are comparable with each other. However, the number of sample points might have affected this condition. Results in the 75% treatment concentration is less scattered than that of the 100% treatment concentration showing the diversity of the results. The three sample points could not entirely show the antibacterial properties of the 100% treatment concentration.

Conclusion. *Dioscorea alata* peel extracts treatment concentrations were effective against selected gram-positive bacteria: *S. aureus* at concentrations 50%, 75%, and 100%, and *B. subtilis* at 75% and 100%. However, selected gram-negative bacteria, *E. coli* and *E. aerogenes* were resistant against *D. alata* peel extracts at all concentrations (25%, 50%, 75%, and 100%). The use of ethanol as a solvent was sufficiently efficient in the extraction of the specific bioactive compounds in *D. alata* peels which attribute to its antibacterial properties.

Recommendations. In the duration of the study, some problems were observed. Therefore, for the improvement of future studies similar or of the same nature as the study, it is recommended that researchers should note the diffusion rate of the extract, weight of plant material, and amount of solvent used in the experiment, and compute for the percent yield. Concentration of the antibiotics used as the positive control must also be noted so that results may be compared to antibiotics currently present in the market through an appropriate susceptibility table. Fractionation of solvents is highly recommended as some solvents may extract certain compounds better than others. For the storage, the temperature of storing must be noted. Other bacteria strains may also be tested, and other antibacterial assays should be explored. Lastly, adding more sample points might also be considered for better comparison between the extract concentrations. Phytochemical analysis of *D. alata* peel extracts is also suggested.

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