

Antibacterial and Zebrafish-based Screening for Wnt Signal Inhibitory Activities of *Syzygium polycephaloides* (C.B. Robb. Merr.) Bark Extracts

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Syzygium polycephaloides, a plant native to Southeast Asia, is used to cure common illnesses. In this study, the methanolic bark extracts of *S. polycephaloides* was evaluated for their antibacterial activity and screened for their Wnt signal inhibitory activity using zebrafish-based assay. In the antibacterial assay, the extracts were tested against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli* using disk diffusion assay. On the other hand, zebrafish-based screening was used to determine the inhibitory potential of *S. polycephaloides* against the Wnt signal. Zebrafish embryos were treated with LiCl to upregulate the Wnt signal which produces eyeless phenotype. The methanolic extracts had antibacterial activity against the test microorganisms except *P. aeruginosa*. After fractionation, ethyl acetate (EtOAc) and aqueous fractions exhibited wide-spectrum antibacterial activity. For the Wnt signal inhibitory activity screening, the LiCl-treated embryos were rescued to their normal eye development after treatment with the *S. polycephaloides* bark extracts (100 µg/mL). The results suggest that the extracts may have inhibited the Wnt signal. This study shows that the *S. polycephaloides* is a potent source of antibacterial compounds and Wnt inhibitors.

Keywords: disk-diffusion assay; lithium chloride; XAV939

I. INTRODUCTION

Syzygium polycephaloides, which belongs to the family Myrtaceae, is utilized by locals in the Philippines as a cure for common illnesses such as cough and hypertension, and a source of local tropical wine (Taguiling, 2013; Environmental Research and Development Bureau, 2017). The fruit is a good source of natural antioxidant because of its high flavonoid and flavonol content (Santiago *et al.*, 2007). Its seeds contain the compound syzygiol which exhibited inhibition of skin tumor promotion (Nishizawa *et al.*, 1991). Other species of the genus *Syzygium*, particularly *S. cumini* and *S. antisepticum*, are known to possess antioxidant, antidiabetic and antimicrobial properties (Srivastava & Chandra, 2013; Yuan & Yuk 2018).

There is a current crisis on antimicrobial resistance because of misuse, inappropriate prescribing, extensive agricultural use

and availability of few antibiotics (Ventola, 2015). This scenario persists as a global threat, with an annual increase in the Philippines reported strains of antimicrobial resistant microorganisms (Research Institute for Tropical Medicine, 2018). Thus, searching for novel antibiotics and new source of these antibiotics must be undertaken. Plants are still considered as very important resources for drug discovery particularly as an alternative source of antimicrobial agents (Khameneh *et al.*, 2019). A single plant may contain many compounds of interest. There are more than 1000 different phytochemicals isolated and identified from plants which possess antimicrobial activities against human pathogens (Barbieri *et al.*, 2017). These isolated compounds can be considered good leads in drug development. To the best of our knowledge, there are

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limited studies on the biological activities of *polycephaloides*.

The evolutionary conserved Wnt/ β -catenin signaling pathway is involved in stem cell renewal, cell proliferation and tissue homeostasis (Steinhart & Angers, 2018). Aberrant regulations caused by mutations in the Wnt pathway have been implicated in various diseases such as sebaceous skin tumour, type II diabetes, bone density defects and colorectal cancers (Nusse & Clevers, 2017; Zhan *et al.*, 2017). Targetting the Wnt signal pathway has been considered as potential therapeutic strategy against cancer (Anastas & Moon, 2013). Natural compounds in some medicinal plants have been reported to be natural agonists and inhibitors of the Wnt pathway which make them good possibilities for creating treatments that can combat cancer (Fuentes *et al.*, 2015a). Natural compounds such as the cardenolides (Park *et al.*, 2014) and limonoids (Fuentes *et al.*, 2015b) have been recently identified to be potential modulators of the Wnt pathway. In this study, methanolic bark extracts of *S. polycephaloides* were screened for their antibacterial and Wnt signal inhibitory activities.

II. MATERIALS AND METHOD

A. Plant Samples

The bark samples were carefully extracted from a large Igot tree (*Syzygium polycephaloides* [C.B. Robb.] Merr.) from Brgy. Lusad, Burauen, Leyte, Philippines on September 2018. The tree was confirmed in its identification by the head forester in the Department of Environment and Natural Resources – Regional Office 8, Tacloban City, Philippines. Collected bark were air-dried in a net bag, safely contained at room temperature at the UPV Tacloban College.

B. Extraction

The bark sample (100.6 g) was pulverized using a blender and macerated with 750 mL 100% methanol (MeOH). The mixture was left overnight and filtered with cheesecloth and Whatman No. 1 filter paper. Then maceration was conducted again to the residue with 750 mL 100% methanol. The solvent was removed under reduced pressure at 35 °C (BUCHI Rotavapor R-270) which constitute the crude methanolic extract (5.0 g). Crude methanolic extract (1.01 g) was resuspended in 30 mL of 10% methanol and extracted with *n*-hexane (3 × 30 mL) to give 29.3 mg of *n*-hexane extract. The MeOH layer was extracted with

EtOAc (3 × 30 mL) to give the EtOAc extract (47.5 mg). The remaining layer was dried in-vacuo and constitute the aqueous layer (933.4 mg). Extracts were stored in refrigerator until analysis.

Test microorganisms

All test microorganisms were obtained from Department of Science and Technology (DOST)- Region 8, Palo, Leyte, Philippines. *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922) were maintained at Nutrient Agar slants and stored at 4°C.

C. Disk Diffusion Assay

The crude methanolic extract and the solvent fractions were tested for their antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* with the disk diffusion method outlined by the Clinical Laboratory Standards Institute (2009a). Day-old bacterial suspensions were prepared and standardized using 0.5 McFarland Standard to contain approximately 1.5 × 10⁸ colony-forming units (CFU)/mL. Using sterile cotton-swabs, the bacteria were inoculated on the surface of Mueller-Hinton Agar (MHA) and the inoculum was incubated for 5-10 min. Sterile test disks (5 mm diameter, Whatman no.1) impregnated with 10 µg/mL in 10% DMSO of the plant extracts. Positive and negative control disks were eluted with 10 µg/mL streptomycin and 10% DMSO respectively. All plates were incubated at room temperature for 24 h. The resulting zones of inhibition (ZOI) was digitally measured using ImageJ analysis software (Schneider *et al.*, 2012).

D. Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) was determined by the two-fold microdilution method recommended by the Clinical Laboratory Standards Institute (2009b). Microdilutions of active extracts were tested at concentrations from 10 mg/mL halving down six times to reach the lowest concentration of 0.313 mg/mL. Bacterial suspensions of *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis* were prepared and the turbidity was adjusted

by comparison with 0.5 McFarland standard. In a 96-well plate, 50 μ L of bacterial suspension was treated with the control and extracts. The MIC is the lowest concentration of the extract which inhibited the visible growth of the microorganism.

E. Zebrafish Maintenance and Embryo Collection

Wild-type zebrafish (*Danio rerio*) were purchased from a local fish dealer in Tacloban City, Philippines. Male and female zebrafish were placed in separate 7 L fish tanks equipped with aerators (Reed & Jennings, 2011). The zebrafish were maintained in a 14 h light and 10 h dark cycle under an optimal temperature range of 27 to 28.5°C. The zebrafish were fed twice a day, once in the morning and once in the afternoon, using pond flakes, and acclimatized for 2 to 3 months. To induce spawning, a pair of mature male and female zebrafish were placed in a mating tank (2 males: 1 female), while being separated from each other using a horizontal tank divider. The dividers were removed on the onset of sunrise in the following day, and the zebrafish were left for 1 h and 30 min. Embryos were collected using a plastic pipette and were placed in petri dishes with distilled water.

F. Zebrafish-based Assay

Small molecule screening methods were based on the procedure by Robertson et al. (2014). The collected embryos at mid-blastula stage were transferred to a mesh basket to commence the transfer between solutions. Embryos at exactly 4.5 hours post-fertilization (hpf) were immersed in 375 mM LiCl (Unilab) at room temperature for exactly 10 min and were agitated at every minute. Embryos were washed for 20 seconds,

three consecutive times, using room temperature distilled water and were incubated at 28.5 °C for 15 min. Necrotic embryos were removed and ten embryos were placed in each well of a 24-well plate. Lithium-treated embryos were then treated with 50 and 100 μ g/mL in 1% DMSO and 5 μ M XAV939. Embryos were incubated at 28.5°C and were observed for rescue of LiCl-induced eyeless phenotype at prim-5 stage (24 hpf). Developmental defects and deaths were noted and scored at long-pec stage (48 hpf).

G. Data Analysis

All tests were carried out in three replications. The results are expressed as mean \pm standard deviation (n=3).

III. RESULT AND DISCUSSION

Plant extracts obtained from species of the genus *Syzygium* contain documented pharmacological properties (Srivastava & Chandra, 2013; Yuan & Yuk 2018). *Syzygium polycephaloides* which is native to the Indo-Pacific, has little literature characterizing its bioactivity. In this study, *S. polycephaloides* bark extract was evaluated for its potential as source of antibacterials and Wnt signal inhibitors.

The crude methanolic extract showed significant inhibitory activity against all the test bacteria except *P. aeruginosa*. But after fractionation, only the EtOAc and aqueous fractions exhibited wide-spectrum of antibacterial property. The EtOAc fraction has zone of inhibitions ranging from 8.4 to 9.7 mm while the aqueous fraction from 7.7 to 9.3 mm (Table 1).

Table 1. Mean zone of inhibition of the different test bacteria treated with the bark extracts of *S. polycephaloides*

Extracts ^a	Zone of Inhibition (mm) ^b			
	Gram positive		Gram Negative	
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Streptomycin [‡]	23.7 \pm 1.8	22.4 \pm 4.1	19.8 \pm 2.9	21.0 \pm 2.1
10% DMSO	- [†]	- [†]	- [†]	- [†]
Crude extract	8.4 \pm 0.20	8.4 \pm 0.5	8.2 \pm 0.7	- [†]
Hexane	7.8 \pm 0.3	8.4 \pm 0.6	9.0 \pm 0.4	- [†]
EtOAc	9.7 \pm 1.1	9.4 \pm 1.1	8.4 \pm 0.6	8.4 \pm 0.4
Aqueous	7.7 \pm 0.4	8.9 \pm 0.8	9.3 \pm 0.6	8.6 \pm 0.7

^a : The concentration used is 10 mg/mL.

^b : mean diameter of zone of inhibition \pm SD (n = 9); The size of the paper disk is 5 mm.

[‡] : positive control with a concentration of 10 mg/mL

[†] : (-) no zone of inhibition observed

The data also show that antibacterial activity of the *S. polycephaloides* bark extracts are within the range of MIC as compared to bark and leaf extracts of other *Syzygium* species (Table 2). It has similar MIC values with the aqueous crude extract of *Syzygium jambos* bark at 0.5-1.0 mg/mL for *S. aureus* (compared to 2.50 ± 0.00 mg/mL – *S. polycephaloides*), but similar values at >1.0 mg/mL for *E. coli* (compared to 1.88 ± 0.90 mg/mL) (Djipa *et al.*, 2000). *S. cumini* leaf ethanolic extract shows much lower MIC values at 200 µg/mL for *E. coli*, 90 µg/mL for *P. aeruginosa*, and 200 µg/ml for *S. aureus* (de Oliveira *et al.*, 2007). The MIC values of the *S. polycephaloides* crude extract, EtOAc and aqueous are comparable.

Table 2. Minimum inhibitory concentration (MIC) of the *S. polycephaloides* extracts against the tested bacteria

Extracts	MIC ^a (mg/mL)			
	Gram positive		Gram negative	
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Crude Extract	1.56 ± 1.30	1.88 ± 0.90	1.88 ± 0.90	N/A [†]
Hexane	10.00 ± 0.00	5.00 ± 0.00	7.50 ± 3.50	N/A [†]
Ethyl Acetate	2.50 ± 0.00	2.50 ± 0.00	1.88 ± 0.90	10.00 ± 0.00
Aqueous	1.88 ± 0.90	2.50 ± 0.00	1.88 ± 0.90	7.50 ± 3.50

^a: Values are presented as mean \pm SD. n = 2.

[†]: not applicable; No tests were conducted due to absence of ZOI on disk diffusion assay.

It can be said that fractionation did not enhance activity except that EtOAc and aqueous fractions exhibited antibacterial activity against *P. aeruginosa* as compared to the crude extract. The *n*-hexane fractions which comprise a very small part of the crude extract had higher MIC values. The results suggest that slightly polar to polar compounds are responsible for the antibacterial activities of the extracts. Phytochemical screening of the extract showed that the methanolic extract, ethyl acetate and aqueous fractions tested positive for the presence of phenols, flavonoids, and saponins. Phenolic compounds including flavonoids showed diverse mechanism of action against bacteria which include inhibition of the efflux pump, damaging the cell membrane and inhibition of cell wall biosynthesis (Khameneh *et al.*, 2019).

Treatment of zebrafish embryos with LiCl during the transition from dome stage – 30% epiboly at 4.5 hpf resulted in eyeless phenotype. LiCl chloride is a known Wnt activator since it inhibits GSK3 β activity and mimics mutations found in colorectal cancers (Robertson *et al.*, 2014). Wnt pathway activation in zebrafish is expressed through an eyeless phenotype (Nishiya *et al.*, 2014). Hence, finding inhibitors of the pathway by rescuing zebrafish from a lithium chloride-induced eyeless phenotype can show potentially excellent therapeutic agents. In this study, XAV939, a known Wnt signal

inhibitor was used as the positive control (Robertson *et al.*, 2014). Embryos exposed to XAV939 showed 97% of eye recovery with no recorded mortality, but all exhibited defects such as heart edema (Figure 2). *S. polycephaloides* bark extracts exhibited eye-recovery (including with observed defects) though only at $25.6 \pm 5.1\%$ because the bark extracts also exhibited considerable mortality ($35.6 \pm 5.1\%$).

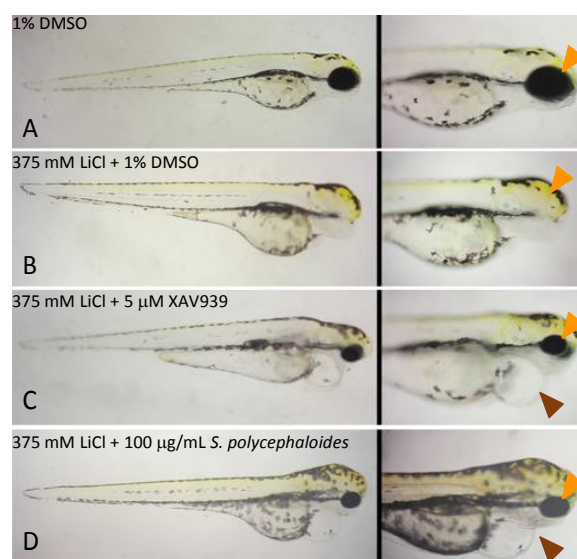


Figure 1. Phenotype of *Danio rerio* embryos observed at 72 hpf after treatment of 1% DMSO (A, control), 375 mM LiCl

(B, activation of Wnt signal). Figures 1.C and 1.D presents the phenotype after treatment of LiCl-treated embryo with 5 μ M XAV939 (C, positive control) and 100 μ g/mL *S. polycephaloides* bark extract (D). Orange arrow indicates eye part while brown arrow indicates developmental defects such heart edema.

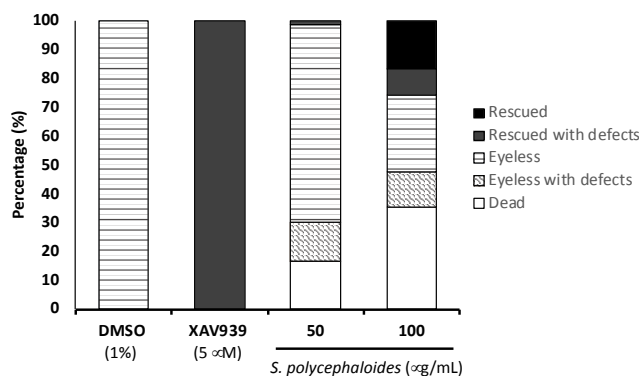


Figure 2. Comparison of observed rescued eye-recoveries, mortality, and defects among LiCl-treated *D. rerio* embryos exposed to 1% DMSO; XAV939 (PC), and *S. polycephaloides* bark, after 48 hpf (n=3). Each replicate has 30 embryos.

But still, the results suggest that *S. polycephaloides* can be a potent source of Wnt inhibitors. Further studies on the isolation of its active compound must be conducted to eliminate the other compounds which have caused high mortality in the zebrafish embryos. As presented earlier, the methanolic crude extract contains phenolic and flavonoid compounds which may be responsible for the inhibitory activity

VI. REFERENCES

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against the Wnt signal of the plant. Quercetin, galangin, apigenin and naringenin are some of the flavonoids reported to inhibit the Wnt signal and exhibited antiproliferative activities against cancer cell lines with active Wnt signal (Fuentes *et al.*, 2015a). To the best of our knowledge, there are no studies on the isolation of compounds present in the bark of *S. polycephaloides*.

IV. CONCLUSION

In conclusion, the methanolic bark extract of *S. polycephaloides* possess antibacterial properties. A wide-spectrum activity is observed for ethyl acetate and aqueous fractions. Wnt signal inhibitory activity was also exhibited as observed by the rescue of eye-development in embryos with upregulated Wnt signal. Further studies on the isolation and characterization of the active compounds are needed to establish the pharmacological importance of *S. polycephaloides*.

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