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Phytochemical screening and *in vitro* antibacterial activity of Syzygium sp.("O-on") crude extracts against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

Identification and subsequent isolation of anti-dengue active compounds in medicinal plants are potentially useful for developing antiviral chemical analogues. This study investigated the potential use of aqueous, methanolic, and ethanolic extracts of Syzygium sp. "O-on" against bacterial co-infection of Staphylococcus aureus and Pseudomonas aeruginosa in dengue. Taxonomic identification of Syzygium sp. "O-on" collected from Mindanao signifies possible new species through their distribution, ecology and conservation status. Phytochemical analysis showed that crude aqueous and organic solvent extracts contain medicinally important bioactive compound. Highest antimicrobial potentials were observed for the methanolic extracts of Syzygium sp. which inhibited 62.5 and 51.16% of the tested microorganisms, respectively, with higher activity against antibiotic-resistant bacteria (83.3%). We suggest that the Syzygium sp. could be potentially exploited to control the behaviors of this pathogen as well as for its utilization as a lead compound in screening for anti-dengue agents based on new antimicrobial targets.

Keywords: anti-dengue, natural products, Pseudomonas aeruginosa, Staphylococcus aureus

INTRODUCTION

Dengue is the most common mosquito-borne viral infection in humans with almost half of the world's population at risk of infection (Ferguson et al. , 2015; Rasool et al., 2011; Sessions et al., 2009). The contemporary worldwide distribution of the risk of dengue virus infection and its public health burden are poorly known (Bhatt et al., 2013). In the Philippines, the Department of Health (DOH) has recorded 131,827 dengue cases from January 1 – December 2, 2017 (Medina, 2018). A total of 13,898 dengue cases were reported nationwide from January 1 to February 17, 2018, this is 33.89% lowered compared to the same time period last year (21, 024), mostly in the region of Region IVA (21.64%), NCR (18.83%), Region III (17.18%), Region I (6.71%), and Region VII (5.89%) (Dengue Disease Surveillance Report, 2018). Although, there are currently no licensed vaccines or specific therapeutics that can stop its rapid emergence and global spread, the Philippine government hopes that a cost-effective vaccine will soon be registered.

Accordingly, this viral infection can be adversely affected by bacterial coinfection (Rice et al., 2012; Palacios et al., 2009; Purcell et al., 2002). However, bacterial coinfection can be easily overlooked in dengue-endemic or -epidemic settings wherein, identification of concurrent bacterial infection in dengue patients would be pivotal for triggering timely antibiotic therapy within the usual context of supportive management (See et al., 2013). Status for vaccine development has been described and emphasized that the only alternative available today to control the disease is through the control of its vector Aedes aegypti (Maria, 2002). Plants and plant-derived products are part of the health-care system with large source of natural antioxidants that might serve as leads for the development of novel drugs. Medicinal plants are known for their potent antioxidant property as they contain bioactive compounds such as carotenoids, benzoic acid, cinnamic acid, folic acid, phenols and flavanoids (Moure et al., 2001). Since natural drugs also possesses activity against A. aegypti by their antiviral mechanism, larvicidal, mosquitocidal

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action and mosquito repellents property, utilization of natural products will provide current approaches for the treatment and management/prevention of dengue (Rasool et al., 2011; Qadir et al., 2015).

In clinical and laboratory practices, cases of dengue virus and bacterial co-infection have shown to worsen the outcome of dengue infection (Nagassar et al., 2012). The reason behind such bacterial co-infection of some human pathogenic bacteria is their ability to reach a high density due to the formation of biofilm-like populations. In microbial biofilm, bacterial cells aggregate on the surface in microcolonies and are embedded in an extracellular matrix whose composition is as variable as that of the biofilm (Moscoso et al., 2006; Davey et al., 2003). Since the occurrence of co-infection by dengue and bacterial has been underestimated, and that few reports have been published so far (See et al., 2013; Trunfio et al., 2017; Araujo et al., 2012), dengue virus and bacterial co-infection should be investigated. This study identifies and evaluates Syzygium sp. as a potential candidate that can be used against dengue. Local people from Northern Mindanao, Philippines utilized this unidentified plant locally known as "O-on" to treat dengue, however, no scientific research has been conducted. They used to harvest the small-to medium-sized trees growing as riparian elements along the banks of a stream were the stems were cut and boiled to treat dengue patients. Some locals collected the branches colored grey when dry and sell it to the market were they thought that it can also cure other diseases aside from dengue. However, due to massive collection and selling of this unknown plant, mass propagation must also be considered. Thus, in this study, characterization of this unidentified plant through morphological, ex situ propagation, phytochemical screening, and in vitro antibacterial assay were done to assess the potential used of O-on as antibacterial co-infection in dengue. Aside that it belongs to Syzygium family used as food preservative and medicinal purposes, it also represents one of the richest source of phenolic compounds such as eugenol, eugenol acetate and gallic acid and posses great potential for pharmaceutical, cosmetic, food and agricultural application (Corteés-Rojas et al., 2014; Sritabutra et al., 2011). Clove (S. aromaticum) for example is one of the most valuable spices and found to be a potential larvicidal agent which is an interesting strategy to combat dengue, a serious health problem in Brazil and other tropical countries (Araujo et al, 2016). In addition, methanolic extract of S. cumini were catechins (759.16 ppm) and rutin (142.24 ppm) bioactive compounds were found caused an increase of platelet counts at both 400 and 800 mg/kg and an increase in leukocyte counts at 800 mg/kg (Bandiola and Corpus, 2018).

METHODOLOGY

Plant Materials

Fresh plant sample was rinsed severally with clean tap water to make it dust and debris free. Then the sample was spread evenly and dried in the shady condition for 3 to 4 days. Dried samples were ground using an electric chopper to reduced particle size and increase surface area for extraction and phytochemical screening.

Plant Identification

Leaves, stems, roots and the reproductive parts was collected for proper identification of the plant. Taxonomic Identification of the unknown species of Syzygium was done using a local taxonomic key of Co's Digital Flora (Pelser et al., 2011), the Kew Royal Botanic Garden's key to species of Syzygium (Royal Botanic Gardens Kew Seed Information Database (SID)) and Craven & Biffin (2010). For identification, phenology, distribution and habitat, and morphological comparison of Syzygium sp. with allied taxa (S. rubicundum, S. nervosum, and S. palodense) were used for characterization, (Shareef et al., 2012).

Ex Situ and Mass Propagation of the Plant

The ex situ and mass propagation of Syzygium sp. was done following the method of Lituañas and Amoroso (2006) with slight modification. Young branches of Syzygium were cut into 4 to 5 nodal segment and planted in different soil media (garden soil, sand and vermicast) under greenhouse condition.

Preparation of Plant Extracts

The dried and powdered samples (each 50g) were extracted successively with double distilled water, ethanol and methanol (each 400 ml) for 10-12 hours using a conventional method. Then the collected solutions were filtered through Whatman No-1 filter paper.

Qualitative Phytochemical Analysis

Phytochemical examinations were carried out for all the extracts, as per standard methods (Yadav & Agarwala, 2011).

Detection of Carbohydrates

Few drops of Benedict's reagent was added to each portion of crude extacts and the mixture was boiled, allowed to stand for two minutes, and the formation of reddish brown precipitate indicates positive result.

Detection of phenols and tannins

About 0.5g each portion crude extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 2% $FeCl_3$ solution were added to 2ml of the fitrate, occurence of a blue-black or blue-green precipitate indicates a positive results.

Detection of flavanoids

About 0.5g of each portion was dissolved in different solvent, warmed and then filtered. Few drops of 2 % NaOH solution were added, an intense yellow color was formed which turned colorless on addition of few drops of diluted HCl acid which indicates the positive result.

Detection of saponins

One gram of each portion was boiled with 5ml of distilled water and filtered. About 3ml of distilled water was added to the filtrate shaken vigrously for about 5 minutes, the formation of stable foam was taken as an indication for the presence of saponins.

Detection of steroids and terpenoid

About 0.2g of each portion were added with 2ml of chloroform and concentrated H_2SO_4 added sidewise, the red color produced in the lower chloroform layer indicates the presence of steriods. For terpenoids, filtrate was dissolved in 2ml of chloroform and evaporated to dryness, and then 2ml of concentrated H_2SO_4, was added and heated for about 2 minutes. The grayish colour indicated the presence of terpenoids.

Detection of alkanoids

Few quantity of each portion was stirred with 5ml of 1% aqueous HCl on water bath and was heated gently. The filtrate was treated with Wagner's reagent (iodo-potassium iodide) and the formation of reddish/redish-brown precipitate indicated the presence of alkaloids.

Assessment of Antibacterial Activity

Syzygium sp. extracts prepared from different solvents was evaluated for antibacterial activity by diffusion and broth dilution assays. To achieve the different pH values, prepared extracts were added with NaOH or HCl solution. Sterile nutrient agar (NB) plates was prepared for bacterial strains and inoculated by a spread method under aseptic conditions. Positive control for pH 5 (NB agar adjusted with HCl solution) and the negative control (sterile water adjusted with HCl solution) while for the positive control for pH 12 (NB agar adjusted with NaOH solution) and the negative control (sterile water adjusted with NaOH solution). Filter paper disc of 5mm diameter (Whatman No. 1 filter paper) was prepared and sterilized. The antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (ZOI in mm) produced by the respective extract at the end of incubation period.

$$ZOI = \frac{\text{Diameter of zone of inhibition} - \text{Diameter of disk}}{\text{Diameter of disk}}$$

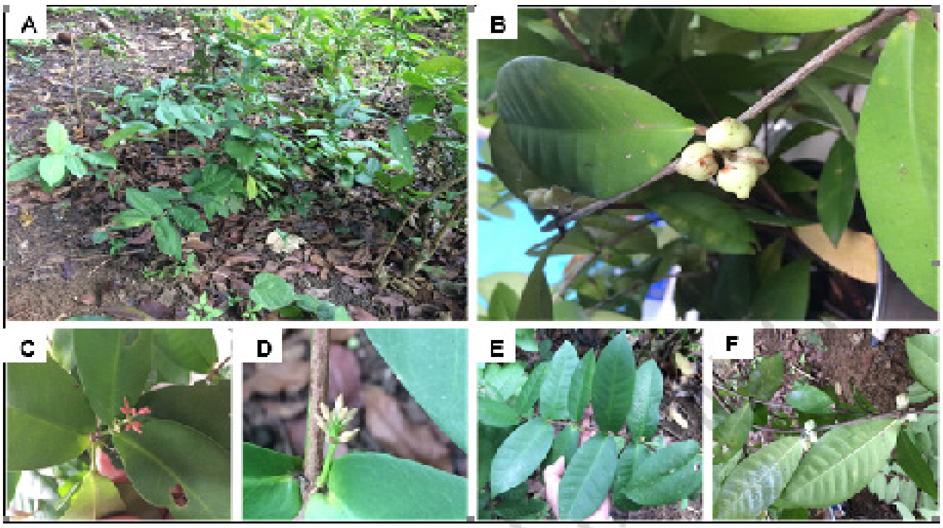

Figure 1. Morphological identification of Syzygium sp. (A) habit, growing in an undershade condition, (B) brachlet with mature fruit, (C) part of brachlet with flower, (D) part of brachlet with immature fruit, and (E&F) brachlet with immature and mature fruit respectively.

Table 1. *Morphological comparison of Syzygium sp. with allied taxa (Shareef et al, 2012)*

Characters	S. rubicundum	S. nervosum	S. palodense	Syzygium sp.
Bark surface	Pale, orange-brown, smooth	Greyish-white or dark brown, smooth	Pale-brown, smooth	Blackish grey, rough
Brachlets	Sharply 4-angular, pinkish-grey when dry	Terete, compressed, swollen at nodes, grayish white when dry	Sharply 4-angular, slightly winged, brownish when dry Crimson	4-angular, grey when dry
Young leaves	Rose-pink	Pink	Crimson	Crimson
Leaf lamina	Narrowly elliptic, 2.5 - 7 x 1.5 -3 cm	Elliptic-obovate, 6-27 x 3-10 cm	Elliptic to elliptic-oblong, 5.5-9.5 x 2.4 - 5.4 cm	Elliptic lanceolate 6.5 - 9.7 x 3.4 - 4.2 cm
Leaf apex	Caudate-acuminate	Obtuse nor obtusely acuminate	Caudate-accuminate	Accuminate
Leaf base	Tapering	Tapering	Obliquely-cuneate	Rounded
Leaf texture	Thinly coriaceous, dull fufuos brown to chocolate-brown beneath on drying	Thinly coriaceous, pale grey-green when dry	Coriaceous, dark-brown above and pale brown-beneath when dry	Thinly coriaceous, dark-brown above to pale-brown be-neath when dry
Lateral nerves	Many, distinct, close and slightly elevated beneath, obscure above, veinlets finely reticulated	10-12 pairs on either side of midrib, dis-tant, 8-9 mm apart, arcuate, shallowly depressed above, distinctly elevated beneath, veinlets faintly reticulated	Many, close, obscure above and distinct beneath, veinlets faintly reticulated	
Petiole Inflores-cence & flower	0.5-0.6 cm long, very slender termi-nal or subterminal or axillary, to 7cm long. Inflorescence branches slender, flowers pedicellate, pinkish-white	1.1-2.3 cm long, slender lateral, to 14 cm long in the axil of fallen leaves; pe-duncle and branches terete, flowers ses-sile, greenish-white	0.2-0.6 cm long, thick and robust terminal or sub-terminal 4.5 - 9 cm long. Inflorescence branches not slen-der, quadrangular, flowers sessile, creamy-white	0.4-0.6 long, thick and robust laterial within nodes, fused to petiole, com-pund inflorescence, creamy-white
Calyx	Repand, cup sharped, 4-lobed persisten, shortly tubinate	Ovoid turbinate becoming campanu-late with short ob-tuse lobes or nearly truncate, calyptrate petals fused, oblong, calyptras apiculate	Campanulate, lobes 4, persistent, deltoid to suborbicular	Tubular
Corolla	Petals fused, calyp-trate, elliptic, calyp-tras without apicule	Petals fused, oblong, calyptras apiculate	Petals fused, calyp-trate, calyptras with-out apicule, obicular to suborbicular	Petals fused oblong calyptras
Fruit	Globose, ca 1cm diam, purple	Globose to ovoid, ca. 1cm diam, purple	Subglobose to obovoid, to 2.2 x 1.8 cm, dark-purple	Globose to oxheart, 1.8 x 2.0 cm, creamy white to deep pinky

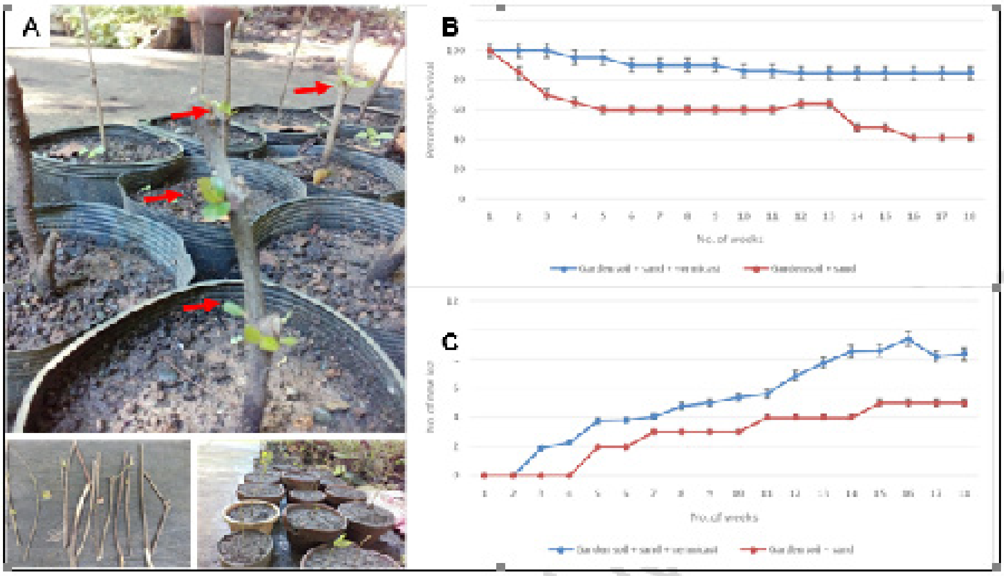

Figure 2. Propagation protocols optimization. (A) Young branches of Syzygium were cut into 4-5 nodal segments and potted in medium composed of 1:1:1 garden soil, sand, and vermicast, and (B) percentage survival of Syzygium sp. and (C) no. of new leaves formed after 18 weeks of planting under greenhouse condition observed grown through cuttings.

RESULTS AND DISCUSSION

Natural products extracted from tissues of terrestrial plants produces innumerate metabolites with distinct biological properties that make them valuable as health products or as structural templates for drug discovery. Local people in Oroquieta City use Syzygium sp. to treat dengue, thus to investigate Syzygium sp. as potential candidate as anti-dengue lead compound, the authors collected a few specimens that were found to be similar to the new species of Syzygium (Myrtaceae) from the southern Western Ghats of Kerala, India (Shareff et al., 2012) but differ in many aspects.

Phenology, Distribution and Habitat

Syzygium sp. known as "O-on" is only from Brgy. Clarin Settlement, Oroquieta City, Misamis Occidental at ca. 18 m elevation. The present population comprises about 47 small- to medium-sized trees and numerous seedlings in its vicinity. It is growing as a riparian element along the banks of a stream. In the present locality, the members of the populations are not yet protected by the legislation of the said barangay. The authors collected a few seedlings from the type locality and are conserving at Central Mindanao University, as part of its ex-situ conservation.

Species of Syzygium are present in virtually all most of Oroquieta City, and are often important components of the biological communities, so the lack of taxonomic resolution presents a serious impediment for a better understanding of ecological processes as well as for its conservation (Fig 1 and Table 1).

Ex situ Conservation of Syzygium sp "O-on

Ex situ conservation of Syzygium sp. through stem cutting exhibited 85% survival after 18 weeks of planting in garden soil, sand, vermicast (1:1:1) potting medium under greenhouse (Fig. 2). The high percentage survival could be attributed to the high NPK content of vermicast. The sand particles provide good aeration and root growth (Lituañas & Amoroso, 2006). However, stem cuttings planted in garden soil and sand (1:1) potting medium showed 41% survival after 18 weeks under greenhouse condition.

In terms of number of new leaves formed, garden soil, sand, vermicast (1:1:1) potting medium revealed more new leaves formed (Fig. 3) and a higher average plant height (Fig. 3B) after 18 weeks of planting in the greenhouse. This indicates that the stem cuttings of Syzygium sp. already adapted to the greenhouse condition and growing vigorously after 18 weeks of planting (Fig.3A).

Phytochemical Analysis

Qualitative phytochemical analysis of Syzygium sp. plant extracts revealed the presence of constituents which are potential for pharmaceutical commercial development (Khoo et al., 2016). Analysis of the plant extracts revealed the presence of medically active compound as summarized in the Table 2. The results revealed the presence of medically active compounds in the plant studied. From the table, it could be seen that, carbohydrates,

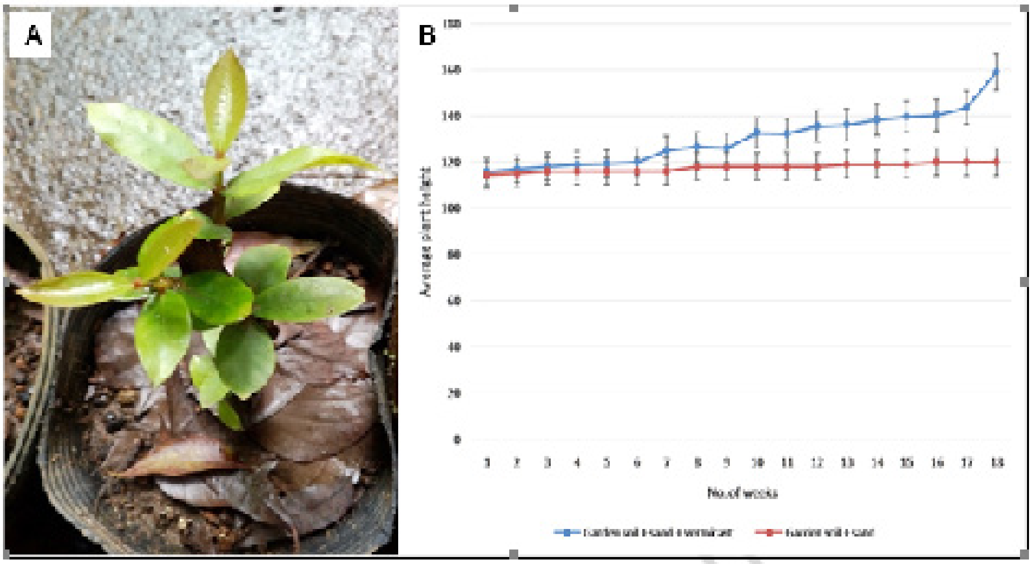

Figure 3. Syzygium adaptation condition. (A) Syzygium sp. growing vigorously after 18 weeks of planting under greenhouse condition and the (B) average plant height of Syzygium sp. after 18 weeks of planting under greenhouse condition.

phenols and tannins, flavanoids, saponins, steroids, terpenoids and alkaloids were present in the extracts.

The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites since they possess biological properties (Singh, 2007). Accordingly, natural antioxidant mainly comes from plants in the form of phenolic compounds such as flavonoid, phenolic acids, tocopherols etc. (Zhao et al., 2014). Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection probable due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Yadav & Agarwala, 2011). Tannins bind to proline rich protein and interfere with protein synthesis and they also are effective antioxidant and show strong anticancer activities (Marjorie, 1996). The plant extracts were also revealed to contain saponins which are known to produce inhibitory effect on inflammation since it has the property of precipitating and coagulating red blood cells (Just et al., 1998). Steroids are used in the treatment of idiopathic thrombocytopenic purpura (IPT) to increase the platelet count which is mediated by auto antibodies (Shashidhara, et al., 2013) have been reported to have antibacterial properties. Alkaloids are important phytochemicals of medicinal plant and can be used as vaccine candidates for viruses since it could serve as important inhibitors to inhibit the replication of dengue virus (DENV) but need further in vitro investigations to confirm their efficacy and drug ability (Qamar et al., 2014). Based from the results obtained on the presence of phytochemical compounds in Syzygium sp. plant, it might be a valuable reservoir for bioactive constituents for substantial medicinal merit.

Antimicrobial Analysis

Since we wanted to examine the co-bacterial infection of dengue, we further tested its antimicrobial activities using varied solvent used for extraction,

Table 2: *Phytochemical constituents of Syzygium sp.*

Phytochemical Screening	Aqueous extract	Methanolic extract	Ethanolic extract
Carbohydrates	+	+	+
Phenols/Tannins	-	+	-
Flavanoids	+	+	+
Saponins	+	+	-
Steroids	+	+	+
Terpenoids	-	+	-
Alkaloids	+	+	+

(+) Positive response (-)Negative Response

Table 3:
Percent inhibition of Syzygium sp. extracts against bacterial co-infection in dengue.

Treatment	Percent Inhibition (%)								
	Different Solvent			pH Level			Different Concentration		
Bacterial Strain	*aqueous*	*methanol*	*ethanol*	*pH5*	*pH7*	*pH12*	*ImM*	*10mM*	*100mM*
S. aureus	55.8	67.51	56.50	53.97	63.16	56.45	56.45	61.42	73.18
P. aeruginosa	53.69	85.08	67.51	60.24	67.15	70.45	60.45	70.85	72.35

pH level and increasing concentration through percent inhibition (Table 3). Result showed moderate inhibition activity with the zone range of 3–5 mm since we did not obtained high purity concentration of the extracts in different solvent used. Maximum inhibition was observed against P. aeruginosa (5 mm) and minimum inhibition against S. aureus (3 mm). Among the solvent tested, methanol extracts of Syzygium sp. showed significant antimicrobial activity against multi-drug resistant clinically isolated microorganisms. In terms of pH level, pH 12 yielded higher percent inhibition in P. aeruginosa while at pH 7 in S. aureus implying that increasing pH level inhibit microbial growth. Accordingly, P. aeruginosa outer member is sensitive when exposed with chitosan nanoparticles when increasing pH values however, increasing the pH from 7.5 to 9 abolished chitosan nanoparticles activity (Aleanizy et al., 2018). On the other hand, the optimal pH at which S. aureus can be detected at pH 6.5 and slight decrease of the probability of growth can be noticed in the pH interval of 7.0-7.5 at more stringent condition (Valero et al., 2009). Ensuring a suitable pH range is important in maintaining the growth stability of the tested microorganism. This study also demonstrated that increasing concentration of Syzygium extracts significantly yielded higher percent inhibition. The antimicrobial activity of the Syzygium may be due to tannins and other phenolic constituents that if undesirable amount can precipitate proteins and inhibit digestive enzymes and play an important role in chelating transitional metals and scavenging free radicals (Chattopadhyay et al., 1998). Although, the mechanism of the action of these plant constituents is not yet fully known, it is clear that the effectiveness of the extracts largely depends on the type of solvent used, pH level and concentration. This observation might indicate that there can be an existence of non-polar residues in the extracts which have higher both antimicrobial and phytochemical activities.

CONCLUSION

In conclusion, our results indicate that the Syzygium sp collected from Misamis Occidental have the potential to control P. aeruginosa and S. aureusgroup behaviors. Morphological characterization of Syzygium sp. compared with its allied taxa differs in several characteristics. It is similar to the new species of Syzygium (Myrtaceae) from the South Western Ghats of Kerala, India but differs from its bark surface when dry, color of the leaves when young, tabular calyx, and oblong calyptras and may represent a new species of Syzygium. Ex situ conservation also showed that cutting method have higher percent survival than using wildlings for massive propagation. Media preparation must also be considered for it propagation were garden soil, sand and vermicast (1:1:1) showed higher % survival under greenhouse condition. Phytochemical analysis also showed that crude aqueous and organic solvent extracts of Syzygium contains medicinally important bioactive compound. The antimicrobial activity of Syzygium sp. methanolic crude extracts were considerably more effective radical scavengers than those using lest polarity solvent, indicating that antioxidant or active compounds of different polarity could be present in leaves of Syzygium sp. Antimicrobial activity varies widely depending on the several factors, test medium, types of pathogens (P. aeruginosa and S. aureus), type of solvent used, pH levels and Syzygium sp concentration sensitive. Although Syzygium sp. contains bioactive compounds and appears to inhibit multiple targets could be a valuable addition to P. aeruginosa and S. aureus group-behavior inhibitors for the development of anti-virulence compounds, further studies on the efficacy of these natural antimicrobial agents in a range of food products as well as evaluation of potential interactions of antimicrobial compounds with components of food matrices must be investigated.

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