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Antimicrobial activity of supercritical fluid extracts of two Philippine medicinal plants, *Psidium guajava* and *Euphorbia hirta*: Implications to community health

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Abstract The study is aimed to determine the antimicrobial activity of supercritical crude extracts of *Psidium guajava* and *Euphorbia hirta* against four bacteria such as *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli*, *Pseudomonas aeruginosa* and the two fungi namely, *Aspergillus niger* and *Candida albicans*. Fresh leaves of *P. guajava* and whole plant of *E. Hirta* were extracted using Supercritical Fluid Extraction at 120 atm with supercritical CO_2 as the extracting solvent. Antibacterial activities of the crude extracts against the six microorganisms were determined by Filter Paper Disc method. Results showed that the crude extracts of the two plants exerted vary degrees of inhibition to the six test microorganisms. *P. guajava* exerted pronounced inhibitory action against the gram-positive bacteria (*B. subtilis* and *S. aureus*) but minimal against the gram-negative species (*E. coli* and *P. aeruginosa*) and fungi (*A. niger* and *C. albicans*). *E. Hirta* extract showed weak bacterial activity against all test microorganisms. Comparison in the antimicrobial activities between these two plants revealed *P. guajava* to be much stronger than *E. Hirta* but only against gram-positive bacteria while both plants have the same minimal inhibitory effect against gram-negative and fungi species. Further, *P. guajava* extract showed stronger bactericidal effect against gram-positive bacteria when compared with the standard antibiotic chloramphenicol which may scientifically validate its effectiveness as alternative herbal remedies to treat ailments common in rural areas. The weak antimicrobial and antifungal actions of *E. hirta* demonstrated in our study may further point to the plant's other medicinal value such as platelet-increasing effect as reported by some authors.
Key Words: Philippine medicinal plants, *Psidium guajava*, *Euphorbia hirta*, supercritical fluid extraction, antimicrobial activity.

Introduction. Man has relied on most plants for its therapeutic values (aside from its uses as food, spices and condiments in foods, timber and clothing) since the beginning of time. In many countries in Asia, Latin America and Africa, knowledge on herbal plants for its medicinal uses is largely dependent on one's knowledge that have been handed over from generation to generation. Nowadays, the role of herbal plants as alternative medicines in treating ailments in many developing countries are gaining popularity because of the high cost that it entails in using modern life-saving medicines, hospitalization and primary healthcare services. Further, in developing countries where modern healthcare facilities are inaccessible to majority of the people especially in rural areas, the use of local herbal remedies is the main solution in treating their illnesses. In fact, the World Health Organization (WHO) reported that more than 80% of the world's population now depends largely on traditional medicine for their healthcare needs. At present, compilation of studies pointing to the importance of medicinal plants in providing natural source of bioactive compounds that are used mainly for medicine purposes are increasing. In addition, literatures and ethnobotanical reports are quite available suggesting that plants are the sleeping giants of pharmaceutical industry (Hamburger & Hostettmann 1991; Akinpelu & Onakoya 2006).

In the Philippines, there are more than 1000 species of plants with medicinal values, however, the Philippines' Department of Health had approved and recommended for use ten medicinal plants only. Out of these ten, only two plants namely, *Cassia Alata* and *Psidium guajava* are antibacterials. Despite this information, the importance of medicinal plants in the Philippines cannot be overlooked because most of these traditional plants are being used as poultices (for wounds, scabies and skin diseases), liniments and topical ointments (Penecilla & Magno 2011).

P. guajava (Family Myrtaceae) commonly known as guava or also locally known as "bayabas" is one of the ten herbal plants recommended for use by the Philippine Department of Health (DOH). It is widely distributed in the Philippines and is common in backyards because it grows well in various soil types. The branches are 4-angled with opposite leaves (Hernandez 1980; Ticzon 1997). It is a small tree that can grow up to 3 meters tall with greenish-brownish smooth bark. The round globular fruit, which starts as a flower, is small and measures 3 to 6 cm long, and when ripe becomes soft and turns yellowish-green (www.philippineherbalmedicine. org/bayabas.htm). As a folklore medication in the country, *P. guajava* fruit, bark and leaves are used. Decoction of fresh leaves is used as an antiseptic wash and aromatic bath, for diarrhea, mouthwash, ulcers, wounds, scabies, and vaginal wash for post-partum care in women. Infusion of fresh leaves is used for wound cleaning to prevent infection, for toothache, gum swelling and nosebleeds. The bark is boiled and drank thrice a day for diarrhea and vertigo. The twigs, chewed at the ends until frayed, are used as alternative for toothbrushing with whitening effect (Seidenschwarz 1994; Ticzon 1997; Balongcod & Balongcod 2011; Olowa et al 2012). In the scientific field, many pharmacological investigations have demonstrated the ability of *P. guajava* leaves, barks and fruits to exhibit antimicrobial, anti-diarrheal, antimalarial, antitussive, hepatoprotective, anti-oxidant, anti-genotoxic and antimutagenic, anti-allergic, anticancer/antitumor, cardiovascular, hypotensive, anti-hyperglycemic, anti-inflammatory/analgesic, antinociceptive, wound healing, antipyretic, spasmolytic and CNS depressant activities (Gutierrez et al 2008; Kamath et al 2008; Joseph & Priya 2011; Kumar 2012). These exhibited activities were attributed to different bioactive compounds isolated from extracts in different parts of guava. In particular, the active flavonoid compound quercetine derivatives, terpenoids, tannins and other glycosides found in leaf extract of *P. guajava* showed strong antimicrobial activities (Arima & Danno 2002; Limsong et al 2004; Akinpelu & Onakoya 2006; El-Mahmood 2009b; Adeyemi et al 2009; Metwally et al 2010; Rattanachaikunsopon & Phumkhachorn 2010; Joseph & Priya 2011; Penecilla & Magno 2011; Banu & Sujatha 2012).

Another important medicinal plant in the Philippines is *Euphorbia hirta* (Family Euphorbiaceae) commonly known as asthma weed or cat's hair and is also locally known as "tawa-tawa". The plant is slender-stemmed and bushy that grows with several tiny branches from the base to the top. It has a height of 2 inches with several tiny flowers bunched together with opposite rhombus leaves blotched with purple and toothed at its edge. The plant thrives in open wastelands, grasslands, pathways and roadsides (Quisumbing 1978). As a folklore remedy in the country, the whole plant is boiled with water and drank thrice daily to lower high fever and dengue fever (Olowa et al 2012), whereas fresh leaves are used to cure dengue fever (Quisumbing 1978) as well as bronchitis, asthma, constipation, dysentery, vomiting, conjunctivitis, colic, skin diseases, thrush, snake bites, and hemostatic (www.stuartxchange.com/AltMed.html). Extract from leaves are also applied on sore eyes while the smoke coming from the burnt leaves are inhaled for asthma relief (Balongcod & Balongcod 2011). Several scientific evaluations on the pharmacological properties of extract of leaves, stem, bark and whole plant have pointed out the efficacy of *E. hirta* as an antimicrobial agent (Sudhakar et al 2006; Ogbulie et al 2007; El-Mahmood 2009a; Okoli et al 2009; Kumar et al 2010b; Sharma et al 2010; Bakkiyaraj & Pandiyaraj 2011; Singh et al 2011; Perumal et al 2012) because of the presence of bioactive compounds such as tannins, saponins, phenolics, flavanoids, cardiac glycosides, anthroquinones and alkaloids. Other pharmacological activities of the plant include antihelminthic (Adedapo et al 2005), antithrombocytopenic (Apostol et al 2012), anti-inflammatory/antiasthmatic (Ogunlesi et al 2009; Das et al 2010), antimalarial (Ogunlesi et al 2009), antioxidant (Jagadeesan et al 2011), antidiabetic (Kumar et al 2010a), antifungal, antidiarrheal, antifertility and antiamoebic (Tona et al 2000).

Supercritical fluid extraction is a technique that uses carbon dioxide for extraction of natural products instead of the traditional organic solvents which are suspected to be carcinogens. Therefore, it has the potential to be environmentally friendly "green" processing technique because it works in closed loop and hence is not a generator of carbon dioxide (which is a growing concern in global warming). Further, it is a potential solvent for replacement of many undesirable organic solvents, which present a threat to environment, health and safety in the work place (Basniwal et al 2005; Nagarsekar et al 2010).

Due to the importance and wide distribution of these two plants and the environmental benefits in employing supercritical fluid CO_2 as extracting solvent, this study was conducted. It aims to determine the presence of antimicrobial activities of these herbs against 4 bacteria and 2 fungi, compare the antimicrobial activities between these two plants and then compare the effectiveness of *P. guajava* and *E. hirta* extracts to standard antibiotics such as chloramphenicol and mycostatin.

By providing a scientific data on the sensitivity of the test organisms to the extracts of *P. guajava* and *E. hirta*, this research can justify and promote the use and application of these herbal preparations in rural communities where such practices are prevalent. In particular, the extracts may prove to be all-natural, safe, effective and inexpensive alternative to costly commercially sold drugs for infectious diseases such as skin diseases, diarrhea and candidiasis that are commonly associated with the six test microorganisms.

Material and Method. Four net bags filled with leaves of *P. guajava* and three net bags filled with whole plants of *E. hirta* were collected from the botanical garden at MSU-Marawi City. The samples were washed thoroughly and then air-dried for 12 days. The dried samples were cut into smaller pieces and homogenized using an osterizer. About 150 grams of the homogenized sample was placed in a cloth bag and loaded in the Supercritical Fluid Extractor machine (Figure 1) using supercritical CO_2 as extracting solvent. Experimental extraction condition was set at 120 atm at 40°C. After an hour of extraction, the supercritical CO_2 extracts (in a form of paste, Figure 2) were subjected for antibacterial and antifungal activities by adopting the Filter Paper Disc method. The crude extract was transferred into a small vial and was reconstituted in 2.5 ml of hexane. Sterile filter paper disc (Advantec) with a diameter of 6 mm was impregnated with 30 µL of the extract, and aseptically dried under a sterile hood. Subcultures of bacteria (24 hour) and fungi (48 hour) were prepared in agar slants. One loopful of the test organism was seeded in 50 mL of melted sterile top agar. Observing aseptic measures, 15 mL of the nutrient agar (bacterial culture medium) or potato-dextrose agar (fungal culture medium) was dispensed into sterile petri dishes and allowed to cool for 15 minutes. Subsequently, 2.5 mL of the seeded top agar was spread over the solidified nutrient agar or potato dextrose agar and allowed to cool for 15 minutes. The dry discs impregnated with the plant extracts were placed on the surface agar plates and incubated at 37^{o}C. Standard discs of chloramphenicol (30 µg/disc) and mycostatin (30 µg/disc) were used as positive controls. Zones of inhibition were measured in millimeters using a ruler after 24 hours for bacterial cultures and 36 hours for fungal cultures. The antimicrobial activity of the *P. guajava*, *E. hirta* and the two standard antibiotics against *Staphylococcus aureus, Escherichia coli*, *Bacillus subtilis, Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans* were recorded based on the diameter of the zone of inhibition (ZOI) exerted by the plants against the test microorganisms. This was done by measuring the clear area (halos) around the disc using the millimeter scale of a transparent ruler. Each experiment was carried out in triplicates.

Figure 1. Supercritical Fluid Extraction (SFE) machine.

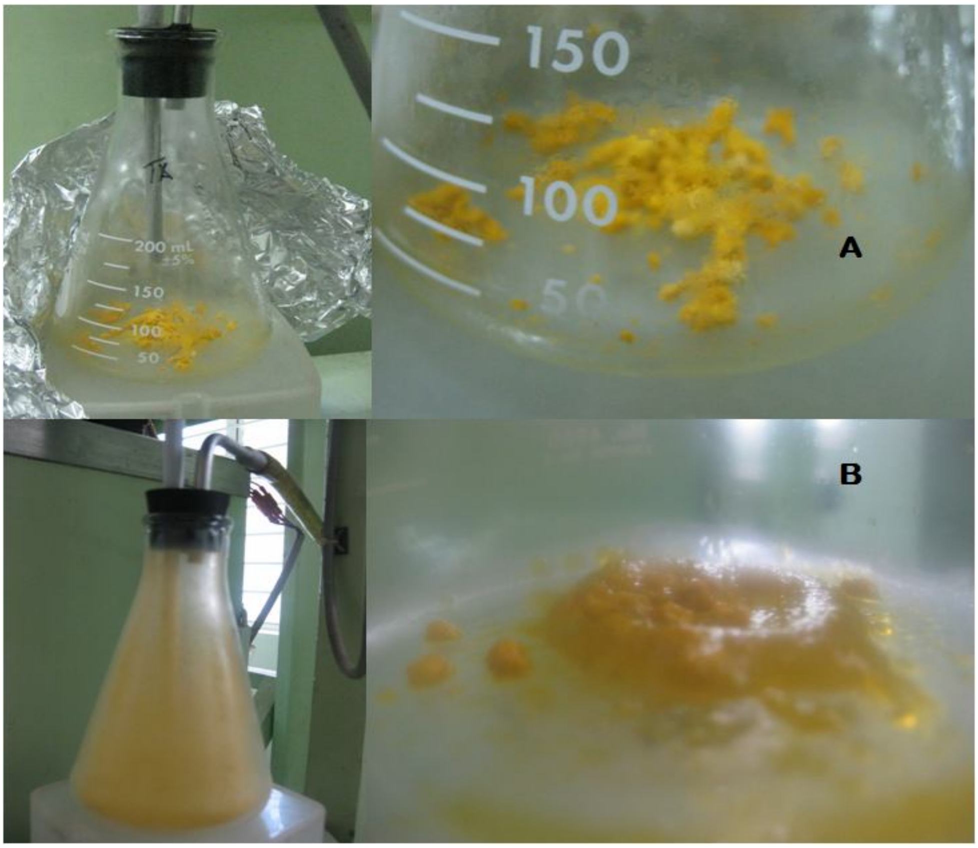

Figure 2. Plant crude extracts of (A) *E. hirta* and (B) *P. guajava* under 120 atmospheric pressure using Supercritical Fluid Extraction (SFE).

Results and Discussion.

Antimicrobial activity, as exhibited by the zone of inhibition, were observed in the supercritical fluid extracts of *P. guajava* and *E. hirta* tested against the four bacteria and two fungi (Table 1). It showed that the tested plants exerted different degrees of antimicrobial activity against the test microorganisms.

Table 1

Antimicrobial activities of supercritical fluid extracts of *P. guajava*, *E. hirta* and standard antibiotics

Microorganisms	*Zones of Inhibition (ZOI) (mm)**			
	P. guajava	*E. hirta*	*Chloramphenicol*	*Mycostatin*
B. subtilis	33.42	9.58	26.58	nd
S. aureus	24.83	9.67	24.75	nd
E. coli	10.58	9.17	21.92	nd
P. aeruginosa	9.75	9.00	22.75	nd
A. niger	7.92	7.75	nd	23.83
C. albicans	8.83	9.25	nd	21.92

mm* - mean of three replicates, nd - not done.

Although *P. guajava* extracts exerted varying degrees of sensitivity to all microorganims, strong antibacterial activities were more pronounced against gram- positive bacteria, *B. subtilis* (Figure 3 A) and *S. aureus* (Figure 3 B), with mean zone of inhibition of 33.42 mm and 24.83 mm, respectively, when compared to the gram-negative species (Figure 4 A, B) and fungi (Figure 5 A, B). Furthermore, antibacterial activity exerted by guava extract towards these gram-positive bacteria were stronger than those of the standard antibiotic chloramphenicol (ZOI: *B. subtilis* = 26.58 mm and *S. aureus* = 24.75 mm).

Our present findings are in accordance with the studies of Sanches et al (2005), Suresh et al (2008) and El-Mahmood (2009b) who reported the leaves of *P. guajava* to have considerable activity against gram-positive bacteria but less for gram-negative species. It is suggested that the strong bactericidal activity exhibited by the leaf extracts against the gram-positive bacteria is possibly due to substances that has protein-degrading activity (Anas et al 2008; Akinpelu & Onakoya 2006) and can complex with bacterial cell walls (Anju et al 2011; Akinpelu & Onakoya 2006). Bactericidal substances usually affect the synthesis of bacterial cell wall and cell membrane resulting to leakage of cytoplasmic content and hence, cell death (Tortora et al 2001). It has been demonstrated that tannins and flavonoid derivatives quercitine, which are numerous in guava leaves, have protein-binding activities (Owen & Johns 1999; Belemtougri et al 2006) and has the ability to complex with cell walls of bacteria (Cowan 1999; Akinpelu & Onakoya 2006), respectively. Brotz-Oesterhelt et al (2005) further showed an antibacterial compound, acyldepsipeptides (ADEPS), which inhibits gram-positive bacteria via uncontrolled proteolysis. Ali et al (1996) also reported flavonoids to be inhibitory to *S. aureus* and has been used in treatment of inflammed tissues. On the other hand, antibacterial activity exerted by the plant extract against gram-negative bacteria was weak when compared to those of standard chloramphenicol (Table 1). Their less susceptibility may be attributed to their thick murine layer in the cell wall which may have prevented the entry of most of the environmental substances such as antibiotics and plant-based biocides into the cell (Khandhasamy et al 2008; Tortora et al 2001). In addition to this barrier, its periplasmic space may also have enzymes which can breakdown foreign molecules that are introduced from the outside. With this, guava extract can be categorized as bactericidal to the two gram-positive bacteria suggesting that the plant may contain active chemical components that can kill bacteria. In contrast, guava extract can be categorized as bacterisotatic to the two gram-negative species having only the ability to stop the growth or multiplication of the microorganisms but not necessarily harming or killing them.

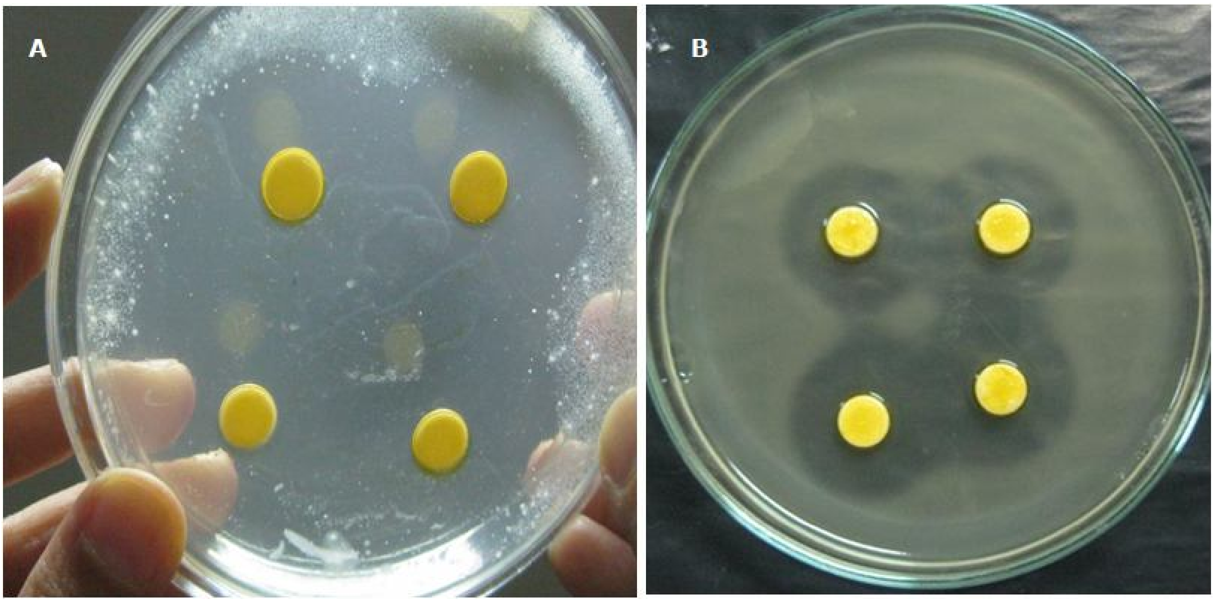

Figure 3. Antimicrobial assay plate for the antibacterial activity of *P. guajava* on the growth of (A) *B. subtilis* and (B) *S. aureus* showing wide zone of inhibition.

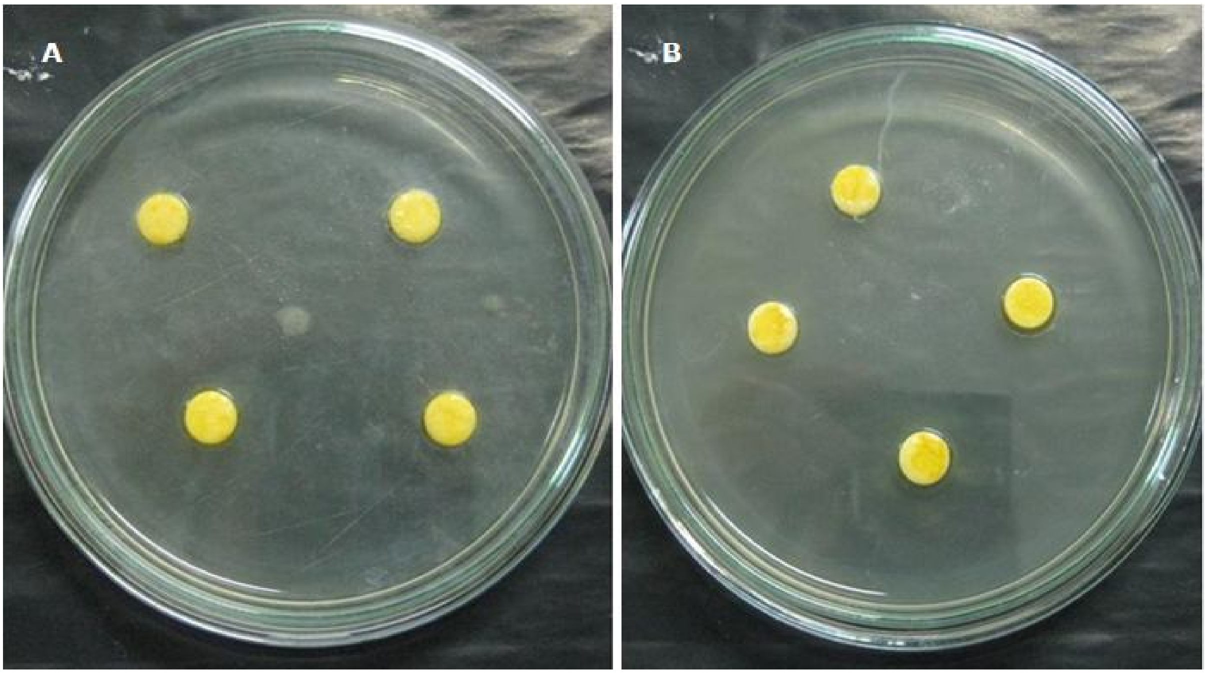

Figure 4. Antimicrobial assay plate for the antibacterial activity of *P. guajava* on the growth of (A) *E. coli* and (B) *P. aeruginosa* showing minimum zone of inhibition.

The antifungal activity exhibited by the guava extract was not particularly strong when compared to those of the standard mycostatin (Table 1) and this in agreement with the study of Egharevba et al (2010) and Singh et al (2011) who both demonstrated guava leaf extract in having no activity against fungi isolates. However, our results are in contrary to the studies of Metwally et al (2010) and Anju et al (2011) who showed leaf guava extracts having strong antifungal activity. Our present results further support the knowledge of *P. guava* leaf extracts possessing strong antibacterial properties rather than pronounced antifungal activity although their antimycotic action could not be ruled out.

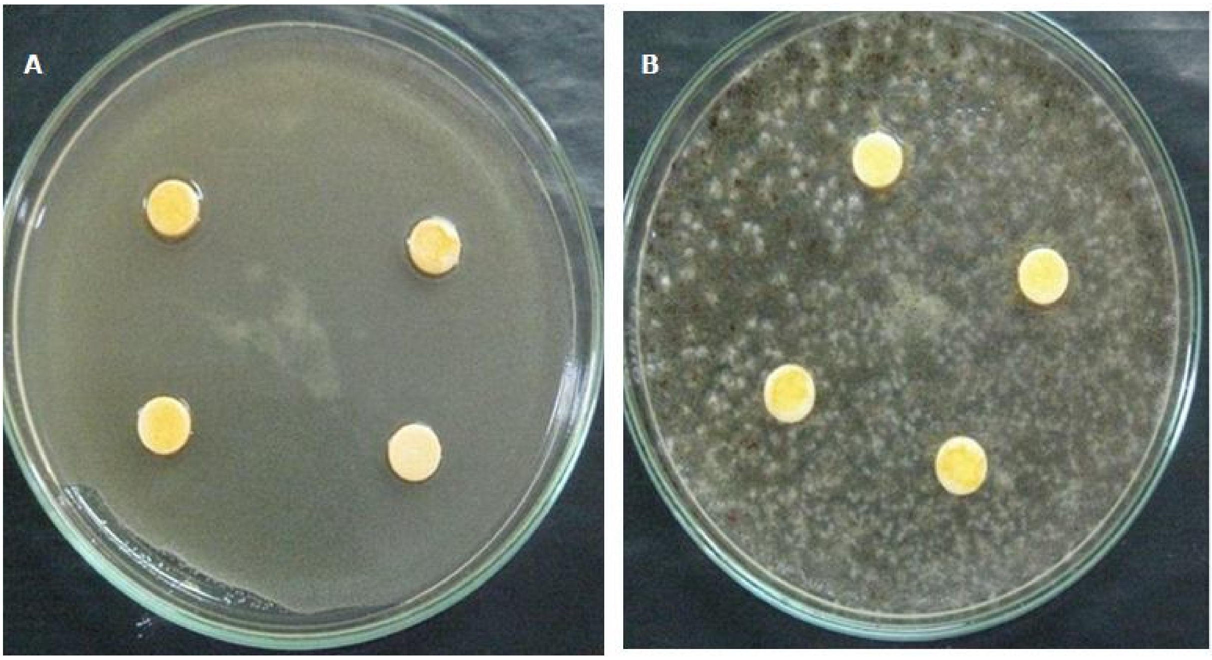

Figure 5. Antimicrobial assay plate for the antibacterial activity of *P. guajava* on the growth of (A) *C. albicans* and (B) *A. niger* showing minimum zone of inhibition.

E. hirta extracts exerted minimal antimicrobial activity against the four bacteria (Figure 6 A-D) and two fungi (Figure 7 A, B) when compared to those of the standard antibiotic mycostatin. This is evident by their minimum zone of inhibition that ranges between 9-9.67 mm and 7.75-9.25 mm, respectively (Table 1). With this, *E. hirta* may be categorized as bacteriostatic and mycostatic to the four bacteria and two fungi, respectively.

The present study is in accordance with the study of Bakkiyaraj & Pandiyaraj (2011) who demonstrated low antimicrobial activity exerted by the plant extract against the fungus *C. albicans*. Although our findings showed bacterial effect but in mininimal activity, it is in contrary with the studies of Mohamed et al (1996), Ogbulie et al (2007), Suresh et al (2008), El-Mahmood (2009a) and Bakkiyaraj & Pandiyaraj (2011) who demonstrated *E. hirta* leaf extracts exerting pronounced antimicrobial action against gram-positive and gram-negative bacteria as well as the fungus *A. niger* (Mohamed et al 1996).

Since the present study shows that the plant extract could not provide promising results on its antibacterial and antifungal activities, it can be suggested that the plant extract may be promising in other activities such as its platelet-increasing effect or antithrombocytopenic as reported by Apostol et al (2012). In the Philippines, the fresh leaves (Apostol et al 2012) and whole plant (Olowa et al 2012) of *E. hirta* are traditionally used as a cure for dengue fever.

Comparison between the inhibitory actions against these six microorganisms by the extracts of *P. guavaja* and *E. Hirta* revealed that guava extracts are more potent against gram-positive bacteria (Table 1). However, the minimal inhibitory actions of both plant extracts were the same against gram-negative bacteria and fungi species.

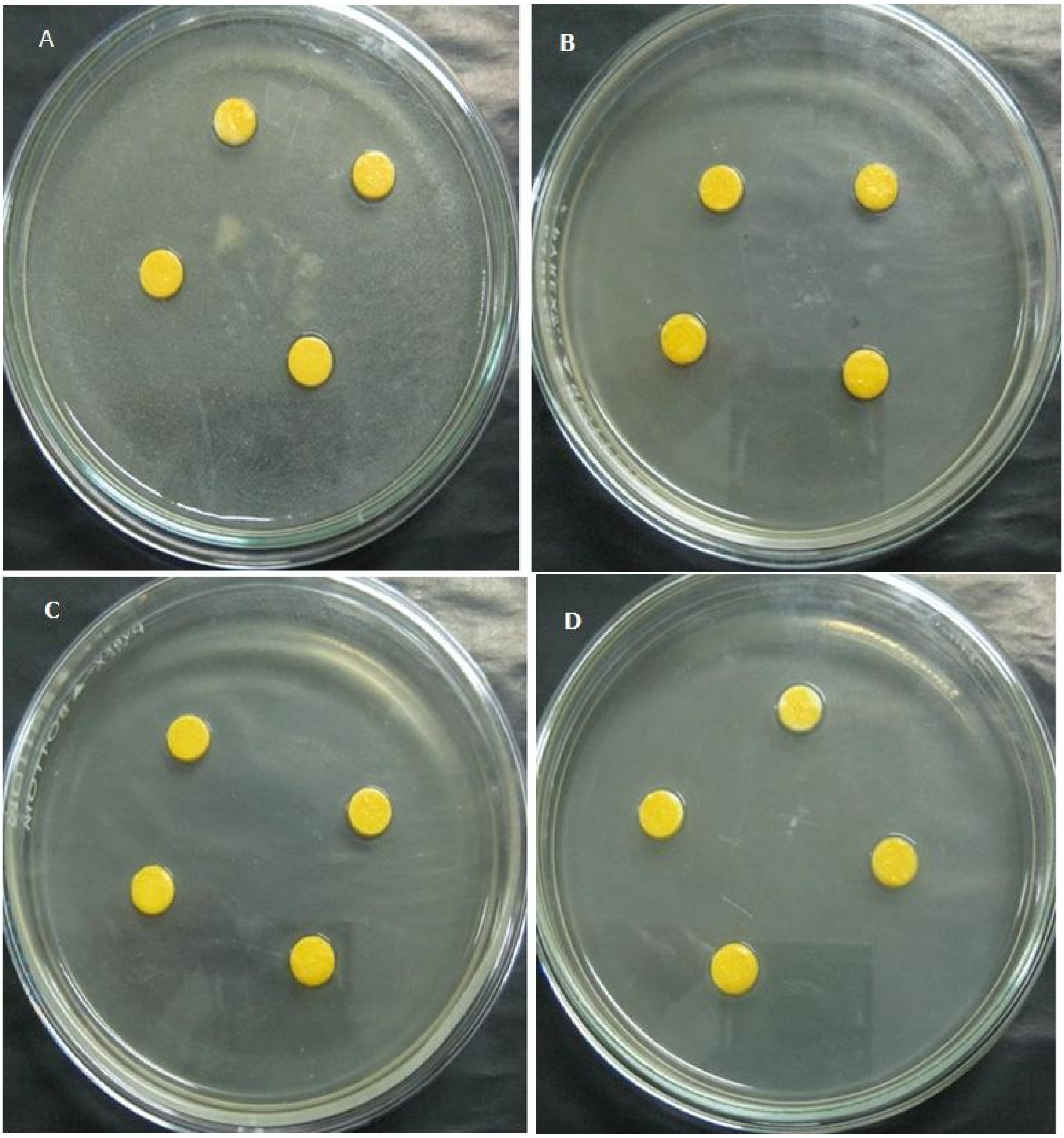

Figure 6. Antimicrobial assay plate for the antibacterial activity of *E. hirta* on the growth of (A) *B. subtilis*, (B) *S. aureus*, (C) *E. coli* and (D) *P. aeruginosa* showing minimum zone of inhibition.

Implications to Community Health. In the Philippine social stratification system, it is a fact that a majority of the Filipinos belong to the lower class, many of whom could not likely appropriate a fraction of their budget for health maintenance. The statistics released by the National Statistics Coordination Board indicates that approximately 24 out of 100 Filipino families did not earn enough to satisfy their basic food and non-food requirements. It is in this junction that the Department of Health strengthens its community health programs. The use of herbal medicine is part of its health promotion in the community level and functions in the encouragement of the Filipino populace to make use of whatever is available within their immediate environment and in their respective communities. As the use of locally available medicinal plants and herbs has been advocated (many of which just grow in the household backyard and fields), their effectiveness in the treatment of common ailments as attested to by the National Science

Development Board (Cuevas 2007) has led to continuing research on the medicinal properties of those already identified and promoted herbs and plants.

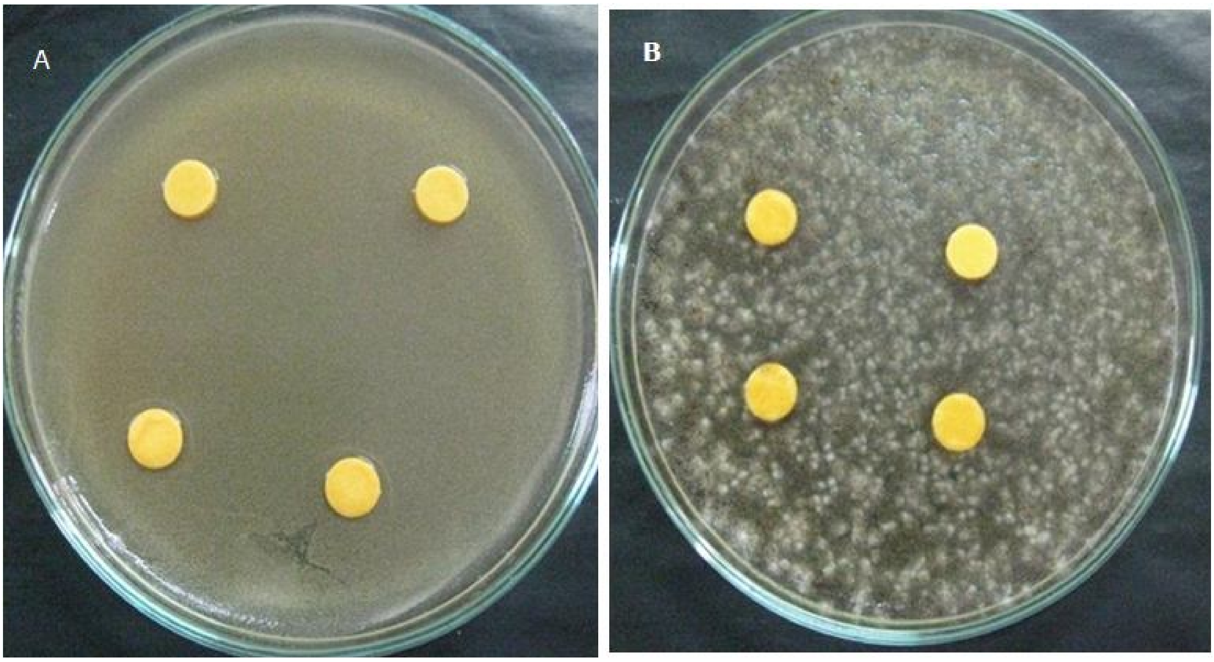

Figure 7. Antimicrobial assay plate for the antibacterial activity of *P. guajava* on the growth of (A) *C. albicans* and (B) *A. niger* showing minimum zone of inhibition.

P. guajava is fortunately included as one of the ten approved herbal medicines of the Department of Health (DOH) in the Philippines. *E. hirta* on the other hand, though not one of those approved herbal medicines yet, has been already widely used around the country, amongst rural and urban community dwellers alike who would be more than willing to share their good and positive claims of the healing experiences they have had with it; whether used independently in the home-setting or synergistically with prescribed pharmacological medicines in the hospital setting in the form of fluid replacement therapy. At the heart of this study is the "community," the core of the Philippine society, with the premise of providing a better alternative of low cost medicines but with equal potency with those synthetic medicines available in the market nowadays. This study is deemed to eventually motivate researchers and students to advance the restoration of health by promoting alternatives integrated in every health teaching plan. Since traditional use of plants with therapeutic values are encouraged as alternative medicines, hence the proper conservation and sustainable use of such plant resources and the incorporation of the traditional knowledge with scientific findings by local communities should be enhanced through education.

Conclusions. The present findings on the effectiveness of *P. guajava* extract as strong antimicrobial agent against gram-positive bacteria strongly supports the traditional/folkloric knowledge of local users about their selection of this plant sample as treatment of various ailments caused by this type of bacteria. Since our study revealed the extract of *P. guajava* to be stronger than the commercially sold chloramphenicol, it will further support the use of these plant-based medicines as an effective and strong alternative medicine. In a country where hospitals and modern-life saving drugs are inaccessible to the majority of the people living in rural areas, local herbal remedies that are enhanced by scientific study can help them immensely. With this knowledge at hand, the way to aid them is to teach them the appropriate preparation and application of these plant-based medicines in a proper and aseptic way so as to ensure absence of secondary infection. Moreover, the weak antimicrobial and antifungal actions of *E. hirta* demonstrated in our study further point to the plant's value in platelet-increasing effect or antithrombocytopenic as reported by some authors. Considering that the Philippines is frequently experiencing bouts of dengue fever, there is therefore a need for detailed scientific study of the plant's medicinal uses in order to ensure and vouch for the effectiveness and safe use of *E. hirta*.

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