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TOTAL PHENOLICS, TOTAL FLAVONOIDS, ANTIOXIDANT ACTIVITY AND ANTIBACTERIAL PROPERTY OF PROPOLIS PRODUCED BY THE STINGLESS BEE, *Tetragonula biroi* (Friese), FROM LAGUNA AND QUEZON, PHILIPPINES

Joy Line T. Cumbao[1], Paul Lloydson J. Alvarez[1*], Ma. Desiree Belina-Aldemita[1], Jose Rene L. Micor[1], Mark Rickard N. Angelia[1], Analinda C. Manila-Fajardo[2] and Cleofas R. Cervancia[2]

[1] Institute of Chemistry and [2]Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna
* Corresponding author: pjalvarez@up.edu.ph

ABSTRACT

The total phenolics (TP) and total flavonoids (TF) of the ethanolic extracts of *Tetragonula biroi* (Friese) propolis obtained from selected municipalities in Laguna and Quezon, Philippines were determined using Folin-Ciocalteau and aluminum chloride colorimetric methods, respectively. The antioxidant activities were determined using ferric ion reducing antioxidant power (FRAP), hydrogen peroxide scavenging activity (HPSA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Values obtained for Laguna propolis in each assay were 255.22 ± 14.75 mg GAE/g, 1.91 ± 0.42 mg QE/g, 5.21 ± 3.08 µmol Fe^{2+}/g, 12.73 ± 1.45 (IC_{50}) and 15.49 ± 1.13 (IC_{50}), respectively. Those for Quezon propolis were 308.04 ± 119.55 mg GAE/g, 5.30 ± 1.43 mg QE/g, 24.22 ± 16.88 µmol Fe^{2+}/g, 11.39 ± 2.33 (IC_{50}) and 14.94 ± 3.49 (IC_{50}), respectively. Statistical analyses showed that the samples only differed significantly in TF and FRAP values. This explains the similarity in the *in vitro* partial inhibitory activity and reactivity of the samples against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*.

Key words: antioxidant activity, flavonoids, polyphenols, propolis, *Tetragonula biroi*

INTRODUCTION

Propolis, also known as bee glue, is the sticky dark colored material made by bees from different plant parts combined with bee enzyme. Aside from being a useful building material, it has been used to protect the hive from intruders and pathogens (Bankova et al., 1999). This characteristic made it a popular traditional medicine in many countries; curing both internal and external diseases. It has also served as a health food as it was believed to cure inflammation, aging, heart disease, diabetes and even cancer (Nagai et al., 2003). Studies have reported biological and pharmacological activities of propolis, mainly antibacterial, antiviral and anti-inflammatory (Gulcin et al., 2008; Trusheva et al., 2010). The activities reported can be due to the chemical composition of propolis, which is composed mainly of polyphenols, flavonoids and their derivatives. Alvarez et al. (2013) have identified several flavonoids and phenolic compounds in stingless bee propolis from the Philippines using tandem liquid chromatography-mass spectrometry (LC-MS). Relating the composition of propolis to

its antioxidant capacity has become progressively more important since the increasing number of pathological diseases and disorders due to oxidative stress made the search for more natural antioxidants important as synthetic antioxidants are found to have toxic effects (Gulcin et al., 2010).

This study thus aimed to quantify the total polyphenol and flavonoid contents of propolis extracts, correlate the extent of antioxidant properties with their total polyphenol and flavonoid contents, and assess its antimicrobial property.

MATERIALS AND METHODS

Sample Collection

Propolis samples were collected from colonies of *T. biroi* located in selected municipalities of Laguna (Los Baños, Alaminos and Sta. Cruz) and Quezon (Lucban, Tiaong and Atimonan). Propolis samples were collected from stingless bee colonies reared in coconut shell. Using a hive tool, the propolis was scraped from the inner portion of the coconut shell. The samples were air-dried and allowed to freeze overnight prior to pulverization through the use of blender. After the samples were pulverized, the material was air-dried for another 12 h prior to extraction using ethanol.

Extraction from Propolis

Polyphenols and flavonoids were extracted from the propolis samples using the method described by Popova et al. (2004). A 50.0 g pulverized propolis was added with 150 mL of 70% ethanol. It was allowed to stand for 7 d at room temperature with constant stirring. The resulting mixture was decanted. The decantate was centrifuged at 2000 rpm for 10 min to completely remove the remaining residues. The supernatant liquid was obtained and labeled as ethanolic extract of propolis. The extracts were concentrated through the use of Buchi RE111 rotary evaporator equipped with Buchi 461 water bath.

Total Polyphenol Content Using Folin-Ciocalteau Colorimetric Method

The total polyphenol content of the propolis ethanolic extracts was determined using the Folin-Ciocalteau colorimetric method described by Singleton et al. (1999) with some modifications. A 0.25 mL propolis extract placed in a volumetric flask was added with 17.5 mL water followed by 1.25 mL 2 N Folin-Ciocalteau reagent. It was then added with 3.75 mL 10% Na_2CO_3 solution and diluted to mark using distilled water. The solution was allowed to stand for 2 h in the dark and then the absorbance was measured at 771 nm. Calibration curve was generated using gallic acid standard solutions ranging from 10 to 120 mg/L. All values were reported as milligram gallic acid equivalent (GAE) per gram of propolis. Total polyphenol content of the propolis extracts was calculated using the formula:

$$Polyphenol\ content\ \left(\frac{mg\ GAE}{g\ propolis}\right) = \frac{\left(concentration, \frac{mg}{L}\right) x\ volume\ of\ sample\ x\ DF}{mass\ of\ propolis\ in\ extract, g}$$

Total Flavonoid Content Using Aluminum Chloride Colorimetric Method

The total flavonoid content of the propolis ethanolic extracts was determined using the aluminum chloride colorimetric method described by Chang et al. (2002) with some modifications. A 1.00 mL propolis extract was mixed with 4 mL methanol followed by 1 mL 10% $AlCl_3$. The mixture was allowed to stand for 5 min. After incubation, the absorbance of the solution was measured at 430 nm. Calibration curve was generated using quercetin standard solutions ranging from 10 to 100 mg/L. All values were reported as milligram quercetin equivalent (QE) per gram of propolis. Total flavonoid content of the propolis extracts was calculated using the formula:

$$Flavonoid\ content\ \left(\frac{mg\ QE}{g\ propolis}\right) = \frac{\left(concentration, \frac{mg}{L}\right) x\ volume\ of\ sample\ x\ DF}{mass\ of\ propolis\ in\ extract, g}$$

Ferric Reducing Ability Power (FRAP) Assay

The ferric reducing ability power of the propolis ethanolic extracts was determined using the method described by Escudero et al. (2008) with some modifications. A 200 µL propolis extract was reacted with 1.5 mL freshly prepared FRAP reagent (0.300 M acetate buffer (pH 3.6), 0.01 M 2,4,6-tris(1-pyridyl)-5-triazine (TPTZ) in 40 mM HCl, and $FeCl_3 \cdot 6H_2O$ mixed at a volume ratio of 10:1:1. It was then mixed thoroughly and incubated for 4 minutes. The absorbance of the solution was measured at 597 nm. Calibration curve was generated using ferrous sulfate standard solutions ranging from 10 to 500 µg/mL. All values were expressed as µmol $FeSO_4 \cdot 7H_2O$ or ferrous sulfate equivalence per gram of propolis. FRAP value for the propolis samples was calculated using the following formula:

$$FRAP\ value\ \left(\frac{mg\ standard}{g\ propolis}\right) = \frac{\left(concentration, \frac{mg}{L}\right) x\ volume\ of\ sample\ x\ DF x\ \left(\frac{1}{MM_{ferrous\ sulfate}}\right)}{mass\ of\ propolis\ in\ extract, g}$$

Hydrogen Peroxide (H_2O_2) Scavenging Activity Assay

The hydrogen peroxide scavenging activity of the propolis ethanolic extracts was determined using the method described by Ruch et al. (1989) with some modifications. A solution of 20 mL 40 mM H_2O_2 was initially prepared in 0.1 M phosphate buffer (pH 7). Then, 0.6 mL of the H_2O_2 solution was mixed with 1000 µL sample (propolis extract or the standard α-tocopherol, gallic acid, and quercetin) in

3.4 mL phosphate buffer. Absorbance of the solution was then measured at 230 nm. Results for the H_2O_2 scavenging capacity of the propolis extract and standard compounds were expressed as the percentage of the scavenged H_2O_2 as shown in the formula:

$$Scavenged\ H_2O_2\ (\%\ RSA) = \left(1 - \frac{A_{\lambda 230-s}}{A_{\lambda 230-c}}\right) x\ 100$$

where:

$A_{\lambda 230-s}$= absorbance of the sample
$A_{\lambda 230-c}$= absorbance of the control (H_2O_2 in buffer solution)

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The DPPH radical scavenging activity of the propolis ethanolic extracts was determined using the method described by Gulcin (2009) with some modifications. A 0.1 mM solution of DPPH was prepared in ethanol. Then, 0.5 mL of this solution was added to 1.5 mL of sample (propolis extract or the standard α-tocopherol, gallic acid, and quercetin). The solution was mixed through a vortex mixer and incubated in the dark. After 30 min, absorbance of the solution was measured at 517 nm. Percentage DPPH scavenging capacity of the propolis extract and standard compounds was calculated using the following equation:

$$Scavenged\ DPPH\ (\%\ RSA) = \left(1 - \frac{A_{\lambda 517-s}}{A_{\lambda 517-c}}\right) x\ 100$$

where:

$A_{\lambda 517-s}$= absorbance of the sample
$A_{\lambda 517-c}$= absorbance of the control (DPPH in ethanol)

***In Vitro* Determination of Antimicrobial Property of Propolis**

Antimicrobial analysis was performed using disc diffusion method in accordance with the test reference under United States Pharmacopoeia 30-NF 25, 2007 <87> Biological Reactivity Tests, *in vitro*. Inoculate was prepared by initially growing 24 cultures of each test organism (*Staphylococcus aureus* Rosenbach and *Escherichia coli* (Migula) Castellani and Chalmers) in separate tubes, followed by immediate inoculation of 10 mL tryptic soy broth. Tubes were then incubated at 35°C for 24 h.

Inoculum with volume equal to 0.2 mL was transferred into small Petri dish followed by addition of 15 mL melted nutrient agar. It was mixed well, until a uniform distribution of inoculum was observed, and allowed to solidify. Paper disc (10 mm) was dipped in propolis extract and excess liquid was removed by tapping it into the sides of the container. It was then gently overlaid on the surface of the prepared agar. The same procedure was repeated for all the extracts and negative control (ethanol). Plates were then incubated at 35°C for 24 h prior to observing clear zones, measuring the diameter of the zone of inhibition in millimeters, and determining the reactivity of propolis extracts toward the test organisms.

Statistical Analyses

The resulting values were presented as mean ± standard deviation of three determinations and statistically analyzed using SPSS (version 16.0, SPSS Inc). Statistical analyses employed in this study are: One-way analysis of variance (ANOVA) and Tukey's honestly significant difference test for all pairwise comparisons. Results with $p < 0.05$ were regarded as significant.

RESULTS AND DISCUSSION

Total Polyphenol (TP) and Total Flavonoid (TF) Content

Ethanolic extracts of propolis produced by *T. biroi* obtained from selected municipalities in Laguna and Quezon were found to have a distinct aromatic odor and varying intensities of amber-like color. Extracts from Tiaong exhibited the lightest color while those from Lucban and Atimonan were the darkest (Figure 1). One reason for the observed difference is the complexity of chemical composition of each extract (Bankova et al., 1999). A darker color of the extract is an implication that there is a greater number of biologically active components such as polyphenols and bioflavonoids present in the sample. This was supported by the calculated values for the TP and TF of each extract (Table 1).

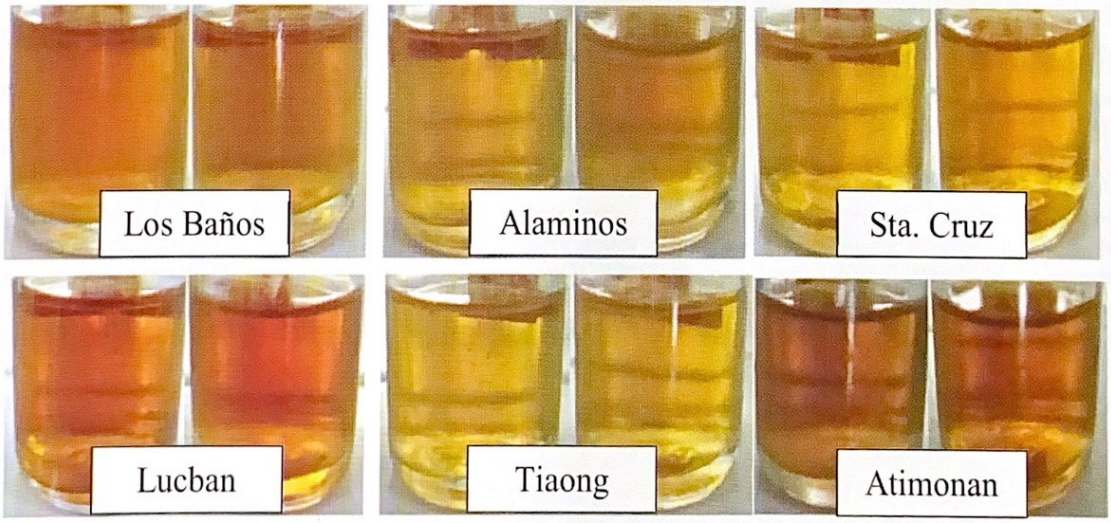

Figure 1. Ethanolic extracts of propolis samples obtained from Laguna (upper row) and Quezon (lower row), Philippines.

Table 1. Total phenolics (TP) and total flavonoid (TF) contents of propolis extracts produced by *Tetragonula biroi* obtained from selected municipalities in *TP and TF values were expressed as mg GAE/ g propolis and mg QE/ g propolis, respectively.

Province	Municipality	TP*	Average TP*	TF*,**	Average TF*,**
Laguna	Los Baños	269.16 ± 8.95	255.22 ± 14.75	2.34 ± 0.32^{c}	1.91 ± 0.42^{b}
	Alaminos	239.76 ± 7.93		1.51 ± 0.03^{d}	
	Sta. Cruz	256.73 ± 3.65		1.87 ± 0.08^{cd}	
Quezon	Lucban	387.45 ± 14.15	308.04 ± 119.55	6.43 ± 0.04^{a}	5.30 ± 1.43^{a}
	Tiaong	170.54 ± 6.27		3.69 ± 0.19^{b}	
	Atimonan	366.12 ± 16.15		5.77 ± 0.27^{a}	

*TP and TF values were expressed as mg GAE/ g propolis and mg QE/ g propolis, respectively.
**Values in the same column followed by the same letter are not significantly different by Tukey's honestly significant difference test ($p < 0.05$).

The TP values ranged from 170.54 ± 6.27 to 387.45 ± 14.15 mg GAE/ g propolis while the TF values ranged from 1.51 ± 0.03 to 6.43 ± 0.04 mg QE/ g propolis. Differences in the TP values among samples were found to be insignificant. TF values, on the other hand, were significantly different among samples ($p < 0.05$).

Samples from Lucban and Atimonan had the highest TF. The observed differences in the TF composition of the propolis samples can be attributed to several factors such as geographical location, vegetation or the local flora present in the collection site, and the season during sample collection. Considering the geographical location of the municipalities where propolis were collected, samples obtained from Lucban, Los Baños and Alaminos were expected to have the higher TP and TF because these areas were located near mountains, namely: Mt. Banahaw and Mt. Makiling. However, slight deviations from the expected results were obtained. Atimonan propolis samples exhibited a higher TF compared to Los Baños and Alaminos, mainly because of the vegetation and season during the sample collection. Comparing the vegetation present in Alaminos and Atimonan, the availability of plants where resins were collected by stingless bees is greater in the area of Atimonan compared to Alaminos. The apiary in Alaminos was located in an area surrounded by residential houses and not by plants which can be a good source of resins. Considering the season of collection, propolis samples from Atimonan and Los Baños were collected during the month of December and May, respectively. According to Finstorm and Spivak (2010), propolis does not only protect bees against microbial growth, it also serves as a proof for uncontrolled air flow inside the hive. Given that the vegetation around two of the sampling sites were comparable, it would be logical that the TF of Atimonan samples would be higher. During cold season, more insulation is needed by bees. As the demand or the need for propolis in the hive increases, the frequency of resin collection would also increase. This would then contribute to the complexity of the components of the propolis made by bees.

Propolis samples obtained from Alaminos exhibited the lowest value (numerically) of TF even if the geographical location, vegetation or local flora in the collection site and season during sample collection were considerably good. This can be mainly due to the low quality of propolis samples gathered which is a result of the weak colony inhabiting the hive. It was observed that even though the requirement for propolis is high because of the cold season, colonies were not proactively gathering resins for propolis production because of continuous rainfall. In addition, most of the hives were infested with ants and wasps which also contribute to the weakening of the colonies.

Antioxidant Property of Propolis Extracts

Antioxidants are defined as any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of the said substrate. In biological systems, Antioxidant capacity is a measure of the ability of a certain compound to inhibit or eliminate the damage caused by the unwanted reactive oxygen species naturally present in the cell (Miguel et al., 2011). Antioxidant capacity is considered as the best described property of every group of polyphenols and flavonoids. The following assays support the values obtained for TP and TF.

Ferric reducing ability power (FRAP) assay

FRAP assay, as described by Escudero et al. (2008), was used to assess the antioxidant activity of the propolis samples. The FRAP values of propolis extracts are shown in Table 2.

Table 2. Ferric Reducing Ability Power (FRAP) values of propolis extracts produced by *Tetragonula biroi* obtained from selected municipalities in Laguna and Quezon, Philippines.

Province	Municipality	FRAP values*	Average
Laguna	Los Baños	18.44 ± 0.15^{c}	5.21 ± 3.08^{b}
	Alaminos	12.29 ± 0.07^{e}	
	Sta. Cruz	14.90 ± 0.16^{d}	
Quezon	Lucban	42.97 ± 0.12^{a}	24.22 ± 16.88^{a}
	Tiaong	10.23 ± 0.09^{f}	
	Atimonan	19.47 ± 0.40^{b}	

*FRAP values were expressed in µmol $FeSO_4 \cdot 7H_2O$/ g propolis.

The FRAP values ranged from 10.23 ± 0.09 to 42.97 ± 0.12 µmol Fe^{2+}/g propolis. There were significant differences in the FRAP values of propolis extracts. Highest FRAP value was exhibited by the propolis sample obtained from Lucban, Quezon. Differences in the observed value for the FRAP assay of propolis extracts can be attributed to the composition of the samples and rate of redox reaction. Chemical composition, specifically the flavonoid content of the samples, affects the FRAP value exhibited by the extracts. Higher value of TF gives higher FRAP value. Rate of redox

reaction, on the other hand, greatly affects the FRAP especially if the sample has high concentrations of slow-reacting polyphenols which require longer reaction time for detection.

Scavenging activity assay

Scavenging activities of three positive controls (α-tocopherol, quercetin and gallic acid) and the propolis samples were determined through HPSA and DPPH assays. The calculated IC_{50} (concentration of inhibitor producing 50% inhibition) values of the positive controls and propolis extracts are shown in Tables 3 and 4, respectively.

Table 3. Calculated values for IC_{50} of tocopherol, quercetin and gallic acid determined through HPSA and DPPH assay.

Control	HPSA IC_{50} values*	DPPH IC_{50} values*
α-tocopherol	12.54	15.97
quercetin	11.76	17.59
gallic acid	15.16	18.36

*IC_{50} values were expressed in µg/mL.

Table 4. Calculated values for IC_{50} of propolis extracts produced by *Tetragonula biroi* obtained from different municipalities in Laguna and Quezon, Philippines using HPSA and DPPHRSA assays.

Province	Municipality	HPSA IC_{50} values*	Average HPSA IC_{50} values*	DPPH IC_{50} values*	Average DPPH IC_{50} values*
Laguna	Los Baños	11.26 ± 0.05	12.73 ± 1.45	14.34 ± 0.18	15.49 ± 1.13
	Alaminos	14.15 ± 0.37		15.53 ± 0.32	
	Sta. Cruz	12.79 ± 0.26		16.60 ± 0.15	
Quezon	Lucban	9.77 ± 0.01	11.39 ± 2.33	12.61 ± 0.06	14.94 ± 3.49
	Tiaong	14.06 ± 0.15		18.95 ± 0.05	
	Atimonan	10.35 ± 0.08		13.24 ± 0.46	

*IC_{50} values were expressed in µg/mL.

A lower IC_{50} value indicates a stronger "antioxidant power". This is due to the fact that a low value for the said parameter means that a lower concentration of the antioxidant is required to effectively scavenge 50% of the whole population of the radical or the substrate. Polyphenols greatly affect the scavenging activity.

The HPSA IC_{50} values of the samples ranged from 9.77 ± 0.01 to 14.15 ± 0.37 µg/mL and the DPPH IC_{50} values ranged from 12.61 ± 0.06 to 18.95 ± 0.05 µg/mL. Differences in the IC_{50} values for both HPSA and DPPH among samples were found to be insignificant. Compared to the IC_{50} values of the positive controls, the results imply that the samples definitely have significant H_2O_2 and DPPH radical scavenging activities.

Antibacterial Property of Propolis Extracts

As confirmation of the values obtained in the chemical tests, *in vitro* antibacterial property of the extracts showed positive results. *In vitro* test showed no inhibitory action in the control and inhibition zones were formed in all test samples. Reactivity was mild indicating that the inhibitory effect was only limited under the specimen. The reactivity was similar for both *E. coli* and *S. aureus* (Table 5).

Table 5. Inhibitory activity of various propolis extracts from different municipalities in Laguna and Quezon, Philippines, against *Escherichia coli* and *Staphylococcus aureus*.

Province	Test Sample (Propolis Extract)	*Escherichia coli*			*Staphylococcus aureus*		
		Total mean zone of inhibition (mm)	Reactivity*	Inhibitory activity	Total mean zone of inhibition (mm)	Reactivity*	Inhibitory activity
-	**negative control**	0	0	(-)	0	0	(-)
Laguna	**Los Baños**	10	2	++	10	2	++
	Alaminos	10	2	++	10	2	++
	Sta. Cruz	10	2	++	10	2	++
Quezon	**Lucban**	10	2	++	10	2	++
	Tiaong	10	2	++	10	2	++
	Atimonan	10	2	++	10	2	++

*Reactivity ratings: 0-none (no detectable zone around or under specimen), 1-slight (some malformed or degenerated cells under specimen), 2-mild (zone limited under the specimen), 3-moderate (zone extends to 10 mm beyond specimen), 4-severe (zone extends greater than 10 mm beyond specimen)

These findings would imply that the zone of inhibition (10 mm) was limited under the specimen only and few bacterial growths were observed within the zone of inhibition. Photographs of the Petri dishes used in this analysis are shown in Figures 2 and 3.

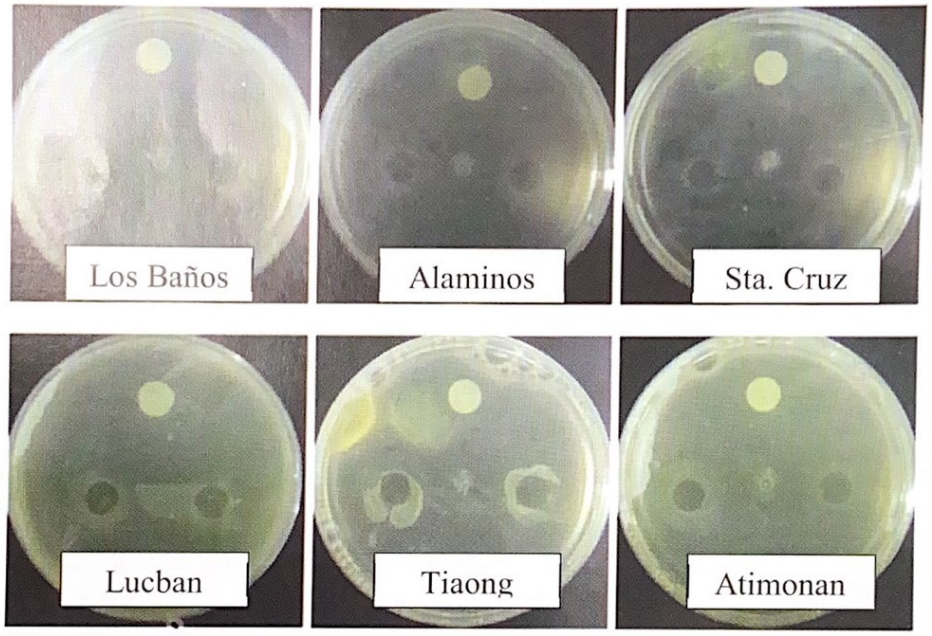

Figure 2. Zone of inhibition of propolis samples collected from different municipalities in Laguna (upper row) and Quezon (lower row), Philippines, against *Escherichia coli.*

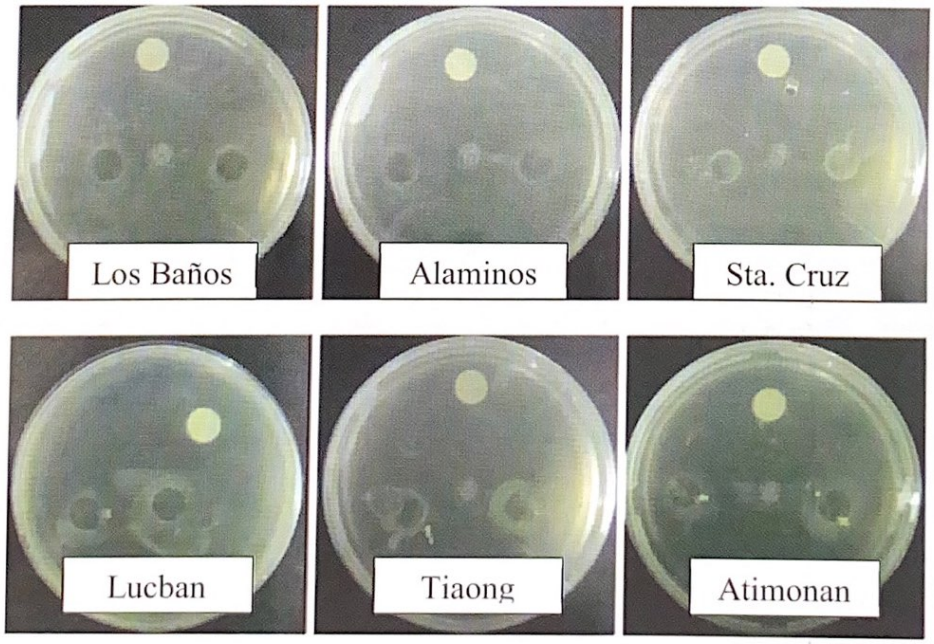

Figure 3. Zone of inhibition of propolis samples collected from different municipalities in Laguna (upper row) and Quezon (lower row), Philippines, against *Staphylococcus aureus.*

A similar observation was observed by Najafi et al. (2007). They found that propolis from Iran and China also showed inhibitory action toward *S. aureus* although comparisons can only be made on a qualitative level as they did not provide a numerical value for the zone of inhibition. On the other hand, the antibacterial activity of the Laguna and Quezon samples was relatively weaker than that observed by Katircioglu and Mercan (2006) from their Turkey propolis samples. They have recorded eight mm radius zone of inhibition beyond the specimen of *S. aureus*. The relatively weak antibacterial activity of Laguna and Quezon samples can be explained by the concentration of the propolis extract used. The samples were not subjected to rotary evaporation, thus, a lot of solvent used in extraction was still present. However, even with such low concentration, the extracts still exhibited a level of antibacterial property.

CONCLUSION

Total phenolics and total flavonoids of the ethanolic extracts of *T. biroi* (Friese) propolis obtained from Laguna and Quezon were determined using Folin-Ciocalteau and Aluminum Chloride colorimetric methods, respectively. The antioxidant activities were determined using Ferric Ion Reducing Antioxidant Power (FRAP), Hydrogen Peroxide Scavenging Activity (HPSA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Statistical analyses showed that the samples only differed significantly in TF and FRAP values. All propolis samples showed the same partial inhibitory activity and reactivity against the gram-negative *E. coli* and gram-positive *S. aureus* using the disc diffusion method

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