



## Preliminary Screening for Various Bioactivities in Honey and Propolis Extracts from Thai Bees

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### ABSTRACT

**Aims:** Honey and propolis have long been used in traditional medicine whilst honey is consumed as food. A screening for various bioactivities in honey from *Apis florea* and *A. andreniformis*, and the crude water and ethanol extracts of propolis from *A. mellifera* and *Tetragonula laeviceps*, from Thailand are reported.

**Study Design:** Cell based study.

**Place and Duration of Study:** Department of Biology, Faculty of Science, Chulalongkorn University, between June 2010 and April 2011.

**Methodology:** Various components such as protein, sugar, gluconic acid were assayed in honey while total sugar, reducing sugar, total polyphenol and flavonoid content were assayed in crude propolis. Samples were tested for *in vitro* antimicrobial, *in vitro* antiparasitic and antiproliferative activities.

**Results:** The crude propolis extracts showed good bioactivities. Antibacterial activity was found against *Bacillus cereus* (a model Gram-positive bacteria) in the water extracts of propolis from *T. laeviceps* (TLW) and *A. mellifera* (AMW), with MIC values of 50 and 100 µg/ml, respectively, whilst against *Escherichia coli* (a model Gram-negative bacteria), TLE

revealed some 24.0% growth inhibition. Most interestingly, the ethanol extract of propolis from *T. laeviceps* (TLE) displayed a strong anti-malarial activity with a MIC of 4.48 µg/ml against *in vitro Plasmodium falciparum* growth, whilst AMW revealed a high inhibition of *Mycobacterium tuberculosis* growth (74.3%). Furthermore, TLW (50 µg/ml) provided the highest anti-Herpes Simplex Virus type 1 replication activity at 33.0% without any sign of cytotoxicity to the host Vero cells. Finally, *in vitro* anti-proliferation activity against four cancer cell lines in tissue culture was noted with IC<sub>50</sub> vales ranging between 25.5 - 29.3 and 26.8 – 49.5 µg/ml for TLE and AME, respectively.

**Conclusion:** Overall, the propolis of Thai *A. mellifera* and *T. laeviceps* exhibit diverse and some novel bioactivities worthy of further enrichment and characterization.

*Keywords:* Honey; propolis; anti-malarial; antiproliferation; antimicrobial activity.

## 1. INTRODUCTION

Propolis and honey are economically viable bee products that can be of significant importance to local communities. Propolis is variable in appearance but is typically a dark brown and sticky substance that is mainly derived from plant resins, whilst honey is a bee-processed form of plant nectar using  $\alpha$ -glucosidase. Since ancient times, propolis has been used as glue and a general-purpose sealer due to the relatively high wax content of its composition, and honey has long been consumed as food due to its high proportion of monosaccharides. In addition, both have been reported to provide bioactivities useful in traditional or alternative medicine. For example, both propolis and honey are reported to have anti-proliferative (Pichichero et al., 2010; Valente et al., 2011), anti-bacterial (Koru et al., 2007; Sherlock et al., 2010), anti-viral (Al-Waili, 2004; Diaz-Carballo et al., 2010), anti-oxidative (Giorgi et al., 2011; Gulcin et al., 2010), anti-diabetic (Al-Waili, 2004; Kang et al., 2010) and anti-inflammatory (Ahmad et al., 2009; Sforcin, 2007) activities, amongst others. When analyzed by gas chromatography-mass spectrophotometry (GC-MS), high-performance liquid chromatography (HPLC) and capillary zone electrophoresis, the main components directly involved in the bioactivities mentioned above were found to be flavonoids (which could be divided into the three classes of flavonols, flavones and flavanones) and phenolic acids and their derivatives (Bankova, 2005).

Hegazi et al., (2000) reported that aromatic acids and carbonic acids with a benzoic ring in the aliphatic chain displayed potent antimicrobial activity, especially against *Bacillus subtilis* and *Pseudomonas aeruginosa*. In addition, caffeic acid phenethyl esters (CAPE) showed the strongest antiproliferative activity. Other than CAPEs, galangin, xanthomicrol and chrysin also showed good antiproliferative activity (Hernandez et al., 2007). In addition, the higher antioxidant activity in Turkish honey was found to be related to the levels of polyphenolics in it (Kucuk et al., 2007).

Honey and propolis bioactivities are not only due to the presence of compounds from the chemical groups mentioned above, but also to other factors that play an important role. For instance, in honey, the antimicrobial activity is also due to the reduced water activity ( $a_w$ ) that results from the high osmolarity, and the presence of hydrogen peroxide, glucose oxidase and catalase, as well as the low  $p^H$  (Effem, 1988; Weston, 2000). It has also been reported

that non-peroxide factors, such as lysozyme, play an important role in the antimicrobial activity (Snowdon and Cliver, 1996).

Typically, honey is widely consumed raw and neat, although it is also consumed in lesser amounts after cooking or after dilution and fermenting, and so most research has focused upon the bioactivities of raw honey (Basualdo et al., 2007; Kucuk et al., 2007; Silici et al., 2010). In contrast, propolis cannot be used in its nascent form due to the mass of inedible materials, such as wax, in its composition, but can be subjected to various solvent extractions. Research into the bioactivity and composition of propolis extracts have suggested that the extraction methods are likely to influence the resultant bioactivities, as to be expected given the range of chemical structures and thus polarities. Indeed, various organic solvents have been used in order to solubilize different compounds (Koru et al., 2007, Li et al., 2010; Najafi et al., 2007).

The bioactivities of both propolis and honey depend mainly on the vegetation at the collection sites, the season in which it was collected, geography and other factors (Basualdo et al., 2007; Choi et al., 2006; Koru et al., 2007; Kucuk et al., 2007). However, there has been no evaluation of the bioactivities from bee products in Thailand. In this research, we report the nutritional composition and various bioactivities, such as the *in vitro* inhibition of proliferation of cancer cells, *Plasmodium falciparum* (malaria) and Herpes simplex virus, as well as the anti-microbial activity found in raw honey and in the water and ethanolic extracts of propolis from bees in Thailand. The benefit of this work may apply to the pharmaceutical industry and health-food in the future, and may promote the bee industry and increase the income of small scale local bee farmers in Thailand.

## **2. MATERIALS AND METHODS**

### **2.1 Honey Collection**

Honey from *Apis florea* was collected from an apiary in Samut Songkram province while honey from *A. andreniformis* was collected from a wild forest in the Kanchanaburi province in June, 2010. Samples were stored at RT until use. Propolis from *A. mellifera* hives was collected from an apiary in Lopburi province, whilst that from *Tetragonula laeviceps* was collected from an apiary in Samut Songkram province in June, 2010.

### **2.2 Propolis Extraction**

The propolis from *A. mellifera* and *T. laeviceps* was extracted by water and ethanol according to the method of Najafi et al., (2007) and diluted where applicable into DMSO (ethanolic extracts) or water (aqueous extracts).

### **2.3 Determination of Components in Honey**

Gluconic acid levels were determined according to method of Mullin and Emmons (1997). The protein, fat, ash, crude fiber, invert sugar and reducing sugar levels, as well as the acidity, were determined according to the AOAC methods (A.O.A.C., 2005) 991.20, 989.05, 938.08, 978.10, 923.09, 923.09 and 935.57, respectively. The total carbohydrate, total calories and calories from fat were determined by calculation following the methods outlined in AOAC (A.O.A.C., 2005). Glucose and fructose levels were determined by the AOAC

method 982.14 (A.O.A.C., 2006), whilst moisture was evaluated according to AOAC method 925.45 (A.O.A.C., 2008).

## **2.4 Determination of Four Groups of Components in Crude Propolis**

Total sugar was measured by the phenol/sulphuric acid method (Dubois et al., 1956), and reducing sugar by the dinitrosalicylic acid method (Miller, 1959). The total polyphenol and flavonoid contents were determined by the Folin-Ciocalteu colorimetric method (Singleton et al., 1999) and the method of Woisky and Salatino (Woisky and Salatino, 1998), respectively.

## **2.5 Bioactivity Assay**

### **2.5.1 In Vitro antimicrobial activity**

Anti-microbial activity was evaluated by two methods. Firstly, the agar well diffusion method (Perez et al., 1990) was performed with honey: water (v/v) ratios of 1:0, 3:1, 1:1, 1:3 and 0:1 (solvent control), added at 200 µl to the central hole (1 cm diameter) in 11 cm LB-agar plates. After culturing the seeded test bacterial suspension for 4 hr, the inhibition zone (in cm) was measured. The bacterial strains used were the Gram-negative *Escherichia coli* and the Gram-positive *Staphylococcus aureus*. The second anti-microbial assay was performed using the resazurin microplate assay (Webster et al., 2010), using the same *E. coli* and *S. aureus* strains plus the Gram-positive *Bacillus cereus* and, as a model yeast pathogen of humans, *Candida albicans*.

The anti-*Mycobacterium tuberculosis* assay was performed using the standard green fluorescent protein microplate assay as reported (Changsen et al., 2003), using the *M. tuberculosis* strain H37Ra. Finally, the anti-HSV-1 (Herpes simplex virus type I) proliferation assay was performed by the green fluorescent protein-based assay as reported (Dixon et al., 2009; Hunt et al., 1999).

### **2.5.2 In Vitro antiplasmodial assay**

The screening of the samples for potential ability to inhibit the growth of *Plasmodium falciparum* strain K1, as an assay for anti-malarial activity, was performed using the microculture radioisotope technique as reported (Desjardins et al., 1979).

### **2.5.3 Antiproliferative activity**

The presence of potential anti-cancer cell specific proliferation or cytotoxic activity was screened for *in vitro* using the resazurin microplate assay as reported (Webster et al., 2010), against three cancers derived cell lines in tissue culture. The selected cancer cell lines were the MCF7-breast cancer, KB-oral cavity cancer and the NCI-H187-small cell lung cancer. Furthermore, human leukemia cell line HL-60 was likewise screened as above except using the luminescent based ATP detection assay (ATPLite assay system, Perkin Elmer, cat.# 6016943) as described.

### 3. RESULTS AND DISCUSSION

#### 3.1 Results

##### 3.1.1 Principal nutritional composition of the honey and propolis extracts

The principal as detected components in the *A. florea* and *A. andreniformis* honey are summarized in Table 1. In general, the values of all 16 parameters in both types of honey were broadly similar. Both types of honey are relatively very acidic ( $p^H$  3.8 and 4.3 in *A. florea* and *A. andreniformis*, respectively), which likely helps in the antimicrobial activity. Furthermore, if considering the total calorific value, honey is a good source for providing energy (297.5 and 274.5 kcal / 100 g for *A. florea* and *A. andreniformis* honey, respectively) but is not a good source for fat (0.00 and 0.03% in *A. florea* and *A. andreniformis* honey, respectively) or crude fiber (0.00% in both type of honey), whilst the carbohydrate is essentially monosaccharides.

Normally, the ash content and the color of honey are related, with light-colored honeys having an ash content of below 0.1% (w/v) (Gomez Pajuelo, 1995). Here, the percentage of ash in the honey from *A. florea* and *A. andreniformis* were fairly high at 1.35 and 0.81% (w/v), respectively, which is congruent with the dark color of both types of honey.

**Table 1. Components in Thai *A. florea* and *A. andreniformis* honey**

Properties	<i>A. florea</i>	<i>A. andreniformis</i>
Moisture % (v/v)	24.30 ± 0.02	30.60 ± 0.18
Protein % (w/v)	1.71 ± 0.01	2.05 ± 0.01
Fat % (w/v)	0.00	0.03 ± 0.00
Ash % (w/v)	1.35 ± 0.02	0.81 ± 0.02
Crude fiber % (w/v)	0.00	0.00
Total carbohydrate % (w/v)	72.70 ± 0.10	66.50 ± 0.10
Total calories (Kcal/100 g)	297.50 ± 0.40	274.50 ± 0.33
Calories from fat (Kcal/100 g)	0.00	0.27 ± 0.01
Invert sugar % (w/w)	2.89 ± 0.03	2.42 ± 0.01
Reducing sugar % (w/w)	51.60 ± 0.61	59.30 ± 1.10
Glucose % (w/w)	23.70 ± 0.08	26.50 ± 0.23
Fructose % (w/w)	27.90 ± 0.08	32.80 ± 0.55
Acidity (as lactic acid) % (w/w)	0.92 ± 0.00	0.94 ± 0.03
Gluconic acid % (w/w)	2.46 ± 0.02	2.91 ± 0.02
Proline (µg/µl)	1.80 ± 0.01	2.40 ± 0.02
pH	3.80 ± 0.01	4.30 ± 0.02

Remark: Data are shown as the mean ± 1 S.D. and are derived from 3 replications.

As expected, both types of honey were rich in monosaccharides, principally as glucose followed by fructose, which can be absorbed directly into the digestive tract of the consumer.

The components for raw honey were measured directly, whereas the level of the parameters of the four measured components of propolis, the total sugar, reducing sugar, total polyphenol and flavonoid levels, were measured from the crude water and ethanolic extracts, as outlined in the methods section, and are summarized in Table 2. Reducing sugar was only found in the water extract, as expected for these strongly hydrophilic or polar molecules, and was some 1.5-fold higher in the water extract from *T. laeviceps* propolis than in that from *A. mellifera*. Whilst the amount of total sugars was only slightly higher (1.05-fold) in the *A. mellifera* propolis (water extract) than that in the *T. laeviceps* propolis, they significantly differed in their likely polarity, being mainly found in the water extract in *T. laeviceps* (water: ethanol solubility ratio of 6.06:1 (w/w)), and so presumably mostly polar molecules, compared to almost equally solvated level in *A. mellifera* (water: ethanol solubility ratio of 0.99:1 (w/w)), and thus a likely higher proportion of less polar sugars.

**Table 2. The level of four different types of components found in the water (W) and ethanolic (E) extracts of propolis from *T. laeviceps* (TL) and *A. mellifera* (AM) at one location in Thailand**

Components ( $\mu\text{g/ml}$ )	TLW	TLE	AMW	AME
Total sugar	$1.405 \pm 0.11$	$0.232 \pm 0.24$	$1.472 \pm 0.95$	$1.494 \pm 0.21$
Reducing sugar	$42.35 \pm 0.08$	ND	$63.92 \pm 0.08$	ND
Total polyphenol	$0.574 \pm 0.12$	$16.88 \pm 0.12$	$0.710 \pm 0.12$	$2.818 \pm 0.12$
Flavonoid	$0.044 \pm 0.16$	$0.257 \pm 0.26$	$0.079 \pm 0.16$	$0.661 \pm 0.38$

Remark: The data came from the methods in "Determination of components in crude propolis" of Materials and Methods.

ND represents for no available data.

Data are shown as the mean  $\pm$  1 S.D. and are derived from 3 replications.

As expected given their low polarity, a significantly higher level of total polyphenols were found in the ethanolic extractions of the propolis from both bee species than in the water extraction, although the water: ethanol (v/v) partition ratio varied just over 7.4-fold, being 1:29.4 and 1:3.96 in *T. laeviceps* and *A. mellifera*, respectively, again suggesting potentially different components with different polarities. Assuming the total extraction of all the polyphenols, or at least an equal efficiency between propolis samples was attained, then there is a striking difference in the levels between propolis from the two bee species, with some six-fold higher total ethanolic soluble polyphenols in the propolis from *T. laeviceps* than in that from *A. mellifera*. This contrasts with the flavonoid levels in the propolis extracts, which are higher in *A. mellifera* than in *T. laeviceps* (2.57-fold in the ethanolic extract), although a differential solubility ratio ((w/w) water: ethanol) was also noted between the two types of propolis, being 1:5.84 and 1:8.37 in *T. laeviceps* and *A. mellifera*, respectively. However, the level of total sugars observed in the propolis extract from *A. mellifera*, both in the water and ethanolic extracts, was higher than in that from *T. laeviceps*.

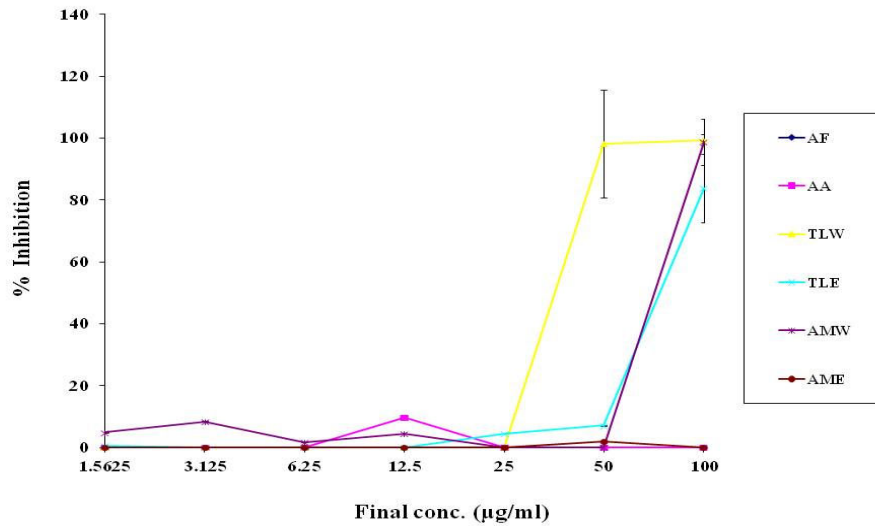
Thus, both the honey and the water and ethanolic extracts of the propolis of these Thai honey bee species have some nutritional benefit and potentially may be of use for consumption.

### **3.1.2 Anti-bacterial activity of the honey and propolis extracts**

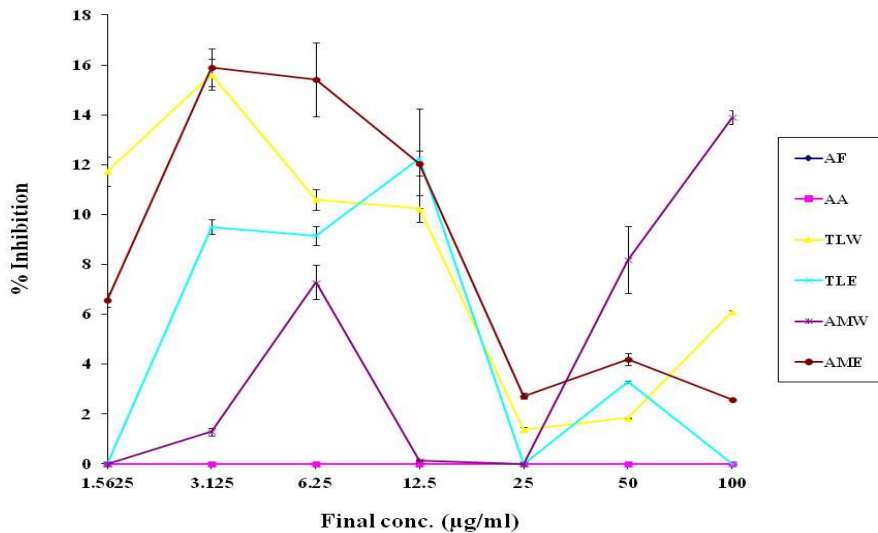
The raw honey from *A. florea* and *A. andreniformis* and of the water and ethanolic extracts of propolis from *A. mellifera* and *T. laeviceps* were diluted to final concentrations of 1.5625,

3.125, 6.25, 12.5, 25, 50 and 100  $\mu\text{g} / \text{ml}$  and then tested for anti-bacterial activity against the Gram-positive *B. cereus* and *S. aureus*, and the Gram-negative bacteria *E. coli* (Figure 1). The honey from both *A. florea* and *A. andreniformis* revealed no significant antibacterial activity against all three tested bacterial isolates (Figure 1a – c), at least within this concentration range of 0 – 400  $\mu\text{g} / \text{ml}$ . Thus, if any such bioactive components are present in honey, unless masked by other components, they are likely to be at low levels relative to the mass of sugar.

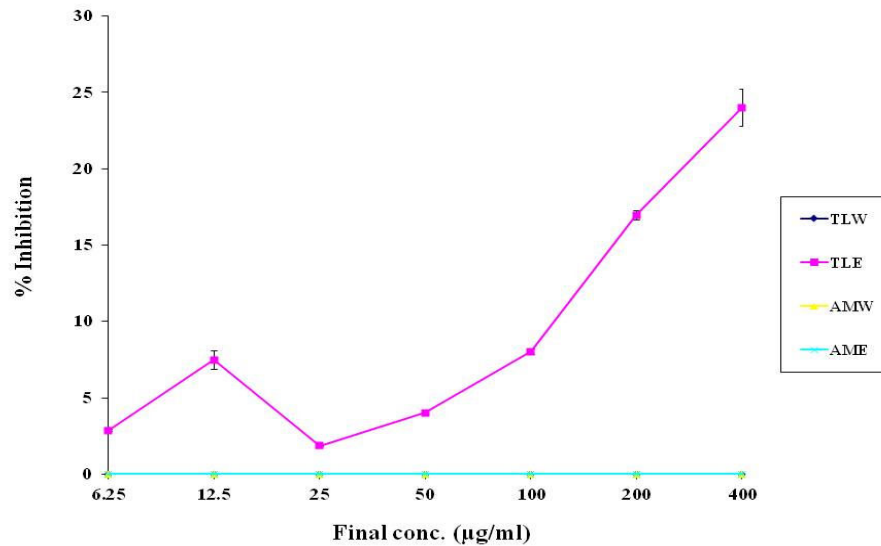
**A**



**B**



C



**Fig. 1. Anti-bacterial activity of the water (W) and ethanolic (E) extracts of propolis from *T. laeviceps* (TL) and *A. mellifera* (AM), and the honey from *A. florea* (AF) and *A. andreniformis* (AA) against (A) *B. cereus*, (B) *S. aureus* and (C) *E. coli***

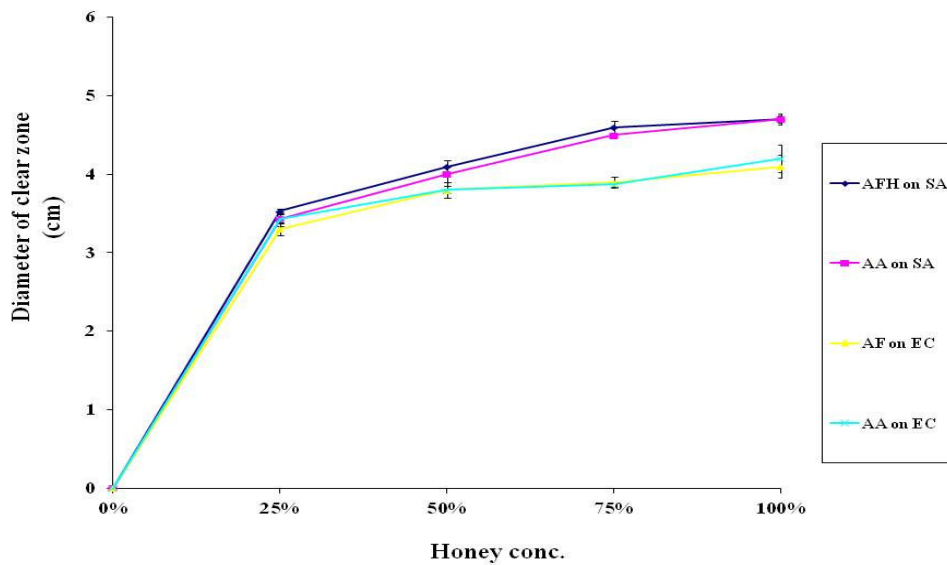
*DMSO* at 1% (v/v) was used as the solvent (negative control) while (A & B) vancomycin or (C) ampicillin was used as a positive control.

Data are shown as the mean  $\pm$  S.D. and are derived from 3 repeats.

In contrast, the four propolis extracts showed at least some antibacterial activity on at least one bacterial isolate, but varied between bacteria, extraction solvent and bee species. The water extract from *T. laeviceps* was the most active in terms of antibacterial activity against the two Gram-positive bacteria, displaying the highest inhibition level (98.2%) and the lowest MIC (50  $\mu\text{g} / \text{ml}$ ) level of *B. cereus* (compared to a MIC of 2  $\mu\text{g} / \text{ml}$  for vancomycin) and the joint highest inhibition level (~16%) and lowest MIC (3.13  $\mu\text{g} / \text{ml}$ ) of *S. aureus* (compared to a MIC of 0.5  $\mu\text{g} / \text{ml}$  for vancomycin). However, no antibacterial activity was noted with this extract against the Gram-negative *E. coli*, for which the control ampicillin showed an MIC of 8  $\mu\text{g} / \text{ml}$ . Thus, overall for all three bacterial isolates tested the ethanolic extract of propolis from *T. laeviceps* was the most active, being the only extract to show any activity against the Gram-negative *E. coli* with an almost biphasic dose-dependent inhibition (initial peak of ~7% at 12.5  $\mu\text{g} / \text{ml}$  and then an increasing inhibition from ~3% to ~24% with increasing doses from 50 to 400  $\mu\text{g} / \text{ml}$ ), and a reasonable inhibition of *B. cereus* (~80% at 100  $\mu\text{g} / \text{ml}$ ) and *S. aureus* (~12% at 12.5  $\mu\text{g} / \text{ml}$ ). Although the apparent sensitivity of the three bacterial strains varied, with the Gram-negative *E. coli* being the least sensitive to these propolis extracts, the two Gram-positive bacteria were markedly different in their sensitivity. *B. cereus* was insensitive to any of the four extracts at concentrations of 25  $\mu\text{g} / \text{ml}$  or below, with inhibition noticed at 50 (97% for one extract) and 100  $\mu\text{g} / \text{ml}$  (at 80 – 97% inhibition for three extracts), whilst inhibition of *S. aureus* was clearly biphasic with sensitivity at low concentrations (0 – 12.5  $\mu\text{g} / \text{ml}$ ) but not at 25  $\mu\text{g} / \text{ml}$  followed by increasing inhibition with increasing propolis extract doses at and above 100  $\mu\text{g} / \text{ml}$  but to a much lower level (~7-16% inhibition).



The honey from both *A. florea* and *A. andreniformis* showed no antibacterial activity against all three tested bacterial strains at the 0 - 400  $\mu\text{g} / \text{ml}$  range (Figure 1), as already mentioned above. It might be possible that the chosen range of concentrations (1.56 - 400  $\mu\text{g} / \text{ml}$ ) was too low to attain a sufficient concentration of the bioactive component(s) if they are very minor components. Thus, the screening of the two honey samples was performed but at much higher concentrations (100, 75, 50 and 25% (v/v) honey diluted in water) using the agar well diffusion method against *S. aureus* and *E. coli*. Honey from both bee species provided broadly similar levels of inhibition to both tested bacteria at a 25% (v/v) dilution (Figure 2). Further increases in the honey concentration caused a slight further increase in the level of inhibition (diameter of the zone of growth inhibition) with both types of honeys showing the same inhibition level as each other at each concentration, but with *S. aureus* being slightly more sensitive than *E. coli*.



**Fig. 2. Diameter (in cm) of the clear growth inhibition zone for *S. aureus* (SA) and *E. coli* (EC) induced by the addition of 200  $\mu\text{l}$  of the indicated concentration of *A. andreniformis* (AA) and *A. florea* (AF) honey**

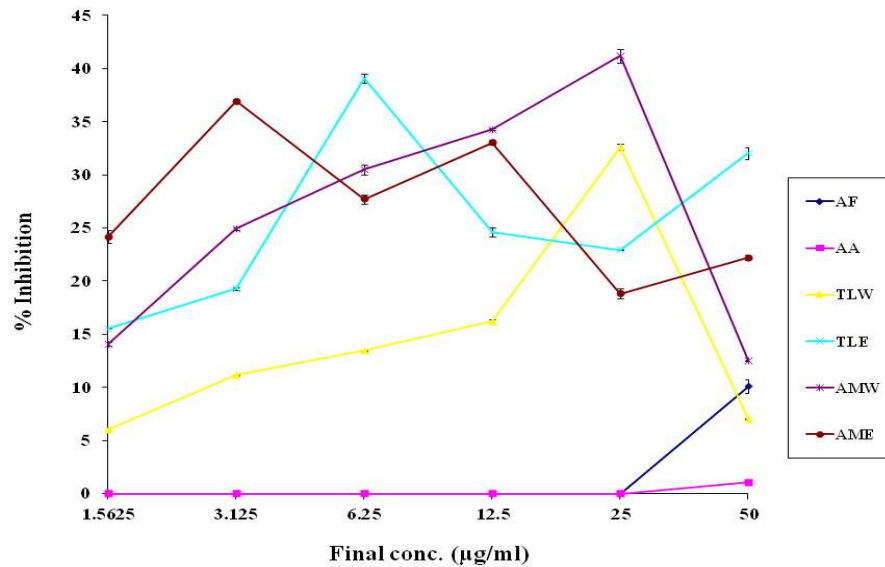
Data are shown as the mean  $\pm$  S.E. and are derived from three replications. Significant differences between means were analyzed by One Way ANOVA (SPSS program) at  $p \leq 0.05$ .

### **3.1.3 Anti-fungal activity of the honey and propolis extracts**

In addition to the two species of Gram-positive and one species of Gram-negative bacteria evaluated above, the potential presence of any antifungal activity in the two honey and four propolis extracts was screened at 0 - 50  $\mu\text{g} / \text{ml}$  against *Candida albicans* as a representative pathogenic yeast. All four propolis extracts revealed moderate maximal inhibition levels (30 - 40%) of *C. albicans* growth (Figure 3), with this being broadly dose-dependent up to a maximal inhibition level at 25  $\mu\text{g} / \text{ml}$  for the water extract of *T. laeviceps* (~31% inhibition) and *A. mellifera* (~41% inhibition). In difference, the ethanolic extracts of *A. mellifera* and *T. laeviceps* propolis showed a maximal inhibition of *C. albicans* at 3.13 and 6.25  $\mu\text{g} / \text{ml}$  (~36 and 37% inhibition), respectively, and then declined at higher doses.

In contrast to the propolis extracts, the two types of neat honey showed either no detectable inhibition of *C. albicans* (*A. andreniformis*), or only a weak inhibition (*A. florea*; <10% at 50 µg/ml and no detectable inhibition below this dose).

Among the four selected microbes in this study (two Gram-positive and one Gram-negative bacteria plus one yeast) that were used to screen for antiproliferative activity, the percentage of growth inhibition, used to determine the degree of antimicrobial activity, is likely to depend on the type and concentration of the test sample, and on the microbial species / isolate screened against, etc.



**Fig. 3. Antifungal activity of the water (W) and ethanolic (E) extracts of propolis from *T. laeviceps* (TL) and *A. mellifera* (AM), and the honey from *A. florea* (AF) and *A. andreniformis* (AA) against *Candida albicans***

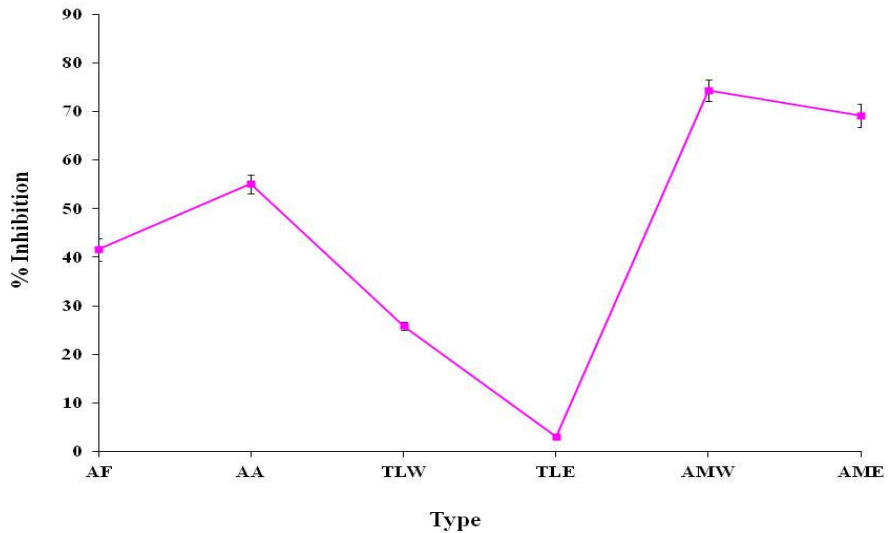
*DMSO* at 0.5% (v/v) was used as a negative control while amphotericin B ( $IC_{50} = 0.030 \mu\text{g} / \text{ml}$ ) was used as the positive control.

Data are shown as the mean  $\pm$  1 S.D. and are derived from 3 replications.

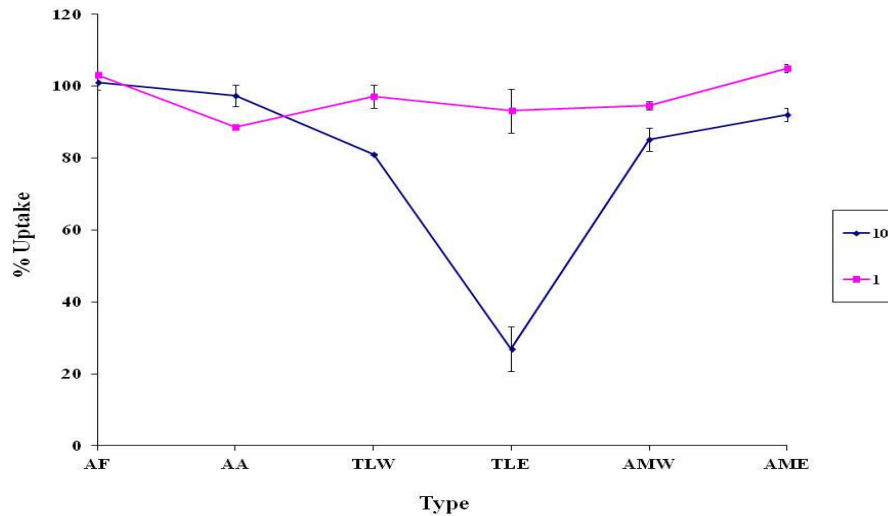
### **3.1.4 Anti-Tb (*Mycobacterium tuberculosis*) activity of the honey and propolis extracts**

To determine the growth inhibition of *Mycobacterium tuberculosis*, the same two honey types and four propolis extracts were evaluated at a final concentration of 50 µg/ml. Surprisingly, a good inhibitory activity against *M. tuberculosis* was revealed, especially that induced by the water and ethanolic extracts of the *A. mellifera* propolis, which yielded 74.3 and 69.1% inhibition of *M. tuberculosis* growth, respectively (Figure 4A).

**A**



**B**



**Fig. 4. Antiproliferation activity of the water (W) and ethanolic (E) extracts of propolis from *T. laeviceps* (TL) and *A. mellifera* (AM), and the honey from *A. florea* (AF) and *A. andreniformis* (AA) against (A) *Mycobacterium tuberculosis* strain H37Ra and (B) *Plasmodium falciparum* strain K1**

(A) DMSO at 0.5% (v/v) was used as the negative control while rifampicin, streptomycin, isoniazid and ofloxacin were used as positive controls with MIC values of 0.003 - 0.012, 0.156 - 0.313, 0.023 - 0.046 and 0.391 - 0.781  $\mu\text{g/ml}$ , respectively. (B) DMSO at 0.1% (v/v) was used as the negative control while dihydroartemisinin was used as the positive control.

Data are shown as the mean  $\pm$  1 S.D. and are derived from 3 replications. Means with a different letter above them are significantly different ( $P < 0.05$ ).

### **3.1.5 Anti-malarial (*Plasmodium falciparum*) activity of the honey and propolis extracts**

Malaria is a debilitating disease caused by parasites in the Genus *Plasmodium* that is widely spread across the tropics, including the often-fatal form in humans caused by *P. falciparum* infection. The main problems of malaria treatment, especially in undeveloped and developing countries, which are indeed those that are mostly at risk, are the high cost medicine and the increasing spread of resistance, including in *P. falciparum*, to available prophylactics. It is thus of importance although challenging to find alternative effective medicines including new prophylactics.

The microculture radioisotope technique is widely used to determine the anti-malarial activity of test compounds and is interpreted by monitoring [<sup>3</sup>H] hypoxanthine uptake as a measure of the parasite growth (Desjardins et al., 1979). In this study honey from *A. florea* and *A. andreniformis*, the water and ethanol extracts from the propolis of *A. mellifera*, plus the water extract of *T. laeviceps* propolis all revealed no significant anti-proliferation activity against *P. falciparum* strain K1 at 10 µg / ml (Figure 4B). However, the ethanolic extract of *T. laeviceps* propolis revealed a significant inhibition of [<sup>3</sup>H] hypoxanthine uptake (6.8% and 73.1% inhibition at 1 and 10 µg / ml, respectively), with an IC<sub>50</sub> value of 4.48 µg/ml. Although this is high compared to dihydroartemisinin (IC<sub>50</sub> = 3.8 nM), the propolis is an unpurified crude extract in contrast to the dihydroartemisinin, and thus *T. laeviceps* propolis is a promising source for anti-malarial activity.

### **3.1.6 Anti-viral (Herpes Simplex Virus Type 1) activity of the honey and propolis extracts**

Viral infections in humans present persistent drug resistance problems compared to other organisms, due in a large part to their high mutation rates, even in DNA viruses. Given that bee products have been linked to reduced viral infections, including herpes viral infections (Nolkemper et al., 2010), we attempted to find out whether the honey and crude propolis extracts exhibited any anti-viral activity against Herpes simplex virus type 1 (HSV-1). None of the four propolis extracts or the two honey types was found to exhibit any detectable cytotoxicity to the viral replication host cell, the Vero cell line (Table 3), and thus there was no significant compounding problem in the assay. Neither honey sample, nor the *A. mellifera* propolis extracts, showed any detectable or significant inhibition of HSV-1 viral replication, but in contrast both the water and ethanol extracts of the *T. laeviceps* propolis showed moderate (33.0%) and weak (18.6%) inhibition. However, whether this reflects a compound(s) with a weak bioactivity and so is of low interest, or an active component(s) with a high specific activity that is at a very low concentration in the crude propolis extraction, awaits bioactivity-directed enrichment.

### **3.1.7 Anti-cancer cell proliferation activity of the honey and propolis extracts**

Currently, cancer is the leading cause of death in humans driving a strong interest in drug development. Here, the MCF7-breast cancer, NCI-H187-small cell lung cancer, KB-oral cavity cancer and human leukemia cell line (HL-60) were used as representative cell lines to screen for any *in vitro* cytotoxicity (HL60) or antiproliferative (other 3 cell lines) bioactivity. The ethanolic extracts of *A. mellifera* and *T. laeviceps* propolis generally yielded the highest level of inhibition of proliferation, whilst the water extracts of both propolis types typically induced the lowest level of proliferation inhibition across the three cell lines (Figure 5A). Both

honey types induced a low (~15 – 30%) level of proliferation inhibition across all three cell lines.

Thus, the highest level of proliferation inhibition was seen with the ethanolic extract of *A. mellifera* propolis on the KB cell line (93% at 50 µg / ml), with an IC<sub>50</sub> value of 26.8 µg/ml, some ~167- and ~64-fold higher than that for the pure doxorubicine and ellipticine (0.16 and 0.42 µg / ml), respectively. Indeed, the ethanolic extracts of *A. mellifera* and *T. laeviceps* propolis induced the next two highest inhibition of proliferation levels on the NCI-H187 cell line at some 80% and 78% inhibition respectively, with IC<sub>50</sub> values (49.5 and 25.5, µg / ml respectively) some 900- and 463-fold lower than pure doxorubicine (0.055 µg / ml) and 110- and 57-fold lower than pure ellipticine (0.450 µg / ml). The MCF7 cell line was somewhat resistant, revealing, for example at a dose of 50 µg / ml, only ~11 – 30% inhibition for all tested samples, whilst the KB cell line was somewhat more susceptible.

However, in terms of the *in vitro* cytotoxicity to the HL-60 cell line in tissue culture the species-source of the propolis was apparently more important. The highest cytotoxicity was induced by the ethanolic, and then the water extract of *T. laeviceps* propolis (67.7% and ~46% inhibition at 50 µg / ml, respectively), with an IC<sub>50</sub> value (29.3 µg / ml) for the ethanolic extract that was some 390- and 9.1-fold lower than that of the control doxorubicine (0.075 µg / ml) and ellipticine (3.22 µg / ml), respectively (Figure 5B). In contrast, the water and ethanolic extracts of the *A. mellifera* propolis were not significantly different from the two types of honey with a weak cytotoxicity of less than 18% at 6.25 µg / ml and at 25 - 50 µg / ml. The double values arise from an apparent reduced cytotoxicity at 12.5 µg / ml for all tested honey and propolis extracts (Figure 5B).

**Table 3. Cytotoxicity against Vero cells (African green monkey kidney) and anti-proliferation activity against HSV-1 (Herpes simplex virus type 1) found in the water (W) and ethanolic (E) extracts of propolis from *T. laeviceps* (TL) and *A. mellifera* (AM), and in honey from *A. florea* (AF) and *A. andreniformis* (AA)**

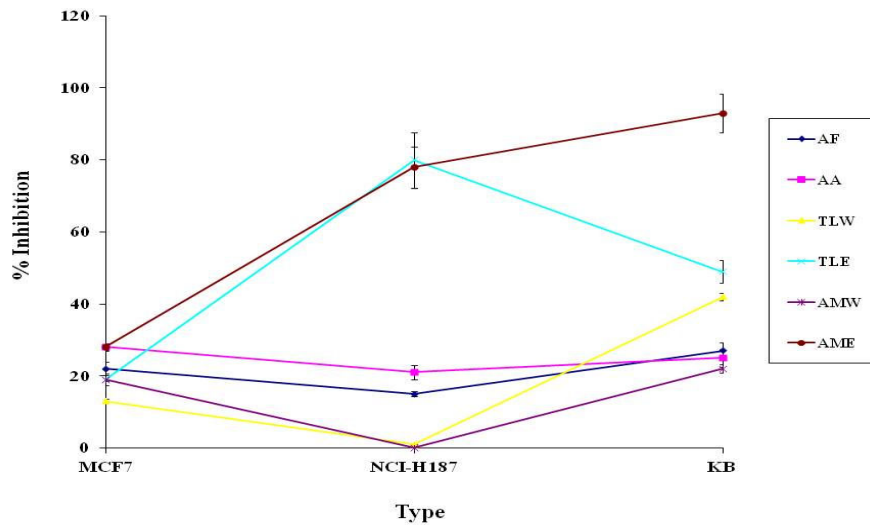
	<b>Cytotoxicity</b>	<b>% Inhibition</b>	<b>IC<sub>50</sub> (µg/ml)</b>
AF	Non-cytotoxic	ND	ND
AA	Non-cytotoxic	ND	ND
TLW	Non-cytotoxic	33.0 ± 1.18	ND
TLE	Non-cytotoxic	18.6 ± 0.97	ND
AMW	Non-cytotoxic	ND	ND
AME	Non-cytotoxic	1.09 ± 0.77	ND
Ellipticine	ND	ND	1.06 ± 0.04
Acyclovir	ND	ND	5.31 ± 0.66

<sup>a</sup>All compounds were tested at a final concentration of 50 µg / ml.

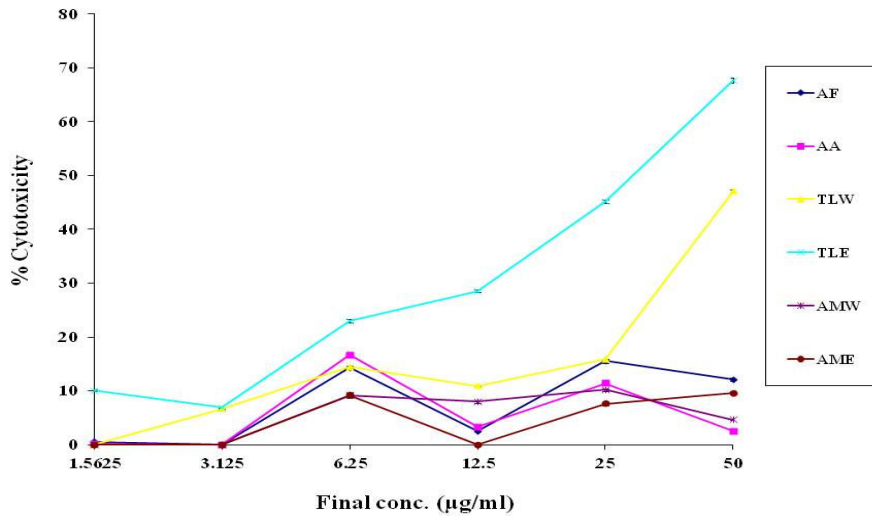
DMSO at 0.5% (v/v) was used as a negative control. Ellipticine and acyclovir was used as the positive control for the cytotoxicity and anti-HSV tests, respectively. ND represents for no available data.

Data are shown as the mean ± 1 S.D. and are derived from 3 replications.

**A**



**B**



**Fig. 5. *In vitro* (A) anti-proliferative activity against the indicated three cancer cell lines and (B) cytotoxic activity against the HL-60 cell line of the water (W) and ethanolic (E) extracts of propolis from *T. laeviceps* (TL) and *A. mellifera* (AM), and the honey from *A. florea* (AF) and *A. andreniformis* (AA).**

The honey and propolis extracts were used at (A) a final concentration of 50 µg/ml or (B) as indicated in the 0 - 50 µg/ml range. DMSO at 0.5% (v/v) was used as a negative control whilst ellipticine and doxorubicine were used as positive controls.

Data are shown as the mean  $\pm$  1 S.D. and are derived from 3 replications.

### 3.2 DISCUSSION

Bee products (honey, royal jelly, pollen and propolis) have been ascribed with several interesting bioactivities (Koc et al., 2011). However, bioactivities depend mainly on external factors including geographical regions (Sawaya et al., 2010). The aim of this study was to screen raw honey from two native Thai bee species, *A. florea* and *A. andreniformis*, and the crude water and ethanolic extracts of propolis from another two bee species, *A. mellifera* and *T. laeviceps* that are widely used in traditional medicine for their potential bioactivities.

Due to the Codex Alimentarius standard (Bogdanov, 1999), the chemical components in the honey from *A. florea* and *A. andreniformis* were assayed in order to check that these two types of Thai honey were of a sufficient quality for human consumption. Almost all the parameters met the standard (Table 1), except for the percentage of moisture, which at 24.3 and 30.6% for the honey from *A. florea* and *A. andreniformis*, respectively, were higher than the maximum level allowed by international regulations of 16 - 23.4% (Finola et al., 2007). The high water content of these two types of honey in this study may, however, simply be due to the fact that they were harvested in the rainy season (June), and so it remains to be evaluated if they would be acceptable in the dry season. Additionally, the degree of hive maturity and climatic factors might play their roles. Ojeda De Rodriguez et al. (2004) reported that the average (w/w) ratio of fructose: glucose in honey is approximately 1.2, in accord with the ratios seen in these two types of honey at 1.18 and 1.24. The higher content of fructose in honey from *A. andreniformis* (32.8%) than *A. florea* (27.9%) will affect the sweetness of the flavor and the granulation of the honey, since glucose is less water soluble than fructose. Manikis and Thrasivoulou (2001) suggested that if the (w/v) ratio of glucose: water is less than 1.7 then a slow crystallization or granulation of the honey will occur. Here, a slow crystallization of both types of honey was observed, in accord with their glucose: water ratio being 0.98 and 0.87 in *A. andreniformis* and *A. florea*, respectively.

The propolis extracts of *T. laeviceps* and *A. mellifera*, and especially the honey samples from *A. florea* and *A. andreniformis* induced only a relatively low to moderate level of inhibition of the tested pathogenic microorganisms at relatively high doses. Indeed, only the water extracts of *T. laeviceps* and *A. mellifera* propolis showed any appreciable inhibition (MIC of 50 and 100  $\mu\text{g} / \text{ml}$  against *B. cereus*) (Figure 1). However, the obtained data does suggest that there is an inhibitory tendency of these samples. Given that the bioactive compounds may be very dilute in these crude products or extractions, they may still be a new source for the development of antibiotic agents, assuming the development of viable enrichment protocols. Certainly this data is consistent with Koru et al. (2007), that Gram-positive bacteria (both aerobes and anaerobes) are the most sensitive to antibiotic agents.

Interestingly, propolis was found to be highly active against malaria, *Plasmodium falciparum*. This is the first such report of this activity in bee products to our knowledge. According to the WHO (WHO, 2008), a sample is deemed to be highly active, promising or of moderate anti-plasmodial activity if the  $\text{IC}_{50}$  is less than 5  $\mu\text{g} / \text{ml}$ , 5 - 15  $\mu\text{g}/\text{ml}$  or 15 - 50  $\mu\text{g}/\text{ml}$ , respectively, whilst samples with an  $\text{IC}_{50}$  value higher than 50  $\mu\text{g}/\text{ml}$  are classified as inactive. Here the ethanolic extract of propolis from *T. laeviceps* had an  $\text{IC}_{50}$  value of 4.48  $\mu\text{g} / \text{ml}$  and would, therefore, be classified as highly active for anti-plasmodial activity. Malaria has long been a major public health problem, especially in tropical regions like Thailand, and increasing resistance to existing prophylactics is a spreading problem that limits both drug effectiveness and increases the cost (WHO, 2008). Finding new potential prophylactics, including from native medicinal natural products is, therefore, of some importance.

At present, chemoprevention is a rapidly growing area of oncology which focuses on the prevention of cancer by using both natural and synthetic agents. The results of this study are not incongruent with the notion that propolis seems to be a possible source for a new natural chemo preventative agent. The cytotoxicity tests to normal cells revealed no evidence of cell cytotoxicity to the non cancer derived Vero cell line but they were cytotoxic to the human leukemia cell line (HL-60).

Overall the ethanolic extracts of propolis from these Thai *A. mellifera* and *T. laeviceps* provided the highest percentage of inhibition of various human pathogens. Li et al. (2010) reported that the flavonoid content of propolis plays a very important role in bioactivities, whilst Birt et al. (2001), reported epidemiological and preclinical evidences to support that polyphenols isolated from propolis could possess chemopreventative properties of cancer. This accords with the results presented here that the ethanolic extracts of propolis from *T. laeviceps* and *A. mellifera* were shown to contain a relatively high total polyphenol (16.88 and 2.82 µg/ml, respectively) and reasonable flavonoid (0.26 and 0.66 µg/ml, respectively) levels. The higher contents in the ethanol extracts of propolis than in the water extracts of the same propolis is expected given the low polarity of these compounds.

In the future, the bioactivity directed enrichment / purification of the active compounds, and further screening of their bioactivities, from the raw honey and the propolis extracts should be performed in order to remove the inert compounds as well as any compounding interactions in order to determine their full potential. That may yield a more suitable phytomedicine for local use.

#### **4. CONCLUSION**

Propolis and honey from Thai bees presented the interesting bioactivities, especially the antiplasmodial activity. The best bioactivity in each assay was found with a different extraction method for the propolis and / or a different bee species of the test product. Thus, it is likely that the active compounds varied and depended on the sample type and bee species, plant sources and potentially season, the condition of the samples and extraction solvent used, amongst other factors.

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#### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.



## REFERENCES

- A.O.A.C. (2005). Official Methods of Analysis of the AOAC International. Association of Official Analytical Chemists, Washington, D.C.
- A.O.A.C. (2006). Official Methods of Analysis of the AOAC International. Association of Official Analytical Chemists, Washington, D.C.
- A.O.A.C. (2008). Official Methods of Analysis of the AOAC International. Association of Official Analytical Chemists, Washington, D.C.
- Ahmad, A., Khan, R.A., Mesaik, M.A. (2009). Anti inflammatory effect of natural honey on bovine thrombin-induced oxidative burst in phagocytes. *Phytother. Res.*, 23, 801-808.
- Al-Waili, N.S. (2004). Natural honey lowers plasma glucose, C-reactive protein, homocysteine, and blood lipids in healthy, diabetic, and hyperlipidemic subjects: comparison with dextrose and sucrose. *J. Med. Food*, 7, 100-107.
- Al-Waili, N.S. (2004). Topical honey application vs. acyclovir for the treatment of recurrent herpes simplex lesions. *Med. Sci. Monit.*, 10, MT94-98.
- Bankova, V. (2005). Recent trends and important developments in propolis research. *Evid. Based Complement. Alternat. Med.*, 2, 29-32.
- Basualdo, C., Sgroy, V., Finola, M.S., Marioli, J.M. (2007). Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Vet. Microbiol.*, 124, 375-381.
- Birt, D.F., Hendrich, S., Wang, W. (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol. Ther.*, 90, 157-177.
- Bogdanov, S. (1999). Honey quality, methods of analysis and international regulatory standards: review of the work of the International Honey Commission. *Mitt. Geb. Lebensmittelunters. Hyg.*, 90, 108-125.
- Changsen, C., Franzblau, S.G., Palittapongpim, P. (2003). Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. *Antimicrob. Agents Chemother.*, 43, 3682-3687.
- Choi, Y.M., Noh, D.O., Cho, S.Y., Suh, H.J., Kim, K.M., Kim, J.M. (2006). Antioxidant and antimicrobial activities of propolis from several regions of Korea. *LWT.*, 39, 756-761.
- Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D. (1979). Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.*, 16, 710-718.
- Diaz-Carballo, D., Ueberla, K., Kleff, V., Ergun, S., Malak, S., Freistuehler, M., Somogyi, S., Kucherer, C., Bardenheuer, W., Strumberg, D. (2010). Antiretroviral activity of two polyisoprenylated acylphloroglucinols, 7-epi-nemorosone and plukenetione A, isolated from Caribbean propolis. *Int. J. Clin. Pharmacol. Ther.*, 48, 670-677.
- Dixon, M.W., Peatey, C.L., Gardiner, D.L., Trenholme, K.R. (2009). A fluorescent protein-based assay for determining gametocyte production in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.*, 163, 123-126.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Roberts, D.A., Smith, F. (1956). Colorimetric methods for determination of sugars and related substances. *Anal Chem.*, 28, 350-356.
- Effem, S.E.E. (1988). Clinical observations on the wound healing properties of honey. *Br. J. Surg.*, 75, 679-681.
- Finola, M.S., Lasagno, M.C., Marioli, J.M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chem.*, 100, 1649-1653.
- Giorgi, A., Madeo, M., Baumgartner, J., Lozzia, G.C. (2011). The relationships between phenolic content, pollen diversity, physicochemical information and radical scavenging activity in honey *Molecules*, 16, 336-347.
- Gomez Pajuelo, A. (1995). El color de la miel. *Vida Apícola.*, 73, 20-25.

- Gulcin, I., Bursal, E., Sehitoglu, M.H., Bilsel, M., Goren, A.C. (2010). Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey. *Food Chem. Toxicol.*, 48, 2227-2238.
- Hegazi, A., Abd El Hady, F., Abd Allah, F. (2000). Chemical composition and antimicrobial activity of European propolis. *Z. Naturforsch.*, 55, 70-75.
- Hernandez, J., Goycoolea, F.M., Quintero, J., Acosta, A., Castaneda, M., Dominguez, Z., Robles, R., Vazquez-Moreno, L., Velazquez, E.F., Astiazaran, H., Lugo, E., Velazquez, C. (2007). Soronan propolis: chemical composition and antiproliferative activity on cancer cell lines. *Planta Med.*, 73, 1469-1474.
- Hunt, L., Jordan, M., De Jesus, M., Wurm, F.M. (1999). GFP-expressing mammalian cells for fast, sensitive, noninvasive cell growth assessment in a kinetic mode. *Biotechnol. Bioeng.*, 65, 201-205.
- Kang, L.J., Lee, H.B., Bae, H.J., Lee, S.G. (2010). Antidiabetic effect of propolis: reduction of expression of glucose-6-phosphatase through inhibition of Y279 and Y216 autophosphorylation of GSK-3 $\alpha/\beta$  in HepG2 cells. *Phytother. Res.*, 24, 1554-1561.
- Koc, A.N., Silici, S., Kasap, F., Hormet-Oz, H.T., Mavus-Buldu, H., Ercal, B.D. (2011). Antifungal activity of the honeybee products against *Candida* spp. and *Trichosporon* spp. *J. Med. Food*, 14, 128-134.
- Koru, O., Toksoy, F., Acikel, C.H., Tunca, Y.M., Baysallar, M., Guclu, A.U., Akca, E., Tuylu, A. O., Sorkun, K., Tanyuksel, M., Salih, B. (2007). *In vitro* antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens. *Anaerobe.*, 13, 140-145.
- Kucuk, M., Kolayli, S., Karaoglu, S., Ulusoy, E., Baltaci, C., Candan, F. (2007). Biological activities and chemical compositions of three honeys of different types from Anatolia. *Food Chem.*, 100, 526-534.
- Li, F., Awale, S., Tezuka, Y., Esumi, H., Kadota, S. (2010). Study on the constituents of Mexican propolis and their cytotoxic activity against PANC-1 human pancreatic cancer cells. *J. Nat. Prod.*, 73, 623-627.
- Manikis, I., Thrasivoulou, A. (2001). The relation of physico-chemical characteristics of honey and the crystallization sensitive parameters. *Apiacta.*, 36, 106-112.
- Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31, 426-428.
- Mullin, W.J., Emmons, D.B. (1997). Determination of organic acids and sugars in cheese, milk, and whey by high performance liquid chromatography. *Food Res. Int.*, 30, 147-151.
- Najafi, M.F., Vahedy, F., Seyyedin, M., Jomehzadeh, H.R., Bozary, K. (2007). Effect of the water extracts of propolis on stimulation and inhibition of different cells. *Cytotechnology*, 54, 49-56.
- Nolkemper, S., Reichling, J., Sensch, K.H., Schnitzler, P. (2010). Mechanism of herpes simplex virus type 2 suppression by propolis extracts. *Phytomedicine*, 17, 132-138.
- Ojeda De Rodriguez, G., Sulbaran De Ferrer, B., Ferrer, A., Rodriguez, B. (2004). Characterization of honey produced in Venezuela. *Food Chem.*, 84, 499-502.
- Perez, C., Pauli, M., Bazerque, P. (1990). An antibiotic assay by the agar well diffusion method. *Acta Biologica et Medicine Experimentalis*, 15, 113-115.
- Pichichero, E., Cicconi, R., Mattei, M., Muzi, M.G., Canini, A. (2010). Acacia honey and chrysin reduce proliferation of melanoma cells through alterations in cell cycle progression. *Int. J. Oncol.*, 37, 973-981.
- Sawaya, A.C., Abdelnur, P.V., Eberlin, M.N., Kumazawa, S., Ahn, M.R., Bang, K.S., Nagaraja, N., Bankova, V.S., Afrouzan, H. (2010). Fingerprinting of propolis by easy ambient sonic-spray ionization mass spectrometry. *Talanta.*, 81, 100-108.

- Sforcin, J.M. (2007). Propolis and the immune system: a review. *J. Ethnopharmacol.*, 113, 1-14.
- Sherlock, O., Dolan, A., Athman, R., Power, A., Gethin, G., Cowman, S., Humphreys, H. (2010). Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. *BMC Complement. Altern. Med.*, <http://biomedcentral.com/1472-6882/10/47>.
- Silici, S., Sagdic, O., Ekici, L. (2010). Total phenolic content, antiradical, antioxidant, and antimicrobial activities of Rhododendron honeys. *Food Chem.*, 121, 238-243.
- Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.*, 299, 152-178.
- Snowdon, J.A., Cliver, D.O. (1996). Microorganisms in honey. *Int. J. Food Microbiol.*, 31, 1-26.
- Valente, M.J., Baltazar, A.F., Henrique, R., Estevinho, L., Carvalho, M. (2011). Biological activities of Portuguese propolis: protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth in vitro. *Food Chem. Toxicol.*, 49, 86-92.
- Webster, D., Lee, T.D., Moore, J., Manning, T., Kunimoto, D., LeBlanc, D., Johnson, J.A., Gray, C.A. (2010). Antimicrobial screening of traditional medicinal plants using the microplate resazurin assay. *Can. J. Microbiol.*, 56, 487-494.
- Weston, R.J. (2000). The contribution of catalase and other natural products to the antibacterial activity of honey: a review. *Food Chem.*, 71, 235-239.
- Woisky, R.G., Salatino, A. (1998). Analysis of propolis: some parameters and procedures for chemical quality control. *J. Apic. Res.*, 37, 99-105.
- World Health Organization. (WHO). 2008. World Malaria Report. Available at: <http://www.who.int/malaria/wmr2008>. Accessed 28<sup>th</sup> January 2011.