

Antibacterial Activity and Antioxidant Capacity of Malaysian Tualang Honey

Aminu Shehu¹, Mohd Adzim Khalili Rohin², Aniza Abd Aziz³, Salwani Ismail⁴

^{1,3,4} Faculty of Medicine, Universiti Sultan Zainal Abidin (UniSZA), Medical Campus, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Terengganu, Malaysia

² Faculty of Health Sciences, Universiti Sultan Zainal Abidin (UniSZA), Gong Badak Campus, Kuala Terengganu, Terengganu, Malaysia

Abstract: Honey is known to possess therapeutic potential including antimicrobial activity and antioxidant. The aim of this study was to evaluate the antibacterial activity and antioxidant capacity of Malaysian tualang honey against five clinically important Gram positive and Gram negative bacteria. Broth dilution method was used to evaluate the antibacterial activity of Tualang honey. Different concentrations of honey (1.56mg/ml – 100mg/ml) in two-fold dilutions were tested against each bacterium. Minimum Inhibitory Concentration (MIC) was determined by visual inspection and spectrophotometric (MIC₉₅) readings at 620nm. Minimum bactericidal concentration (MBC) was also obtained by culturing the bacteria on nutrient agar plates. The antioxidant capacity of Tualang honey was also determined by estimating the total phenolic content, flavonoid content and radical scavenging activity (DPPH). The MIC of Tualang honey by visual inspection and spectrophotometric (MIC₉₅) reading ranged from 3.125mg/ml – 12.5mg/ml and 6.25mg/ml – 25mg/ml respectively. The MBCs values against the five tested bacteria ranged from 12.5mg/ml to 50mg/ml. The lowest and highest MBCs values of Tualang honey were against *S. enterica* Serovar Typhimurium (12.5mg/ml) and *E. coli* (50mg/ml). The total phenolic and flavonoids content of Tualang honey were found to be 275.6±12.5mg gallic acid/kg honey and 71.8±11.3mg quercetin/kg honey respectively. The radical scavenging activity (DPPH) had inhibition concentration (IC₅₀) of 460±16.3µg/ml. Malaysian tualang honey has both bacteriostatic and bactericidal activities against clinically important Gram positive and Gram negative bacteria. The presence of phenolics and flavonoids in the Tualang honey are responsible for its antioxidant capacity.

Keywords: Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), phenolic, flavonoid, radical scavenging activity

1. Introduction

Honey has been used since the ancient times as a food product as well as traditional medicine to treat varieties of ailments due to its healing properties. Currently, functional and biological properties (antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, anticancer) of honey has been scientifically proven [1-6].

Tualang honey is a Malaysian multifloral jungle honey collected from the combs of Asian rock bees (*Apis dorsata*) which build hives on branches of tall tualang tree (*Kompassia excelsa*) located mainly in the north western region of Peninsular Malaysia [7]. Tualang honey has a dark brown appearance and it contains a polyphenol compound which contributes to its high antioxidant activities [8]. However, a limited scientific finding about its microbiological activity has been published [9-10].

The antioxidant capacity of honey is due to the presence of more than 150 polyphenolic compounds, including flavonoids, phenolics acid, flavonols, catechins and cinnamic acid derivatives as well as enzymatic and non-enzymatic substances [11-12]. Flavonoids and phenolic acids are polyphenols which are detected in honey and act as free radical scavengers, peroxy-radical scavengers and as metal chelators [13].

Tan *et al.* [14] examined the bacterial properties of tualang honey against wound and enteric microorganisms in comparison with manuka honey from New Zealand, in which he concluded that tualang honey exhibited variable activities against many different microorganisms. Tumin *et*

al. [15] has studied antibacterial activities of five local honeys (tualang, hutan, gelam, pucuk daun and Ee Feng Gu) and found them to be effective against various pathogenic bacteria strains tested.

In our study, the antibacterial activity of Tualang honey against clinically significant Gram positive and Gram negative bacteria were determined. The total phenolic, flavonoids and radical scavenging activity as part of antioxidant of Malaysian tualang honey have also been quantified.

2. Materials and Methods

2.1 Tualang honey Sample

Tualang Honey used in this study was supplied by Federal Agricultural Marketing Authority (FAMA), Malaysia. It was harvested from *Apis dorsata* bees' nectar on the Tualang tree in the Rain Forest of Kedah in Peninsular Malaysia in November 2014. Prior to submitting to us for analysis, the honey was subjected to gamma irradiation at a dose of 25 kGy. The honey was stored in a dark at a room temperature. All the chemicals and solvents used were of analytical grade.

2.2 Bacteria

Cultures of bacteria were supplied by Microbiology Laboratory, Medical Campus, University Sultan Zainal Abidin (UniSZA), Kuala Terengganu, Malaysia. All bacteria were of standard strains (ATCC, US) comprising two Gram-positive bacteria; *Staphylococcus aureus* (ATCC 9144) and *Staphylococcus epidermidis* (ATCC 14990) and three Gram-

negative bacteria; *Escherichia coli* (ATCC 85218), *Pseudomonas aeruginosa* (ATCC 14149) and *S. enterica* Serovar *Typhimurium* (ATCC 14028).

2.3 Inoculum Preparation

The inoculums was prepared by picking 3-5 morphologically identical colonies from overnight growth with an inoculating wire loop and suspended in 4-5ml of sterile peptone water and incubated at 35-37°C for 24hrs. The turbidity of the actively growing culture was adjusted with sterile peptone water to matches 0.5 McFarland standard (10^6 Colony Forming Unit (CFU)/ml) and then further diluted to obtain a final concentration of 5.0×10^5 CFU/ml [16].

2.4 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of Tualang honey was determine according to Vollekova *et al.* [17] and Usman *et al.* [18] with some minor modifications. Stock solution of 100mg/ml was prepared by dissolving 10g of honey in 100ml of DMSO. Two fold serial dilutions were made to obtain honey concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.56mg/ml. 0.2ml suspensions of the organism was inoculated into the tubes containing Muller – Hinton broth (MHB) and different honey concentration. For each assay, control wells included: (a) wells containing broth only (without honey and inoculum); (b) wells containing broth and inoculum (without honey) and (c) wells containing broth and honey (without inoculum). All the test tubes were incubated for 24hrs at a temperature of 37°C. Growth was observed by visual inspection and by measuring the optical density (OD) at 620nm using spectrophotometer. The OD was measured immediately after visual reading. The lowest concentration of honey that prevented the growth of each microorganism, as detected by lack of visual turbidity compared to a negative control was recorded as MIC. All tests were performed in triplicate and were repeated five times to ensure the reproducibility of the results.

The growth inhibition for the test wells at each honey dilution was determined by the formula:

Percent inhibition = $[1 - (\text{OD test well} - \text{OD corresponding negative control well}) / (\text{OD viability control well} - \text{OD broth only well})] \times 100\%$. The minimum and maximum values were 0% and 100%, respectively.

2.5 Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by taking a loopful of the culture medium from each test tube (from the broth MIC assay) that showed no apparent growth and sub-culturing on fresh Nutrient agar plates. After incubation at 35°C for 24 h, the MBC was read as the least concentration showing no growth on the nutrient agar plates [16].

2.6 Total Phenolic Content

The total phenolic content of the Tualang honey was determined by the Folin-Ciocalteu's reagent [9, 19] with some minor modifications. 500mg of Tualang honey was

first mixed with 5ml distilled water and vortex-mixed for 5minute. This solution (0.5 ml) was then mixed with 2.5 ml of Folin–Ciocalteu reagents and allowed to stand for 5minute and 2ml of 75 g/l sodium carbonate (Na_2CO_3) was then added. After incubation at room temperature for 30minute, the absorbance of the reaction mixture was measured at 760 nm against a blank. Gallic acid (10-250 $\mu\text{g/ml}$) was used as standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of Gallic acid equivalents (GAE)/kg of honey.

2.7 Total Flavonoids Content

The total flavonoid content of Tualang honey was measured using the colorimetric assay [20-21] with slight modifications. 1ml of Honey (20mg/ml) was mixed with 1ml of 2% aluminum trichloride (AlCl_3) (Labosi, Paris, France), followed by the addition of 1ml of potassium acetate. After 6 min, the volume was then increased to 5ml by the addition of 2ml distilled water. The mixture was vigorously shaken to ensure adequate mixing, and the absorbance was read at 415nm after 40 min incubation in a dark. The total Flavonoids content was determined using a standard curve with quercetin as the standard. The mean of three readings was used and expressed as mg of quercetin equivalents (QE)/kg of honey.

2.8 DPPH Free Radical-Scavenging Activity

The scavenging activity of the honey sample for the radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described by Chen *et al.*, [22] and Aljadi and Kamaruddin, [23] with some modifications. Briefly, 0.75ml of the honey solution (0.1g/ml) in warm water was mixed with 1.5ml of 0.09mg/ml DPPH in methanol. The mixtures were left to stand in the dark for 30 minute at room temperature and the absorbance's was measured at 517nm against a blank sample consisting of honey solution with distilled water. The absorbance of a radical blank was also measured using 0.75ml of distilled water. Quercetin was used as positive controls. The radical scavenging activity (RSA) of honey was expressed in terms of percentage inhibition of DPPH radical by honey and was calculated as follows: RSA (DPPH. Inhibition, %) = $[(\text{AB}-\text{AT})/\text{AB}] \times 100$ Where, AB = Absorbance of radical blank (DPPH. without honey) and AT = Absorbance of test sample (DPPH. with honey). The mean of three IC_{50} (concentration causing 50% inhibition) values of honey sample was determined graphically.

3. Results

3.1 Antibacterial activity of Tualang honey

Table 1 shows the MIC values of Malaysian Tualang honey against the five tested bacteria by visual inspection and spectrophotometric (MIC_{95}) reading. The minimum bactericidal concentration (MBC) of tualang honey was also presented.

Under visual inspection, the MICs of Malaysian Tualang honey ranged from 3.125mg/ml – 25mg/ml. Tualang honey had lower MIC value of 3.125mg/ml (indicating better

activity) against *S. enterica Serovar Typhimurium* while the highest MIC value of 25mg/ml was recorded against *E. coli*. Two organisms (*S. aureus* and *P. aeruginosa*) demonstrated the same MIC value of 12.5mg/ml and *S. epidermidis* with 6.25mg/ml (Table 1).

The spectrophotometric inhibition of at least 95%(MIC₉₅) ranged from 6.25mg/ml – 25mg/ml. Malaysian tualang honey had the lowest MIC₉₅ value when tested against *S. typhimurium* (6.25mg/ml) while the highest was against *E. coli* (25mg/ml). The remaining bacterial species (*S. aureus*, *S. epidermidis* and *P. aeruginosa*) revealed similar MIC₉₅ of 12.5mg/ml (Table 1).

The MIC of tualang honey obtained by visual inspection and spectrophotometric (MIC₉₅) showed the same reading for three bacteria (*S. aureus*, *E. coli* and *P. aeruginosa*). Two bacteria (*S. epidermidis* and *S. enterica Serovar Typhimurium*) had an increased spectrophotometric MIC₉₅ values compared to visual MIC reading (Table 1).

The bactericidal activities of Malaysian tualang honey as measured by MBC were recorded to be one reading higher than the spectrophotometric (MIC₉₅) readings. The MBCs values ranged from 12.5mg/ml to 50mg/ml against the five bacteria tested (Table 1). The lowest and highest MBCs values of tualang honey against *S. enterica Serovar Typhimurium* and *E. coli* were 12.5mg/ml and 50mg/ml respectively. However, three bacteria (*S. aureus*, *S. epidermidis* and *P. aeruginosa*) showed equal MBC value of 25mg/ml (Table 1).

Figure 1 displays the details of the bacterial growth response based on MIC against Malaysian tualang honey at different concentration. All the five bacteria tested in this study showed a dose response activity in various degrees.

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration of Malaysian (MBC) Tualang Honey

S/n	Microorganism	Visual	Spectrophotometric	MBC
		MIC	MIC ₉₅	
1	<i>Staphylococcus Aureus</i>	12.5	12.5	25
2	<i>Staphylococcus epidermidis</i>	6.25	12.5	25
3	<i>Escherichia Coli</i>	25	25	50
4	<i>Pseudomonas aeuroginosa</i>	12.5	12.5	25
5	<i>S. enterica Serovar Typhimurium</i>	3.125	6.25	12.5

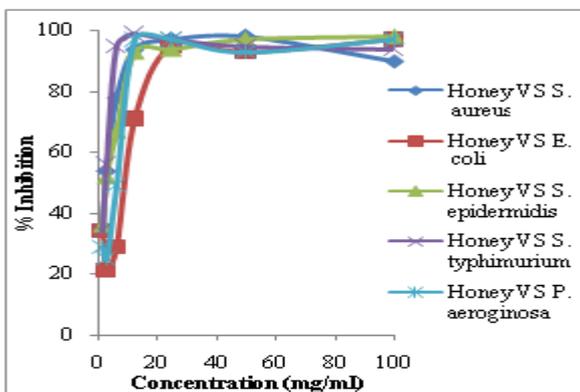


Figure 1: Inhibition of the growth of *E. coli*, *S. aureus*, *S. epidermidis*, *S. typhimurium* and *Pseudomonas aeruginosa* by Tualang honey

3.2 Antioxidant capacity of Tualang honey

Tualang honey contained high amount of phenolic (275.6mg_{gallic acid/kg}), flavonoids (71.8mg_{quercetin/kg}) and radical scavenging activity (460 µg/ml) (Table 2).

Table 2: Antioxidant Capacity of Malaysian Tualang Honey

S/n	Parameters	(Mean ± SD)*
1	Total phenolic content (mg gallic acid/Kg of tualang honey)	275.6±12.5
2	Total flavonoids content (mg quercetin/Kg of tualang honey)	71.8±11.3
3	Radical scavenging activity (DPPH): IC ₅₀ (µg/ml)	460±16.3

*All determinations were carried out in triplicate and the values were expressed as mean ± SD

4. Discussion

Tualang honey is a multifloral jungle honey available in Malaysia. However, its quality and antimicrobial activity are still in the process of investigating and standardizing by many researchers. In this study, we found that Tualang honey has a broad – spectrum of activities against both gram positive and gram negative bacteria. Honey inhibits the growth of bacteria due to its high sugar content (reduced water activity), low pH, generation of hydrogen peroxide (H₂O₂) when diluted and proteinaceous compounds [24].

Visual inspection might have not been accurate because medium containing bacterial growth not detectable by eye would have been described as clear by direct inspection but the growth would have been detectable by the spectrophotometer, as such variations can be seen in MIC values determined by visual inspection and spectrophotometric measurement (i.e. *S. epidermidis* and *S. enterica Serovar Typhimurium*). Visual inspection depends solely on the eye of the observer (viewer) as such variation can be seen from one person to another [14].

In the present study, MIC was performed using broth dilution method and the optical density was measured by means of spectrophotometer. MIC is defined as the lowest concentration of honey that prevents at least 99% of bacterial growth. Otherwise, MIC₉₅ is defined as the concentration of honey required to inhibit the growth of bacteria by 95%. The MIC₉₅ value was included in existing data presentation in order to provide a clearer picture of bacterial growth inhibition pattern. The susceptibility in descending pattern was *S. typhimurium* > *P. aeruginosa*, *S. epidermidis*, *S. aureus* > *E. coli*. Moreover, all the five bacteria tested exhibited higher MBC values than the MIC. The MBC or the lowest concentration of Tualang honey required to kill at least 99% of the bacteria was remarkable especially against *S. enterica Serovar Typhimurium*.

The results of present study were in line with the reports of many researchers [15, 25, 26, 27] which revealed that honey has antimicrobial activity against *S. aureus*, *P.aeruginosa*, *E. coli*, *P. mirabilis*, *Citrobacter ferundi*, *S. faecalis*, *S. flexinari*, *S. typhi*, *S. pyogenus*. The current findings were

also supported by Tan *et al.* [14], who reported that Tualang honey exhibited various activities against different microorganisms.

Tualang honey showed good antibacterial activity against *S. enterica* Serovar *Typhimurium* (6.25mg/ml). This organism is a pathogenic Gram-negative bacteria predominantly found in the intestinal lumen. *S. enterica* Serovar *Typhimurium* causes gastroenteritis in humans and other mammals. In mice, it causes symptoms resembling typhoid fever as in humans. Studying these bacteria in mice could eventually lead to a typhoid vaccine [28].

Honey composed of many different compounds such as polyphenolics, organic acids, vitamins, catalase and glutathione peroxidase which are responsible for its antioxidant activity [23, 29].

The variation in total phenolic and flavonoid content could be due to differences in their composition and geographical origin. The levels of phenolic compounds of tualang honey in this study was within the range previously reported for Tualang honey such as 251.7mg gallic acid/kg [8] and 352.73mg/kg [30]. The total flavonoid content of Tualang honeys is also within the range reported for some Algerian honeys (27 - 71 mg/kg) [13], but higher than that reported for Linen vine honey (25.2 mg/kg); Christmas vine honey (10.9 mg/kg) [31], eucalyptus honey (20–25 mg CE/kg); Sun flower and rape honey (15–20 mg CE/kg); and fir, lavender, ivy and acacia honey (5–10 mg CE/kg), [32-33].

Tualang honey also exhibited high radical scavenging activity (460ug/ml) indicating that it has high antioxidant capacity (Table 2). Flavonoids and phenolic acids are responsible for the antioxidant capacity of honey [13].

5. Conclusion

Malaysian tualang honey demonstrated bacteriostatic and bactericidal activities against both Gram positive and Gram negative bacteria. It was also found to have higher levels of phenolic content, flavonoids content and possessed good radical scavenger activity. The total phenolic content was found to be higher than that of the flavonoids. The presence of high phenolic content and flavonoids are the possible constituent responsible for the antioxidant capacity of Malaysian tualang honey. Further studies should be carried out to identify the actual bioactive compounds responsible for the antibacterial and antioxidant activity of Malaysian tualang honey.

References

- [1] Estevinho L, Pereira AP, Moreira L, Dias LG, Pereira E. Antioxidant and antimicrobial effects of phenolic compounds extracts of northeast Portugal honey. *Food Chem Toxicol* 2008; 46: 3774-3779.
- [2] Gheldof N, Engeseth N J. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *J Agric Food Chem* 2002; 50: 3050-3055.
- [3] Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holerof DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agriculture and Food Chemistry* 2000; 48: 4581-4589.
- [4] Irish J, Carter DA, Blair SE, Heard TA. Antibacterial activity of honey from the Australian stingless bee *Trigona carbonaria*. *Int J Antimicrob Agents* 2008; 32: 89-90.
- [5] Temaru E, Shimura S, Amano K, Karasawa T. Antibacterial activity of honey from stingless honeybees (hymenoptera; apidae; meliponinae). *Polish Journal of Microbiology* 2007; 56: 281-285.
- [6] Wang XH, Engeseth NJ. Antimutagenic effect of various honeys and sugars against trp-p-1. *Journal of Agriculture and Food Chemistry* 2002; 50: 6923-6928.
- [7] Erejuwa OO, Sulaiman SA, Wahab MS, Sirajudeen KN, Salleh MS, Gurtu S. Antioxidant protection of Malaysian tualang honey in pancreas of normal and streptozotocin-induced diabetic rats. *Ann Endocrinol* 2010; 71(4):291–296.
- [8] Mohamed M, Sirajudeen KNS, Swamy M, Yaacob NS, Sulaiman SA. Studies on the antioxidant properties of tualang honey of malaysia. *African Journal of Traditional, Complementary and Alternative medicines* 2010; 7: 59-63.
- [9] Beretta G, Granata P, Ferrero M, Orioli M, Maf. Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. *Anal Chim Acta* 2005; 533: 185-191.
- [10] Adams CJ, Boulton CH, Deadman BJ, Farr JM, Grainger MNC, Manley-Harris M, *et al.* Isolation by HPLC and characterization of the bioactive fraction of New Zealand Manuka (*Leptospermum scoparium*) honey. *Carbohydr Res* 2008; 343(4):651-659.
- [11] Al-Mamary M, Al-Meerri A, Al-Habori M. Antioxidant activities and total phenolics of different types of honey. *Nutrition Research* 2002; 22: 1041-1047.
- [12] National Honey Board. Honey-health and therapeutic qualities. Lashley Street
- [13] Longmont: <http://www.nhb.org>. 2003.
- [14] Khalil MD, Ibrahim Sulaiman, Siti Amrah Alam, Nadia Ramli, Norzihana Mohamed, Mahaneem Bai'e, *et al.* Content and antioxidant properties of processed honey (agromasi1/2) collected from different regions in malaysia. *International Journal of Pharmacy & Pharmaceutical Sciences* 2012; Vol 4, Suppl 3, 214-219.
- [15] Tan HZ, Abdul Rahman R, Gan SH, Halim AS, Hassan SA, Amrah SS, *et al.* The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey. *BMC Complement and Alternative Medicine* 2009; 9:34.
- [16] Tumin N, Abdul Halim NA, Shahjahan M, Noor Izani NJ, Sattar MA, *et al.* Antibacterial activity of local Malaysian Honey. *Malaysian Eur J Pharm Sci* 2005; 3(2):1–10.
- [17] National Committee of Clinical Laboratory Standards. Performance Standards for Antimicrobial Susceptibility Testing. 9th international Supplement 1999; M 100 – Sa, Wayne Pa.

- [18] Vollekova A, Kostalova D, Sochorova R. Isoquinoline Alkaloids from Mahonia aquifolium stem bark is active against Malassezia Sp. *Folia Microbiol* 2001; 46:107 – 111.
- [19] Usman H, Abdulrahman FI, Ladan AH. Phytochemical and Antimicrobial Evaluation of Tribulus terrestris L. (Zygophyllaceae). Growing in Nigeria. *Res. J. Bio. Sci. Medwell Journals* 2007; 2(3):244 – 247.
- [20] Singleton VL, Orthofer R, Lamuela-Ravento RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology* 1999; 299, 152–178.
- [21] Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* 1999; 64:555–559.
- [22] Arvouet-Grand A, Vennat B, Pourrat A, Legret P. Standardisation dun extrait de propolis et identification des principaux constituants. *Journal de Pharmacie de Belgique* 1994; 49, 462–468.
- [23] Chen L, Mehta A, Berenbaum M, Zangerl AR, Engeseth NJ. Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. *J. Agric. Food Chemistry* 2000; 48: 4997–5000.
- [24] Aljadi AM, Kamaruddin MY. Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chemistry* 2004; 85: 513–518.
- [25] Mundo MA, Padilla-Zakour OI, Worobo RW. Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. *Int J Food Microbiol* 2004; 97:1-8.
- [26] Molan PC. The antibacterial activity of honey. *Bee world* 1992; 73(1):5-28. 10.
- [27] Nzeako, Hamdi. Antimicrobial potential of honey. *Medical Sciences* 2000; 2:75-79. 11.
- [28] Kingsley A. Supplements. The use of honey in treatment of infected wounds. Case studies. *BJ of Nursing* 2001; 10(22): Sup 13-Sup 20.
- [29] Garcia-Del Portillo, Francisco, Murray Stein, Finlay B. "Release of Lipopolysaccharide from Intracellular Compartments." *Infection and Immunity. American Society for Microbiology* 1997; 65.1: 24-34.
- [30] Bertonec J, Doberšek U, Jamnik M, Golob T. Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. *Food Chemistry* 2007; 105(2): 822-828.
- [31] Moniruzzaman M., Khalil M., Sulaiman S, Gan S. Advances in the Analytical Methods for Determining the Antioxidant Properties of Honey: A Review. *Afr J Traditional Complement Altern Med* 2012; 9(1):36–42.
- [32] Alvarez-Suarez JM, GonzaLez-Parma AM, Santos-Buelga C, Battino M. Antioxidant Characterization of Native Monofloral Cuban Honeys. *J Agric Food Chem* 2010; 58:9817–9824.
- [33] Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chem* 2005; 91:571–577.
- [34] Ferreira ICFR, Aires E, Barreira JCM, Estevinho LM. Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. *Food Chem* 2009; 114:1438–1443.