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Determination of xanthine oxidase inhibition in stingless bee honey from different botanical origin

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Abstract. Malaysian stingless bee honey or locally known as *kelulut* honey is a natural substance that have been widely explored for their great nutritional and therapeutic values. Modern beekeeping practices in different botanical areas have produced different types of honey that has influence on their physical and chemical composition as well as their biological activity. The main objective of this research is to investigate *in vitro* xanthine oxidase (XO) enzyme inhibition of honey produced by *Heterotrigona itama* species obtained from different botanical origins; (acacia, coconut, mangrove, starfruit, multifruits and multiflowers). In addition, the phytochemical composition was analyzed using spectrophotometric. The result revealed that honey from mangrove area displayed highest phytochemical composition (phenolic and flavonoid) with the value of 34.23 mg GAE/100g and 31.02 mg RE/100g of honey, respectively. The XO inhibitory activity (IC_{50} values) of all tested honeys were ranging from 30 to 185 mg/ml. As a conclusion, *kelulut* honey exhibited good potentials towards the inhibition of xanthine oxidase activities and also suggest a practical value for surveying natural inhibitors for clinical purpose.

Keywords. Stingless Bee Honey; Phytochemical; Inhibition; Xanthine oxidase; Botanical origin.

1. Introduction

Among natural products, honey is one of the healthy foods that have been discovered and used in traditional medicine practices [1]. The botanical and geographical origins as well as bee species has influence on the composition of secondary metabolite in honey [2]. Honey is composed primarily of the sugars glucose and fructose, and contains around 200 bioactive substances such as minerals, amino and organic acid, vitamin, enzymes, proteins, as well as phytochemicals [3].

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Over the past decades, the potential of natural products as modulators of biological functions has been increasingly explored [4]. Although a lot of synthetic and chemical products were used as useful inhibitors, natural products become popular alternative for enzyme inhibitor. Enzyme inhibitors are mainly bioactive secondary metabolites that bind to an enzyme and decrease its bioactivity and catalytic activity. Eventually, blocking enzyme activity can kill a pathogen or restore a metabolic imbalance. Natural enzyme inhibitors are often mediated by its specificity and its effectiveness that designated the absorption desirable to inhibit the enzyme [5].

Honey acts as natural enzyme inhibitors, especially for xanthine oxidase (XO) enzyme. XO catalyses the hydoxylation of hypoxanthine or xanthine to uric acid, and also creates a superoxide radical formed during the metabolic process. This radical has to be neutralized by antioxidant systems and inhibitors which slow down or stop the effect of the level of the enzyme. Clinically, allopurinol has been used as XO inhibitor but its possess side effects such as nephropathy, hepatitis and allergic reactions [6]. Thus, the exploration for new XO inhibitors with a higher therapeutic potential and fewer side effects is needed to fight numerous diseases.

In Malaysia, stingless bee honey is a valuable super food that has nutritional and therapeutic uses. Investigating the bioactivity of different types of honey harvested in different regions with high plant diversity is of a great interest. Thus, the purpose of this research is to investigate the xanthine oxidase (XO) enzyme inhibition of honey produced by *H.itama* species obtained from different botanical origins namely acacia, coconut, mangrove, starfruit, multifruits and multiflowers.

2. Materials and methods

2.1. Collection of honey samples

Honey samples for present investigation have been collected directly from beekeepers during field trips from October to December 2017. Six honey sample from different botanical origins namely acacia, coconut, mangrove, starfruit, multifruits, and Multiflowers were collected (Fig.1). These samples were shipped to the laboratory and stored in sterilized amber glass containers at an ambient temperature ranges from 3^0C to 5^0C in the dark until further analysis. Tualang honey produced by bee species of *Apis dorsata* was used as positive control.

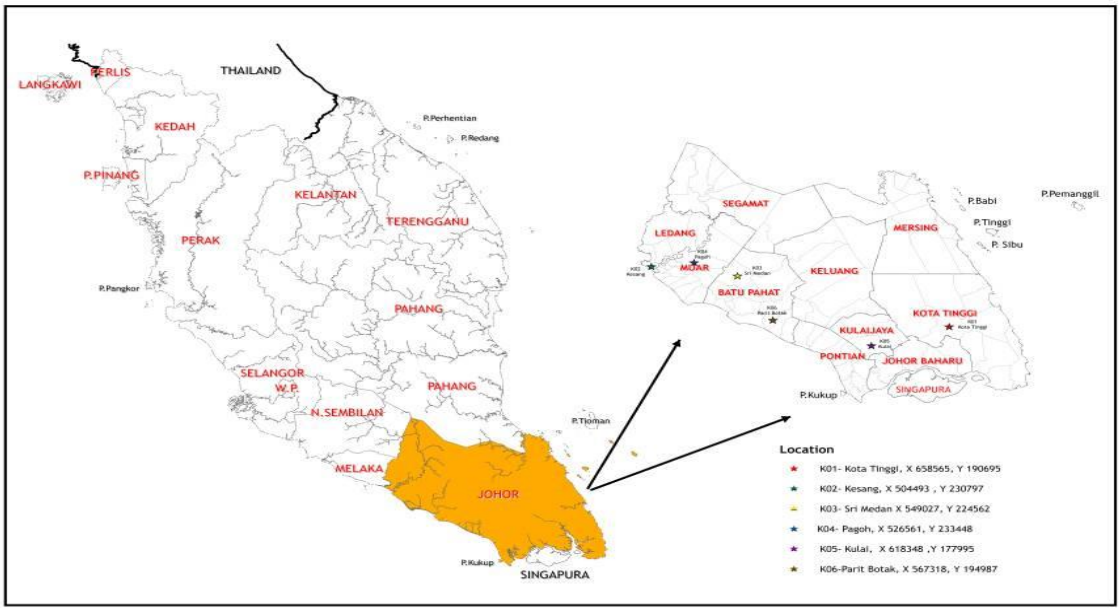

Figure 1. Map of samples distribution in state of Johor, Malaysia

2.2. Sample preparation

About 10 grams of the honey sample was extracted with 50 mL of distilled water in a flask attached to a condenser more than 6 hour at 60°C. The extract was filtered and the final volume was adjusted with distilled water.

2.3. Xanthine oxidase (XO) assay

The XO inhibitory activity was determined spectrophotometrically [5]. The reaction mixture contained an 0.5 mL of extract, 0.77 mL of a phosphate buffer (pH is 7.8) and 0.07 mL of XO, which was freshly prepared before use. After pre-incubation at 25°C for 15 min, the reaction was initiated by the addition of 0.66 mL of a substrate solution into the mixture. The assay mixture was incubated at 25°C for 30 min. The reaction was stopped by the addition of 0.2 mL of 0.5 N HCl and the absorbance was measured at 295 nm. The performance of the assay was verified using allopurinol as the positive control. All the reactions given as IC_{50} values, which defined as the concentration of the extract that inhibits 50% of XO enzyme activity.

2.4. Total phenolic content (TPC)

The total phenolic content were determined using the Folin–Ciocalteu assay [8]. An aliquot of 0.5 mL of extract was mixed with 1.5 mL of 0.2 N Folin–Ciocalteu reagent and 1.0 mL (75 g/L) of sodium carbonate in test tube. The mixture were vortexed for 15 s and allowed to stand for 2 h in the dark at room temperature. The absorbance was measured at 725 nm using the UV-Vis spectrophotometer and the result was expressed as gallic acid equivalents in 100 gram of honey (mg GAE $100g^{-1}$).

2.5. Total flavonoid content (TFC)

The total flavonoid contents were determined using aluminum chloride colorimetric method and the results were expressed as mg of rutin equivalents per 100 gram of honey (mg RE $100g^{-1}$) [9]. About 2 mL of extract were mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution. After incubate for 5 min, 0.6 mL of 10% $AlCl_3$ solution was added and the mixture was allowed to stand for 6 min. Finally, 2 mL of 1 mol/L NaOH solution were added and the absorbance was measured at 415 nm.

2.6. Statistical analysis

All data were analyses using IBM SPSS Statistics 22 and the results were expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to test for statistical significance difference at $p \leq 0.05$ confidence level. Pearson's correlation coefficient (r) was used to determine the correlation between the parameters.

3. Result and discussion

3.1. Inhibition of xanthine oxidase activities

Enzyme inhibitory activities in natural products are quite extensive when compared to other enzyme studies. However, their biological values have not been completely validated. Currently, pharmaceutical research has focused on enzyme inhibition studies due to its potential in leading to discoveries of useful drugs for various physiological conditions. Enzyme inhibitors are molecules that interact in some way with an enzyme to block their activity towards natural substrates [10].

In this study, *in vitro* xanthine oxidase enzyme inhibition result of stingless bee honey was investigated. All honeys extracted from different botanical origins were shown to inhibit enzyme at different concentrations. IC_{50} value for each samples were shown in Table 1. The inhibition values varies from 30 to 185 mg/mL. Lower IC_{50} value indicates higher enzyme inhibition. The highest inhibitory activity of xanthine oxidase was determined in mangrove honey (K03) at 30 mg/mL concentration while the lowest inhibitory activity was found in tualang honey (K07) at 211 mg/mL concentration. When comparing enzyme inhibition and phytochemical, negative correlation was

found between the inhibition of xanthine oxidase and total phenolic (r^2 = -0.904) and total flavonoid (r^2 = -0.003) (Table 2).

Xanthine oxidase enzyme is responsible for oxidative damage that causes a lot of pathological diseases such as gout, hyperuricemia, hepatitis, carcinogenesis and aging [13]. Xanthine oxidase regulation is an important means in the prevention of a lot of diseases. In the previous studies, Sahin [14] investigated the effects of honey on xanthine oxidase inhibition. It was found that the highest inhibitory effect was detected in chestnut honey. The previous study showed that honey exhibited a good potentials towards the inhibition of XO activity, and may contain bioactive constituents useful in the treatment of XO induced diseases. From our study, all the samples which collected from different botanical origin showed the significant inhibition of xanthine oxidase. As a result, honey is an important inhibitor depending on a floral source with the degree of inhibition against xanthine oxidase enzymes. Therefore, the regular consumption contributes to a reduction in inflammatory injuries and strengthening the human immune system.

Table 1. IC_{50} value of XO enzymes inhibitions and phytochemical properties of honeys

Botanical origins	Honey code	Total phenolic content (mg GAE $100g^{-1}$)	Total flavonoid content (mg RE $100g^{-1}$)	Xanthine oxidase-IC_{50} (mg/ml)
Acacia	K01	19.83 ± 0.002	11.56 ± 0.003	185.0 ± 0.002
Coconut	K02	28.72 ± 0.002	23.23 ± 0.002	60.0 ± 0.004
Mangrove	K03	34.23 ± 0.003	31.02 ± 0.002	30.0 ± 0.002
Starfruit	K04	17.02 ± 0.002	8.47 ± 0.002	146.0 ± 0.003
Multifruits	K05	26.79 ± 0.003	13.91 ± 0.002	110.0 ± 0.005
Multiflowers	K06	23.20 ± 0.003	12.35 ± 0.004	105.0 ± 0.001
Tualang	K07	16.37 ± 0.002	5.34 ± 0.004	211.0 ± 0.003

Note: In all the result given, the analysis were performed in triplicate and given as a ± standard deviation.

Table 2. Correlation coefficients of each analysis

		TPC	TFC	XO
TPC	Pearson correlation	1	0.936**	-0.904**
	sig. (2-tailed)		0.000	0.000
TFC	Pearson correlation	0.936**	1	-0.883**
	sig. (2-tailed)	0.000		0.000
XO	Pearson correlation	-0.904**	-0.883**	1

sig. (2-tailed)	0.000	0.000

Note: ** The correlation is significant at a 0.01 level (2-tailed)

3.2. Phenolic and flavonoid contents

The phenolic and flavonoid composition of honey sample influenced by several factors such as floral sources and seasonal, geographical, and environmental condition. The phenolic content of honey's extract from different botanical origins were presented in descending order: mangrove > coconut > multifruits > multiflowers > acacia > starfruit with the value of 34.23, 28.72, 26.79, 23.20, 19.83, and 17.02 mg GAE $100g^{-1}$ of honey, respectively as shown in Table 1 ($p < 0.05$). Meanwhile, the total flavonoid content varies widely from 8.47 to 31.02 mg RE $100g^{-1}$ sample among honey samples ($p < 0.05$). A positive correlation ($r^2 = 0.936$, $p<0.01$) was determined between total phenolic and flavonoid values as shown in Table 2. In comparison with honey produced by different bee species, tualang honey presented lower value in phytochemical composition.

Based on previous study, the northeast Portugal's honey contain total phenolic ranging from 226.16 to 727.77 mg GAE kg^{-1} of honey, whereas about 52.2 to 789.6 mg GAE kg^{-1} of phenolic has been reported for unifloral and multiflorals honeys from Africa [15,16]. Meanwhile, honeys from the northeast of Brazil contained total flavonoid varies from 2.5 to 83.8 mg quercetin/kg of honey [17]. Literature has reported on the ability of phenolic and flavonoid content to inhibit the enzyme activities [18]. These compounds are demonstrated a wide range of biological activities which consider beneficial for human.

4. Conclusion

As summary, we have revealed that honey from different botanical origins have considerable therapeutic effects on enzyme inhibitor. Due to their high level of phytochemical composition, honey from mangrove area was found to be a powerful source for XO inhibitor. The results encourage the use of stingless bee honey in clinical practice as well as to sustain the beekeeping industry in Malaysia.

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