ANTI-MICROBIAL AND ANTI-INFLAMMATORY ACTIVITIES OF CRUDE AND ACIDIFIED FRACTION OF SELECTED MALAYSIAN HONEY SAMPLES

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ABSTRACT

Honey has been reported to have anti-microbial and anti-inflammatory properties, due to the presence of polyphenol and hydrogen peroxide, respectively. To our knowledge, the detail compounds that contribute to the activities are still unknown. Therefore, this study was focused on anti-microbial and the anti-inflammation activities of selected honey samples including Tualang, Acacia and Gelam honey from Malaysia. First, the honey samples were fractionated by acidified water (pH 2) in a solid phase extractor using C18 column. Then, the honey fraction was assayed for anti-microbial and anti-inflammatory activities using well diffusion method and enzymatic cyclooxygenase (COX-1 and -2) assay, respectively. For anti-microbial study, four different microbial strains consisted of gram positive (*Staphylococcus aureus*) and gram negative (*Salmonella typimurium*) bacteria, yeast (*Candida albican*), as well as fungi (*Fusarium oxysporum*) were tested on the honey samples. The result showed that only *Salmonella typimurium* and *Candida albican* were inhibited by crude and acidified fraction of Tualang, Acacia and Gelam honey, but no inhibition was observed for *Staphylococcus aureus* and *Fusarium oxysporum*. The minimum inhibitory concentration (MICs) of the crude honey samples (50.0% w/v) and their fractions (12.5-25.0% w/v) against *Salmonella typimurium* were about two to three times higher than the MICs of Manuka honey and its fraction (6.3-12.5% w/v). However, the MICs of honey samples (3.1-6.3% w/v for crude honey samples and 0.8-3.1% w/v for their acidified fractions) against *Candida albican* appeared to be better, or at least comparable to Manuka honey samples (25.0% w/v for crude honey samples and 3.1% w/v for their acidified fractions). On the other hand, the anti-inflammatory activity of honey samples at 50% of inhibition (IC_{50}) was found to be 0.7-1.7 mg/µL for COX-1 and 0.4-1.3 mg/µL for COX-2. Fractionation did not improve the anti-inflammatory activity of honey samples because their IC_{50} values were increased to 320.0-1080.0 mg/L for COX-1 and 280.0-400.0 mg/L for COX-2. In term of selectivity ratio of COX-1/COX-2, the crude honey samples of Gelam (ratio=3.3) and its fraction (ratio=2.7) appeared to be the most selective COX-2 inhibitor, which was about two times higher than the selectivity of Manuka honey. The anti-microbial and anti-inflammatory properties of honey could be due to the presence of quercetin, chlorogenic acid, acacetin, apigenin-7-o-glucoside, myricetin and coumaryl quinic acid. These phenolic acids and flavonoids were detected in the honey fraction.

ABSTRAK

Madu telah dilaporkan mempunyai sifat anti-mikrob dan anti-inflamatori masing-masing disebabkan oleh kehadiran polifenol dan hidrogen peroksida. Dalam pengetahuan kami, sebatian yang menyumbang kepada aktiviti tersebut masih tidak diketahui. Oleh itu, kajian ini memberi tumpuan kepada aktiviti anti-mikrob dan anti-inflamatori sampel madu terpilih dari Malaysia termasuk Tualang, Acacia dan Gelam. Sampel madu dipecahkan kandungannya menggunakan air berasid (pH 2) di dalam pemecah fasa pepejal kolum C18. Pecahan madu telah dinilai untuk kehadiran aktiviti anti-mikrob dan anti-inflamatori yang dilakukan ke atas sampel pecahan madu menggunakan kaedah masing-masing iaitu telaga resapan dan cerakin enzimatik siklooksigenase (COX-1 dan COX-2). Empat jenis strain mikrob yang berbeza terdiri daripada gram positif (*Staphylococcus aureus*), gram negatif (*Salmonella typimurium*), yis (*Candida albican*) dan juga kulat (*Fusarium oxysporum*) telah diuji ke atas sampel madu. Keputusan menunjukkan bahawa, hanya *Salmonella typimurium* dan *Candida albican* telah direncatkan oleh madu mentah dan pecahan berasid daripada sampel Tualang, Acacia dan Gelam, tetapi tiada perencatan diperhatikan bagi *Staphylococcus aureus* dan *Fusarium oxysporum*. Kepekatan perencatan minimum (MICs) sampel madu mentah (50.0% w/ v) dan pecahannya (12.5-25.0% w/v) terhadap *Salmonella typimurium* adalah kira-kira dua hingga tiga kali lebih tinggi daripada MICs daripada madu Manuka dan pecahannya (6.3-12.5% w/v). Walau bagaimanapun, MICs sampel madu (3.1-6.3% w/v bagi sampel madu mentah dan 0.8-3.1% w/v bagi sampel pecahan berasid) terhadap *Candida albican* adalah lebih baik, atau sekurang-kurangnya setanding dengan sampel madu Manuka (25.0% w/v bagi sampel madu mentah dan 3.1% w/v bagi sampel pecahan berasid). Sebaliknya, aktiviti anti-inflamasi terhadap sampel madu di 50% perencatan (IC_{50}) didapati 0.7-1.7 mg/μL bagi COX-1 dan 0.4-1.3 mg/μL bagi COX-2. Pemecahan tidak meningkatkan aktiviti anti-inflamasi sampel madu kerana nilai IC_{50} telah meningkat kepada 320.0-1080.0 mg/L bagi COX-1 dan 280.0-400.0 mg/L bagi COX-2. Dari segi nisbah selektif COX-1/COX-2, sampel madu mentah dari Gelam (nisbah=3.3) dan pecahannya (nisbah=2.7) adalah merupakan perencat COX-2 yang paling selektif, di mana kira-kira dua kali lebih tinggi berbanding keselektifan madu Manuka. Ciri-ciri anti-mikrob dan anti-inflamasi madu mungkin disebabkan oleh kehadiran kuersetin, asid klorogenik, akasetin, apigenin-7-o-glukosida, mirisetin dan asid kuinik kumaril. Asid fenolik dan flavonoid tersebut telah dikesan di dalam sampel pecahan madu.

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LIST OF ABREVIATIONS

A	-	Acacia
AA	-	Arachidonic Acid
APCI	-	Atmospheric Pressure Chemical Ionisation
ATCC	-	American Type Culture Collection
ATP	-	Adinose trisphosphate
CA	-	California
COX	-	Cyclooxygenase
COX-1	-	Cyclooxygenase-1
COX-2	-	Cyclooxygenase-2
DNA	-	Deoxyribonucleic acid
ELISA	-	Enzyme-Linked Immunosorbant Assay
ESI	-	Electrospray ionization
FAMA	-	Federal Agricultural Marketing Authority
G	-	Gelam
GC	-	Gas Chromatography
GOx	-	Glucose Oxidase Enzyme
H_2O_2	-	Hydrogen peroxide
HCL	-	Hydrochloric acid
HMF	-	Hydroxymethylfurfural
HPLC	-	High-Performance Liquid Chromatography
IC_{50}		Maximal Inhibitory Concentration at 50%
IL1β	-	Interleukin B
IL-6	-	And Interleukin-6
iNOS	-	Inducible Nitric Oxide Synthase
LC-MS/MS	-	Liquid Chromatography Tandem Mass Spectrometry

LOX	-	Lipoxygenase
M	-	Manuka
MA	-	Massachusets
MBC	-	Minimum Bactericidal Concentration
MIC	-	Minimum Inhibitory Concentration
MM6	-	Macro Mac 6
MO	-	Missouri
MRM	-	Multiple Reaction Monitoring
MRSA	-	*Methicillin-Resistant Staphylococcus Aureus*
MSA	-	*Methicillin-Sensitive Staphylococcus Aureus*
NA	-	Not available
ND	-	Not detected
NO	-	Nitric oxide
NSAID	-	Non-Steroidal Anti-Inflammatory Drugs
PAMPs	-	Pathogen-Associated Molecular Pattern
PDA	-	Potato Dextrose Agar
PGD	-	Prostaglndin
PGD_2	-	Prostaglandin D_2
PLA_2	-	Phospholipase A_2
ppm	-	Parts per million
PRRs	-	Pattern Recognition Receptors
RNS	-	Reactive Nitrogen Species
ROS	-	Reactive Oxygen Species
SDA	-	Sabaraud Dextrose Agar
sp	-	Species
SPE	-	Solid Phase Extraction
T	-	Tualang
TLC	-	Thin Layer Chromatography
TNF-α	-	Tumour Necrosis Factor-A
TRIS-HCL	-	Trisbase Hydrochloric Acid
UMF	-	Unique Manuka Factor
UPLC	-	Ultra Performance Liquid Chromatography
USA	-	United State Of America
VCE	-	Vancomycin-Resistant Entrococcus

LIST OF SYMBOLS

Amu/s	-	Atomic mass unit per second
cm	-	Centimeter
eV	-	Electron volt
g	-	Gram
kg	-	Kilogram
kV	-	Kilovolt
M	-	Molarity
m/z	-	Mass to charge ratio
mg	-	Miligram
mg/µL	-	Milligram per microlitre
mg/kg	-	Milligram per kilogram
mL	-	Mililitre
mL/min	-	Mililitre per minute
mm	-	Milimeter
mM	-	Milimolar
MΩ-cm	-	Megaohm-centimeter
N	-	Normality
ppm	-	Parts per million
psi	-	Pound per square inch
V	-	Volt
v/v	-	Volume per volume
w/v	-	Weight per volume
µg	-	Microgram
µL	-	Microlitre

µL/min	-	Microlitre per minute
µm	-	Micrometer
µM	-	Micromolar
-	-	Minus
+	-	Plus
ºC	-	Degree Celcius
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Research Background

This chapter contains the research background, problem statement, significance of study, objectives and scopes of this work under the section of 1.2 to 1.5. General information about honey and its composition, as well as its applications are also discussed in this chapter.

Honey is a thick, liquid form of natural product consisted of carbohydrates, free amino acids, vitamins, trace elements, phenolic compounds, organic acids, proteins and enzymes (Kassim *et al.,* 2010 and Ferreres, 1993). Mostly, honey majority consists of saturated sugars such as fructose (38.0%) and glucose (31.0%) (Gheldof *et al.,* 2002). Honey contains approximately 17.7% of water, 0.1% of total acidity and 0.2% of ashes (Nagai *et al.,* 2006). However, honey compositions are different depending on its environmental and climate condition, as well as processes that it undergoes during processing (Gheldof *et al.,* 2002)

Honey exhibits antioxidant, anti-bacterial, antiviral, anti-microbial and anti-inflammatory activities (Martos *et al.,* 2008). Honey is also being widely used as traditional medicine. Honey is also consumed to provide gastric protection against gastric lesions (Caravaca *et al.,* 2006). It also helps to treat certain illnesses such as athma, cough, skin cancer and to promote wound healing from infection and burns (Cooper *et al.,* 1999; Molan, 1999; Fox, 2002; Molan, 2006).

Honey is a good anti-microbial agent. It was reported that honey could kill various classes of microbes, namely, gram positive and gram negative bacteria, fungi and yeast (Efem *et al.,* 1992; Nasser *et al.,* 2003*;* Halco´n and Milkus, 2004; Omoya and Akharaiyi, 2010). Honey was also reported to inhibit antibiotic resistant microbes such as Meticillin Resistant *Stapylococcus aureus* (MRSA) and Vancomycin Resistant *Enterococcus* (VRE) (Allen *et al.,* 2000). There are several factors that contribute to this anti-microbial effect such as low pH value, osmolarity effect and H_2O_2 content of honey. It is believed that there are compounds that give its anti-microbial property, particularly flavonoids and phenolic acids (Havsteen, 1983).

Many studies showed honey could reduce inflammation and treat inflammatory related disease (Subhramanyam, 1998). Honey could heal inflammation when applied directly on wound, and accelerating wound healing (Subhramanyam, 1998; Molan, 2006). The anti-inflammatroy property of honey is closely related to its flavonoid and phenolic content. For example, galangin and chrysin (flavonoid) were likely to inhibit enzyme that executed inflammation (Raso *et al.,* 2001; Kim *et al.,* 2002). Flavonoids in honey could expel free radicals that might contribute to inflammation (Garcia-Lafuente *et al*., 2009).

1.2 Research Problem Statement

Honey has known for its numerous applications since ancient time. Honey is a potential source of anti-microbial and anti-inflammatory agents. However, scientific information regarding anti-microbial and anti-inflammatory properties of Malaysian honey is very limited. Although, there were many studies done on anti-microbial activity of honey, little research was based on the use of Malaysian honey (Mulu *et al*., 2004; Al-Jabri *et al*., 2003). Therefore, it is important to collect data regarding anti-microbial and anti-inflammatory properties of local honey samples.

Many researches have been done to investigate the group of compounds that responsible for anti-microbial and anti-inflammatory effects of honey (Russel *et al*., 1990). However, the reported compounds that responsible for these effects were varied according to the honey origin. Hence, it is essential to determine the compound in local honey samples that contribute to these biological activities.

Cyclooxygenase (COX) assay has been used to test for anti-inflammatory drugs. However, little information regarding COX assay on honey samples. This is because honey is a complex mixture of compounds. Somehow, this COX assay could be used to have a rapid screening on semi-purified honey fraction for bioactive compound identification.

1.3 Significance of Study

Honey is a natural food that has been consumed since ancient time. It contains about 181 substances (Caravaca *et al*., 2006). Honey has also been used as remedies for health promotion. This indicates that honey has many benefits yet to be explored. In recent years, modern societies have become more conscious about natural treatment for disease fighting and the production of honey related products is increasing. Although some people view the idea as somewhat primitive or ignorant, many remedies are the result of empirical observation since thousands of years.

Data obtained from this research can provide information regarding anti-microbial and anti-inflammatory effect of Malaysian honey. This study also determines the groups of compounds that exhibit anti-microbial and anti-inflammatory effect. The information obtained is essential for the development of new antibiotic and anti-inflammatory drug.

The findings of this study can prove that Malaysian honey is comparable to imported Manuka honey in terms of its ability to prevent microbial infections and inflammation. This can help to boost the quality value of Malaysian honey, thus leading to the increment of Malaysia economy through import and export activity. The increase of Malaysia economy can provide more job opportunities to Malaysian.

1.4 Objectives of Study

The objective of this study was to identify bioactive compounds from Tualang, Acacia and Gelam honey samples for anti-microbial and anti-inflammatory activities based on well diffusion technique and COX assay, respectively.

1.5 Scope of Study

The scopes of this research included:

(i) To fingerprint the acidified fractions collected from honey samples.

(ii) To investigate anti-microbial activity of crude and acidified fraction of honey samples by using well diffusion technique.

(iii) To investigate anti-inflammatory activity of crude and acidified fraction of honey samples by using cyclooxygenase assay.

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