EFFECTS OF TUALANG HONEY SUPPLEMENTATION ON HAEMATOLOGICAL PARAMETERS AND OXIDANTS/ANTIOXIDANTS LEVEL IN ERYTHROCYTES OF RATS EXPOSED TO PETROL VAPOURS

BY

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science

APRIL 2013

AKNOWLEDGEMENTS

I give gratitude to Almighty Allah the Lord of the world for seeing me through this great milestone of accomplishing my study.

I would like to take this opportunity and express my profound gratitude and deep regards to my supervisors, Dr Ang Boon Suen, Associate Professor Dr. Wan Zaidah Abdullah, and Professor Dr. Siti Amrah Sulaiman for their exemplary guidance, monitoring, valuable and constructive suggestions as well as constant encouragement throughout the course of this thesis. The blessing, help, and guidance given by them time to time shall carry me a long way in the journey of life upon which I am about to embark.

I also take this opportunity to express a deep sense of gratitude to all the staffs of Physiology department, Animal Research and Service Centre, and Central Research Laboratory for their cordial support, valuable information and guidance, which helped me in completing this task through various stages. I am obliged to Dr. Friday E. Uboh, Dr Omotayo O. Erejuwa, Mr. Muhammad Salleh, Mr. Nur Mohammad, Md Faizul Ismal Che Adam, Ms. Ang Cheng Yong, Mr. Zulkefli Sanip and Mrs Aminah Che Romli for the valuable assistance provided by them in their respective fields. I am grateful for their cooperation during the period of my study. This acknowledgement cannot be complete without the mention of my colleagues ib the Department of Physiology, USM, all my Nigerian friends/colleagues presently studying in various Malaysian Institutions as well as those in the Department of Physiology, Usmanu Dan Fodiyo University Sokoto, Nigeria, I sincerely appreciate their tremendous support throughout my study.

I would like to acknowledge Universiti Sains Malaysia for providing a short term research grant (304/PPSP/61312005) to support the study. I would also like to acknowledge the Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia for supplying the Tualang honey.

Lastly, I thank my late dad, mum, brothers, and sisters, for their constant prayers, encouragement and support without which this project would not be possible. I am deeply indebted to my wife Asiya A. Sani and my kids Safeeya and Abdulmutallib for their care, love, patience and understanding throughout the course of my study. My mum has been my strongest motivation and inspiration, I therefore dedicate this thesis to her and hope that I have made her proud.

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LIST OF ABBRIVIATIONS

AIDS	Acquired Immuno-deficiency Syndrome
ALP	Alkaline phosphatise
ALT	Alanine transaminases
AST	Aspartate transaminases
BTX	Benzene, toluene, xylene
CAT	Catalase
Cu/Zn-SOD	Copper/zinc superoxide dismutase
DIPE	diisopropyl ether
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DTPA	Diethylenetriaminepentaacetic acid
EC-SOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EPG	Experimental petrol control group
ETBE	Ethyl tertiary butyl ether
ETG-1	Experimental honey test group-1
ETG-2	Experimental honey test group-2
FRAP	Ferric reducing antioxidant power
FSH	Follicle stimulating hormone
GGT	Gamma glutamyl transferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione

GSSG	oxidized glutathione
GST	Glutathione-S-transferases
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCG	Honey control group
IP	intraperitoneal
IQR	Interquartile range
LH	Luteinizing hormone
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	malondealdehyde
ME	Myeloid erythroid
MGG	May-Grunwald-Giemsa
MnSOD	Manganese superoxide dismutase
MTBE	Methyl tertiary butyl ether
NADH	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP+
NCG	Normal control group
NO	Nitric oxide
$OONO^-$	peroxynitrite
ORAC	Oxygen radical absorbance capacity
PCV	Packed cell volume
PWG	Percentage weight gain

RBC	Red blood cell
RDW	Mean corpuscular haemoglobin concentration
RNS	Reactive nitrogen species
RON	Research octane number
ROS	Reactive oxygen species
RS	Reactive species
RVP	Reid vapour pressure
SDS	Sodium dodecyl sulphate
SHR	Spontaneous hypertensive rats
SOD	Superoxide dismutase
STZ	Streptozocin
TAC	Total antioxidant capacity
TAEE	Tertiary-amyl ethyl ether
TAME	Tertiary amyl methyl ether
TAS	Totala antioxidant status
TBA	Tertiary butyl alcohol
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acids reactive substances
TNF-α	Tumour necrosis factor-α
VOC	Volatile organic compounds
WBC	White blood cells
WKY	Wister-Kyoto rats

KASAN DARIPADA SUPLEMEN MADU TUALANG PADA PARAMETER HEMATOLOGI DAN OKSIDAN/ANTIOKSIDAN PERINGKAT DALAM ERITROSIT TIKUS YANG TERDEDAH KEPADA WAP PETROL

ABSTRAK

Petrol digunakan sebagai bahan api untuk pembakaran dalaman enjin. Pelbagai risiko kesihatan tentang pendedahan kepada petrol telah dilaporkan. Ia termasuk penurunan berat badan, hematotoksisiti, tekanan oksidatif, nephrotoksisiti dan keracunan saraf pusat. Suplemen antioksidan seperti vitamin A, C, dan E telah menunjukkan pengurangan kesan toksik wap petrol. Madu mengandungi vitamin, dan polifenol mempamerkan sifat-sifat antioksida yang baik. Kajian ini telah dijalankan untuk mengenalpasti potensi peranan madu dalam perlindungan ke atas kesan buruk pendedahan kepada gasolin terhadap berat badan, parameter hematologi, dan status oksida/antioksidan dalam eritrosit tikus. Sejumlah 56 ekor tikus Sprague-Dawley jantan (berumur 3-4 minggu dengan berat badan 170-230g) telah digunakan dalam kajian ini. Enam tikus telah digunakan pada fasa permulan dan dibahagi secara rawak kepada kampulan kawalan dan kumpulan ujian yang terdedah kepada gasolin (3 ekor tikus setiap kumpulan). Kampulan kawalan didedah kepada udara ambien biasa setiap hari, manakala kumpulan ujian telah didedahkan kepada wap gasolin (11.13 ± 1.1cm3/jam, 6h sehari, 6 hari/seminggu untuk tempoh 11 minggu. Berat badan telah dipantau setiap minggu dan sampel darah diambil pada minggu ke-4, 6, 8, 10, dan 11 untuk kiraan sel-sel darah penuh dan kiraan pembezaan sel darah putih (FBC+ DC). Pada akhir fasa, tikus telah dikorbankan dan sumsum tulang diekstrak untuk peperiksaan sitologi. Baki 50 tikus telah digunakan untuk fasa II dan dibahagi secara rawak kepada lima kumpulan (10 tikus setiap

kumpulan) dan dirawat selama 11 minggu seperti berikut: kumpulan kawalan normal (NCG): dirawat dengan air suling 0.5ml dan terdedah kepada udara ambien biasa, kumpulan kawalan terdedah kepada petrol (EPG): dirawat dengan air suling 0.5ml dan terdedah kepada petrol seperti di atas, kumpulan kawalan suplemen madu (HCG): dirawat dengan madu 1.2g/kg berat badan setiap hari dan terdedah kepada udara ambien biasa, kumpulan ujian terdedah petrol dan dirawat dengan madu 1 (ETG-1): terdedah kepada petrol dan serentak dirawat dengan madu seperti di atas, dan kumpulan ujian pendedahan petrol dan madu 2 (ETG-2): terdedah kepada petrol seperti di atas dan dirawat dengan madu pada dua minggu terakhir eksperimen). Peningkatan berat badan dipantau secara mingguan dan pada penghujung experimen. Sampel darah untuk FBC + DC dan penanda oksidatif diambil secara tusukan jantung; dan sumsum tulang diekstrak untuk sitologi setelah dikorbankan. Keputusan fasa I telah menunjukkan bahawa perbezaan yang ketara dalam peningkatan berat badan dan parameter hematologi mula muncul pada tempoh pendedahan minggu ke 10.

Kajian Fasa II telah menunjukkan bahawa pendedahan kepada petrol adalah berkaitan dengan kemerosotan peningkatan berat badan yang signifikan ($p<0.05$) dan menyebabkan pengurangan min kepekatan hemoglobin korpuskel (MCHC) yang signifikan ($p<0.05$) serta peratusan megakriosit abnormal yang lebih tinggi. Di samping itu, pendedahan kepada petrol tidak menyebabkan perubahan penanda oksidatif yang ketara. Kesimpulannya, kajian menunjukkan bahawa pendedahan kepada petrol menyebabkan kesan buruk pada berat badan, indeks sel darah dan megakriosit dalam sumsum tulang; dan suplimentasi madu berpotensi untuk melindungi kesan-kesan buruk tersebut.

EFFECTS OF TUALANG HONEY SUPPLEMENTATION ON HAEMATOLOGICAL PARAMETERS AND OXIDANTS/ANTIOXIDANTS LEVEL IN ERYTHROCYTES OF RATS EXPOSED TO PETROL VAPOURS

ABSTRACT

Petrol is used as fuel for internal combustion engines. Different health risks including weight loss, haematotoxicity, and oxidative stress have been reported for gasoline. Supplementation with antioxidants such as vitamins A, C, and E has been shown to ameliorate the toxicity effects of gasoline vapours exposure. Honey is a natural product which contains vitamins and polyphenols that possess good antioxidant properties. The present study was carried out to determine the potential protective role of honey against the adverse effects of exposure to gasoline on weight gain, haematological parameters, bone marrow cytology, and oxidants/antioxidants status in erythrocytes of rat. A total of 56 male Sprague-Dawley rats (aged 6-7 weeks, 170-230g) were used in the study. Six rats were used for phase 1 and were randomized and treated as control (exposed to ambient air daily, n=3) and gasoline exposed (exposed to gasoline vapours 11.13±1.1cm^3/h, 6h daily, 6 days/week, n=3) groups. Body weight was monitored weekly and blood sample for full blood count (FBC) and differential counts (DC) was collected at 4th, 6th, 8th, 10th, and 11th week duration. At the end of this phase, the rats were sacrificed and bone marrow was extracted, smeared and stained for cytology. Fifty rats were used for phase II and randomized into five groups (10 per group) and treated for 11 weeks as follows: Normal control group (NCG) - treated with 0.5ml distilled water and exposed to ambient air daily, experimental petrol control group (EPG) - exposed to gasoline +

distilled water as above, honey control group (HCG) - treated with honey 1.2g/kg body weight daily, experimental honey test group (ETG-1)- exposed to gasoline and concurrently treated with honey as above, and experimental honey test group-2 (ETG-2) - exposed to gasoline as above and treated with honey during the last two weeks of the experiment. Weight gain was monitored weekly and at the end of experimental period, blood sample for FBC + DC and oxidative markers was collected via cardiac puncture; and bone marrow was extracted for cytology. The result of phase I established that significant alterations in weight gain and haematological parameters appeared in the gasoline exposed group on the 10^{th} week. The results of phase II showed that exposure to gasoline was associated with significant ($p<0.05$) impairment of weight gain and reduction in mean corpuscular haemoglobin concentration (MCHC) as well as higher percentage of abnormal megakaryocytes. Honey supplementation significantly improved the percentage of abnormal megakaryocytes but did not improve the weight gain and MCHC values. In addition, exposure to gasoline did not cause significant changes in oxidative markers.

In conclusion, this study indicates that exposure to gasoline caused adverse effects on weight gain, blood cell indices and bone marrow megakaryocytes; and that supplementation with honey has the potential to protect against some of the adverse effects.

CHAPTER ONE

INTRODUCTION

1.1 Background

Gasoline is also known as petrol. It is primarily used as fuel for internal combustion engines and also used as diluents, finishing agent, and industrial solvent. It is a petroleum derived mixture of over 500 hydrocarbons that may have between 3 – 12 carbon atoms (Kinawy, 2009). The major sources of exposure to gasoline are oil refineries, oil fields, gasoline filling stations, petrochemical industries, motor mechanical workshops and machines powered by two stroke engines (MacGregor, 1993; Raabe and Wong, 1996). Another important source of exposure to gasoline constituents is gasoline sniffing among poor societies as a cheaper way for mood alterations which has now become a rampant phenomenon (Burbacher, 1993). It has been estimated that about 110 million individuals are exposed to gasoline during the process of refuelling at self-service filling stations (Wixtrom and Brown, 1992). Thousands of individuals working in both oil refineries and gasoline filling stations are continuously exposed to components of gasoline and its by-products (MacGregor, 1993). The composition and characteristics of gasoline depend on the origin of the crude oil, differences in the processing methods and blends, seasonal variations as well as additives such as oxygenates that are required to meet a specific engine performance (Caprino and Togna, 1998). Some of the major components of gasoline of health concern are Benzene, Toluene, and Xylene and are abbreviated as BTX (Periago and Prado, 2005). These compounds are also known as volatile organic compounds (VOCs) (Backer, 1997).

Several studies have reported that significant exposure to benzene exists during self-refuelling of cars at service stations (Egeghy *et al.*, 2000; Pandey *et al.*, 2008b). It has been estimated that for every 30 litres of gasoline containing 5% benzene that is pumped during the process of refuelling, about 700mg of benzene is vaporized (Howard, 1975). Significant levels of other VOCs such as toluene and xylene have been found in the blood of both gasoline station attendants and individuals who self-refuel their vehicles (Backer, 1997; Pandey *et al.*, 2008b; Rekhadevi *et al.*, 2010). The risk of exposure to these VOCs among gasoline station attendants is significantly related to the duration and the volume of gasoline pumped in a given period (Backer, 1997; Periago and Prado, 2005).

There are clear evidences of a casual relationship between occupational exposures to benzene and the occurrence of certain types of leukaemias such as acute myeloid leukaemias (Hayes *et al.*, 1997; Rinsky *et al.*, 2002). There is also an increased frequency of mironucleates in exfoliated buccal cells of gasoline station workers; this could increase the risk of cytogenetic damage among gasoline station workers (Çelik *et al.*, 2003). These findings are supported by other reports which demonstrated significant DNA damage amongst gasoline station attendants in service stations (Pandey *et al.*, 2008; Rekhadevi et al., 2010). Occupational exposures to benzene are also associated with increased frequency of aneuploid sperm (Xing *et al.*, 2010) and decreased levels of antibodies and leukocytes which can impair both humoral and cellular components of immune system (Aksoy *et al.*, 1987; Yin *et al.*, 1987). Long term Exposure to gasoline is also associated with increased risk of developing cancer of the lung and kidney as well as Nasal carcinoma (Lynge *et al.*, 1997).

Experimental animal studies have shown decreased serum level of estradiol and progesterone in female rats; and elevated level of testosterone in male rats exposed to gasoline vapours (Uboh *et al.*, 2007); however, with a longer duration of exposure in a similar study, the serum level of testosterone was significantly reduced and in addition, a distortion of normal histology of testicular tissues was observed (Uboh *et al.*, 2010c). A significant effect on the central nervous system associated with a fluctuation in the levels of monoamine neurotransmitters accompanied with a higher possibility of developing aggressive behaviour was observed in rats exposed to VOCs (Kinawy, 2009). In a similar experiment, impairment of kidney functions manifested by increased levels of serum creatinine, urea, blood urea nitrogen, uric acid, and potassium ion as well as decrease in the levels of serum sodium and chloride ions were observed (Uboh *et al.*, 2008a; Uboh *et al.*, 2010a). Significantly increased activity of liver enzymes such as alanine tansaminase (ALT), aspartate transaminase (AST), gamma-glutamyltransferase (GGT), and alkaline phosphatase (ALP) as well as elevated serum levels of bilirubin in both male and female rats exposed to gasoline vapour was observed and the changes were more severe in the female group than the corresponding male group (Uboh *et al.*, 2005; Uboh *et al.*, 2008c; Uboh *et al.*, 2009b). These findings are supported by the observed increase in size of liver (hepatomegaly) (Uboh *et al.*, 2008c). Gasoline vapour exposure among both male and female rats is associated with decrease in the percentage weight gain and percentage growth rate and the female rats were more affected than the males (Uboh *et al.*, 2008b; Uboh *et al.*, 2008c). An associated decreased levels of haemoglobin (Hb), packed cells volume (PCV) and red blood cells (RBC), and a concomitant increased white blood cells (WBC) count was also observed in both male and female rats exposed to gasoline vapours (Uboh *et al.*, 2008b).

Several experimental animal studies have demonstrated that supplementation with antioxidants reduced the toxic effects of gasoline vapour exposure. Treatment with both water soluble vitamins (e.g. Vitamin C) and lipid soluble vitamins (Vitamins A & E) of rats exposed to gasoline vapour has been shown to reduce the toxicities associated with the gasoline vapour exposures (Uboh *et al.*, 2009b; Uboh *et al.*, 2010c). Administration of vitamin A and E ameliorated the toxic effects of gasoline exposure on Hb, PCV, RBC, and weight of both male and female rats exposed to gasoline (Uboh *et al.*, 2008b; Uboh *et al.*, 2010b); however, the potency of the vitamins in ameliorating the toxicities was observed to be more in female than male rats. Administration of vitamin C was also found to reduce the toxic effects of gasoline vapour exposure on serum testosterone level and testicular tissues (Uboh *et al.*, 2010c). It is therefore believed that the presence of antioxidants may ameliorate the toxic effect of exposure to gasoline vapours (Uboh *et al.*, 2008b).

However, in general, assessment of health risks associated with exposure to gasoline has not been an easy task for scientists; this is because of many factors such as inconsistent results from various reports, imprecise scientific conceptualization in some investigations, and variation in composition of different gasoline formulations (Caprino and Togna, 1998). Several strategies to minimize or curtail the health hazards related to gasoline exposure have been put in place in the recent time. For instance the use of leaded gasoline was replaced by non leaded one. Similarly, some of the potentially toxic compounds were also removed from the gasoline mixture and oxygenates were introduced to enhance engine performance (Schuetzle *et al.*, 1994; Ahmed, 2001). These approaches have resulted in manufacturing of reformulated gasoline mixture devoid of toxic heavy metal additives and with relatively lower

concentrations of toxic compounds like benzene (Constantini, 1993). These strategies however, face a lot of challenges in different countries depending on the laws put in place by the regulatory authorities (Caprino and Togna, 1998). For example, in Europe, Euro II standard recommends that the research octane number [(RON), which is a measure of performance of a fuel and the higher it is, the more the compression a fuel formulation can withstand] of all fuels should be 97 and that Reid Vapour Pressure (RVP), total sulphur, and volume of benzene should not exceed 65 kPa, 500 ppm, and 5% respectively (Tan, 2007a) The European Unions had already moved to this standard since 1995 which was subsequently replaced by Euro III standard and Euro IV standard in 1999 and 2005 respectively (Tan, 2007b). The Euro IV standard further limits the total sulphur to maximum of 50 ppm and volume of benzene to maximum of 1% (FirdausMarzuki; and Isa, 2006). In an attempt to improve the air quality in Malasyia, the Malaysian government proposed to move to Euro II standard by the end of 2007 (Tan, 2007b). During the 4th Petroleum Technology Symposium held in Campbodia in 2006, it was revealed that three out of the six major fuels in Malaysia i.e. Shell, Petronas, BHP, Esso-Mobil, Caltex, and Project were already Euro II compliant (FirdausMarzuki`; 2006). The identity of which of the fuels has moved to the Euro II was however not revealed (Tan, 2007a). It could therefore be expected that the fuel quality would have improved from that time to present.

Honey has a long history of human consumption and is commonly used for both nutritional and medicinal purposes. There is increasing evidence from previous studies that honey has the potential to enhance health or reduce risk of diseases (Tan *et al.*, 2009). Recent study by Mohamed *et al* (2010) has shown that Malaysian

Tualang honey possesses good colour intensity and contains phenolic compounds that possess relatively good antioxidant activity comparable to other types of honey such as Slovenian honey, herb honeys and Romanian honeys. Similarly, in comparison to local honeys such as Gelam honey, Borneo tropical honey, etc; Tualang honey has been found to possess better antioxidant effects and radical scavenging properties (Kishore *et al.*, 2011).

One of the interesting findings about honey is its antioxidant activity (Aljadi and Kamaruddin, 2004; Bogdanov *et al.*, 2008; Omotayo *et al.*, 2010). Omotayo and colleagues have demonstrated in their study that oxidative stress in kidneys of streptozotocin-induced diabetic rats was ameliorated by administration of Tualang honey in a dose dependant manner (Omotayo *et al.*, 2010). In a similar finding, it was shown that Tualang honey exerts some protective effects on pancreas of streptozocin-induced diabetic rats against oxidative stress (Erejuwa *et al.*, 2010a). The antioxidant activity of tualang honey is further confirmed by a related study where effects of a combination of Glibenclimide and Metformin on oxidative stress in pancreas and kidneys of diabetic rats was compared with effect of combination of Glibenclimide + Metformin + tualang honey in another similar group, the findings of which show that, the later combination conferred significant protection against oxidative stress (Erejuwa *et al.*, 2010b; Erejuwa *et al.*, 2011b). However, to date, no available reports exist on the effects of honey supplementation in ameliorating toxicities associated with exposure to gasoline vapours.

1.2 Objectives of the Study

The main objective of this study is to determine the possible protective role of Tualang honey supplementation against the toxic effects of exposure to gasoline

vapours on body weight, haematological parameters and oxidants/antioxidants levels in male rats. The study was divided in to two phases:

Phase 1 (preliminary study): which is aimed to establish the duration of exposure to gasoline that could produce the intended affect on body weight, haematological parameters and bone marrow. The specific objectives for phase I study are:

(a) To determine the effect of exposure to gasoline vapours on percentage weight gain of rats.

(b) To establish the duration of exposure to gasoline sufficient to produce significant changes in haematological parameters.

(c) To determine the effect of exposure to gasoline on bone marrow cytology at the end of the duration established in (i) above.

Phase II (intervention) study: Which is aimed to determine the effect of honey supplementation on body weight, haematological parameters, bone marrow and oxidants/antioxidants levels of rats exposed to gasoline vapours. The pecific objectives for phase II study are:

(a) To determine the effect of honey supplementation on weight changes associated with 11 weeks gasoline vapours exposure in rats.

(b) To determine the effect of honey supplementation on haematological parameters of rats exposed to gasoline vapours for 11 weeks.

(c) To determine the effect of honey supplementation on cytomorphology of bone marrow in rats exposed to gasoline vapours for 11 weeks.

(d) To determine the effect of honey supplementation on oxidants/antioxidants level in erythrocyte lysates of rats exposed to gasoline vapours for 11 weeks.

1.3 Research Hypothesis

It was hypothesized that:

(a) Tualang honey protects against or ameliorates the toxic effects of exposure to gasoline vapours on percentage growth rate of male rats.

(b) Tualang honey significantly protects against or ameliorates the toxic effects of exposure to gasoline vapours on haematological parameters of male rats.

(c) Tualang honey has the potential to significantly protect or ameliorate the toxic effects of exposure to gasoline vapours on bone marrow cytomophorlogy in male rats.

(d) Tualang honey protects against or ameliorates the toxic effects of exposure to gasoline vapours on erythrocyte lysate oxidative markers in male rats.

1.4 Significance of the Study

This study evaluated the possible protective role of honey against haematological, and bone marrow toxicities, as well as oxidative stress associated with exposure to gasoline vapour; hence, it has the potential of offering some solutions for ameliorating and/or preventing toxicities associated with exposure to gasoline vapours among gasoline station workers who are occupationally exposed to constituents of the gasoline, as well as the protective role of honey against such toxicities to the general population. Moreover, no documentation on the potential role of honey in prevention against the toxicity effects of gasoline exists, hence the

present study has provided baseline data which could be further used to explore the potential preventive role of honey in ameliorating such toxicities due to exposure to gasoline.

1.5 Limitation of the Study

The results obtained in this study are based on findings in animal models; thus, they may not reflect the exact situation in humans

CHAPTER 2

LITERATURE REVIEW

2.1 Honey

2.1.1 Definition

Honey is a natural sweet substance, produced by honey bees from the nectar of plants or from secretion of living parts of plants or excretion of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Codex Alimentarius Commission, 2001).

2.1.2 Formation of honey

Honey bees collect their honey from two sources namely, nectar (a sugar solution secreted in the flower nectary) and honeydew (a secretion product of plant-sucking insects with varying sugar concentration excreted as droplets). Honey formation begins when bee foragers collect nectar and honeydew from plants and carry them by means of their honey sac and take to their colony. They subsequently add enzymes from their hypopharyngeal glands and transfer the mixture to the colony bees, which is then passed over to each other by these nurse bees (colony bees) and the honey is finally filled into the combs. During this process, the bees continuously fan with their wings; this reduces the honey's humidity until the water contents reaches 30-40% when the honey is deposited into the combs. At the same time the bees add two additional enzymes i.e. invertase (which transforms sucrose into fructose + glucose) and glucose oxidase, (which oxidizes glucose to gluconic acid and hydrogen peroxide). The hydrogen peroxide acts as an agent against bacterial contamination.

The continuous fanning and relatively warm colony temperature (35^{o}C) reduces further the honey humidity. In addition, the bees also suck out the honey and deposit it back into the combs which also further reduce the water content of the honey. This transformation process occurs in one to three days and by this time the honey is ripe with a humidity of less than 20%; it is at this time that the bees cap the combs thereby preventing absorption of moisture by honey. In general, only under rare situations (high humidity or tropical conditions) can honey with more than 20% water be capped by bees. The water content of honey plays an important role in the quality and storage capacity of honey. It depends on a number of factors such as humidity, temperature, colony strength, hive type and intensity of honey flow (Bogdanov, 2010).

2.1.3 Types of Honey

Classification of honey is based on a number of qualities notably floral sources, geographical origin, technique of processing and production, as well as colour.

2.1.3 (a) Floral origin

Honey is either referred to as: unifloral (when it is mainly made from single plant species); or multifloral (when it contains pollen from more than one plant species without domination by any single plant species). The designation of honey in to such categories is according to the floral or plant source where the whole or major part of the honey comes from and as a guide, it has the organoleptic, physicochemical and microscopic properties equivalent to that source (Molan, 1998; Bogdanov, 2011b; Ramírez-Arriaga *et al.*, 2011).

2.1.3 (b) Geographical or topographical origin

Honey can be assigned the name of a geographical or topographical area provided that it was produced exclusively within the region referred to in the naming (Bogdanov, 2011b; Wang and Li, 2011).

2.1.3 (c) Technique of production

Honey can be designated as extracted honey (when it is obtained by centrifuging decapped honey combs); pressed honey (when it is obtained by squeezing the honey combs) and drained honey (when it is obtained by draining decapped honey combs) (Bogdanov, 2011b).

2.1.3 (d) Processing technique

Honey can be referred to as normal honey (when it is in liquid or crystalline form or a mixture of both); Comb honey (when it is laid up by the bees in newly constructed broodless combs); and cut comb in honey or chunk honey (when it contains one or more sections of comb honey) (Bogdanov, 2011b; Chang *et al.*, 2011; Liberato *et al.*, 2011).

2.1.4 Chemical Constituents of honey

In general, honey contains both chemical and microbiological components. The chemical constituents include carbohydrates, proteins, enzymes, minerals, vitamins, and phenolic compounds, whereas the microbiological constituents include bacteria and yeast (Manzanares *et al.*, 2010; Bogdanov, 2011a).

2.1.4 (a) Carbohydrates

Sugars are the major components of honey, consisting about 95% of honey dry weight. The sugars are mainly fructose and glucose. Other sugars found in honey include maltose, turanose, erlose, melezitose and raffinose (Bogdanov, 2011a; Erejuwa *et al.*, 2011a).

2.1.4 (b) Protein and amino acids

Honey contains relatively small amount of proteins in form of amino acids and enzymes; the latter is added during the process of honey formation. The major enzymes are diastase (amylase), invertase (saccharase, α-glucosidase), glucose oxidase and catalase (Bogdanov *et al.*, 2008; Manzanares *et al.*, 2010; Bogdanov, 2011a). The predominant amino acid is proline, others include phenylalanine, aspertatic acid, glutamic acid, asparagines, serine, glutamine, histidine, glycine, threonine, arginine, □-alanine, α-alanine, gama-aminobutyric acid, tyrosine, valine, tryptophan, leucine, isoleucine, ornithine and lysine (White and Doner, 1980; Pérez *et al.*, 2007).

2.1.4 (c) Vitamins

The amount of vitamins present in honey is variable and probably depends on the source. The major vitamins reported include thiamine (B1), riboflavin (B2), panthothenic acid (B3), ascorbic acid (C) nicotinic acid, and pyridoxine (Haydak *et al.*, 1942)

2.1.4 (d) Phenolic compounds

Honey contains a large number of phenolic compounds in the form of flavonoids (quercetin, kaempferol, galangin, fisetin, myricetin, pinocembrin, naringin, hesperidin, pinobanksin, apigenin, acacetin, chrysin, luteolin, gankwanin, wogonin, and tricetin); phenolic acids (caffeic acid, chlorogenic acid, cinnamic acid, p-coumaric acid, vanillic acid, ferulic acid, p-hydroxybenzoic acid, gallic acid, syringic acid, rosmarinic acid and derivatives) as well as coumarins (coumarin) and tannins (ellagic acid) as summerized in a review recently published by our team (Abubakar *et al.*, 2012).

2.1.4 (e) Organic acids

Honey is generally acidic with gluconic acid being its major acid component. The pH varies according to the type of honey and ranges from 3.3 to 6.5 (Bogdanov, 2011a). Varying concentrations of organic acids have been reported to be found in honey. These include citric, pyruvic, tartaric, galacturonic, gluconic, malonic, malic, citramalic, quinic, sucinic, glycolic, lactic, fumaric, formic, acetic, propionic, and butyric acids (del Nozal *et al.*, 1998).

2.1.5 Physical Properties of Honey

2.1.5 (a) Water content and viscosity

Honey posses water which serves as its quality parameter. The part of water that is bound to sugars is inaccessible to microorganisms hence making it difficult for bacterial contamination. Honey is usually very viscous and its viscosity depends on the water content and on temperature. However, the composition of honey has little

influence on the viscosity (Manzanares *et al.*, 2010; Bertoncelj *et al.*, 2011; Bogdanov, 2011a).

2.1.5 (b) Density

Density is an important physical property of honey. Honey density is 50% more than that of water and it also depends on the water content (Bogdanov, 2011a).

2.1.5 (c) Hygroscopicity

Hygroscopicity is the ability of a substance to attract and hold water molecules from the surrounding environment. Hygroscopicity of honey is one of its important characteristic especially in processing and storage; it increases in high humid conditions. Thus it is necessary to close honey containers tightly when stored in humid areas (Manzanares *et al.*, 2010; Bertoncelj *et al.*, 2011; Bogdanov, 2011a).

2.1.5 (d) Colour

The colour of honey in liquid form varies from clear and colourless to dark amber or black. Honey colour plays an important role in marketing and in deciding its end use. Darker honeys in general are usually for industrial uses, while lighter honeys are marketed for consumption (Bertoncelj *et al.*, 2011; Bogdanov, 2011a).

2.1.6 Biological properties of honey and alternative traditional medicinal values

2.1.6 (a) Antimicrobial property

Honey possesses antibacterial activity against both gram positive bacteria (Streptococcus pyogenes and Staphylococcus aureus) and gram negative bacteria

(Pseudomonas aeruginosa, Shigella flexneri, and Escherichia coli) (Tan et al., 2009). This antibacterial effect may be attributed to relatively high osmotic effect of sugar content of honey, low pH of honey (3.6-4.5), and the presence of hydrogen peroxide as well as its phenolic acids and flvonoids contents (Wahdan, 1998). In another study, it was suggested that the bactericidal effect of honey is through interruption of cell cycle machinery particularly the cell division (Henriques et al., 2010). Honey also possesses antifungal activity against epidermophyton and microsporum species (El-Gendy, 2010).

2.1.6 (b) Anti-inflammatory property

Honey has been reported to have anti-inflammatory property. It reduces both acute and chronic inflammation, although the exact mechanism for its anti-inflammatory activity is not fully established, a number of effects have been reported as reviewed by (Pieper, 2009) for example, a reduction in number of inflammatory cells was observed in biopsy specimens exposed to honey. Application of honey-based ointment was found to significantly improve the condition of patients with atopic dermatitis or psoriasis following two weeks of treatment (Al-Waili, 2003b). It was also reported that honey enhanced relief of cough symptom in children with upper respiratory tract infection (Heppermann, 2009).

2.1.6 (c) Antioxidant property

Honey has been shown to be rich in both enzymatic and non enzymatic antioxidants such as catalase, flavonoids, alkaloids, polyphenols, carotenoid, maillard-reaction products, and vitamins (Haydak et al., 1942; Schepartz, 1966; Gheldof et al., 2002). The antioxidant property of honey is in fact ascribed to its phenolic contents

inclduding the flavonoids (Beretta *et al.*, 2007; Van Den Berg *et al.*, 2008; Khalil *et al.*, 2011; Kishore *et al.*, 2011a). It is believed that some of the constituents of honey may act synergistically to produce its antioxidant effect (Gil et al., 1995), as a significant correlation was observed between the antioxidant strength of two Malaysian honeys and their total phenolic contents; hence this implies that the total phenolic contents of honey play a vital role in their antioxidant property (Aljadi and Kamaruddin, 2004). Furthermore, the amount and type of antioxidant components of honey vary among samples depending on such factors as plant source and colour of the honey; darker honeys have higher antioxidant constituents than brighter honeys (Gheldof *et al.*, 2002). The in vitro antioxidant effect of honey as well as its antiradical property has been studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, oxygen radical absorbance capacity (ORAC) assay, and the ferric reducing antioxidant power (FRAP) assay (Gheldof *et al.*, 2002; Hussein *et al.*, 2011). These assays have been widely used to demonstrate that different varieties of honey from various sources across the globe show high antioxidant activities. Oddo and colleagues have demonstrated a high antioxidant activity in Australian honey (Oddo *et al.*, 2008). In another study, Turkish red pine honey was demonstrated to scavenge DPPH, thus suggesting its antiradical property (Akbulut *et al.*, 2009). Similar antioxidant activities were reported for Peruvian honeys produced by ten species of stingless bees (Rodríguez-Malaver *et al.*, 2009).

A vast number of reports have been documented on the antioxidant properties of a variety of honeys including Ecoudorian stingless bee (Meliponinae) honey (Guerrini *et al.*, 2009), Venezuelan honey from *Apis mellifera* (Vit *et al.*, 2009), monofloral Cuban honeys (Alvarez-Suarez *et al.*, 2010), Portugal honey (Estevinho *et al.*, 2008),

Spanish honey (Pérez *et al.*, 2007), Croatian honeydew honey (Jerković and Marijanović, 2010), American buckwheat honey (Van Den Berg *et al.*, 2008).

2.1.6 (d) Anticancer property

Invitro studies have shown that honey displays anticancer activity against breast, colon and liver cancers (El-Gendy, 2010). It has been reviewed that honey: has the ability to induce apoptosis; possesses antimetastatic activity, control the proliferation of bladder cancer cells and these properties could be attributed to presence of polyphenols in honey (Jaganathan and Mandal, 2009). In a related study, honey was found to cause apoptotic cell death in renal carcinoma (Samarghandian et al., 2011). We have previously reviewed that the bulk of phenolic compounds found in honey have been demonstrated to have anti-leukaemic activities (Abubakar et al 2012).

2.1.6 (e) Immuno-modulatory activity

An in vitro study using human monocytic cell line has shown that three different types of honey (Manuka, pasture, and jelly) have stimulatory effects on the production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), and interleukin-1□ as well as an anti-inflammatory cytokine interleukin-6 (Tonks *et al.*, 2003b). A 5.8 kDa of Manuka honey has also been demonstrated to stimulate cytokine production via TLR4 in the above mentioned type of cells (Tonks et al., 2007). Honey has chemo tactic effect on neutrophil (Fukuda et al., 2008) and also stimulates antibody production during both primary and secondary immune responses (Al-Waili and Haq, 2004). It was demonstrated in a case report of an AIDS patient that honey caused reduction in elevated levels of prostaglandins with a

concomitant increase in nitric oxide levels, and percentage of lymphocytes among other parameters (Al-Waili et al., 2006).

2.1.7 Honey and haematological parameters

Daily consumption of honey for two weeks has been shown to elevate the values of haemoglobin, red blood cells, packed cell volume, monocytes, lymphocytes and eosinophils in healthy human subjects (Al-Waili, 2003a). In a similar study, it was demonstrated that honey exhibited stimulatory effects on T-lymphocytes and monocytes in cell cultures with subsequent release of cytokines, TNF-α, interleukin-1, and interleukin-6 by the monocytes (Abuharfeil *et al.*, 1999; Tonks *et al.*, 2001; Tonks *et al.*, 2003).

2.2 Gasoline

2.2.1 Overview of gasoline Composition

Present in gasoline are over 500 hydrocarbons that may have between 3 – 12 carbons (Backer, 1997; Kinawy, 2009). The inevitable exposure to this substance in our daily life cannot be over stressed (Uboh *et al.*, 2008b). Although, the composition and characteristics of different batches of gasoline depend on the origin of the crude oil, differences in the processing methods, and blends (Raza *et al.*, 1995), some of the major components of gasoline of health concern are Benzene, Toluene, and Xylene abbreviated as BTX (Yin *et al.*, 1987; Hayes *et al.*, 1997; Periago and Prado, 2005); and also known as volatile organic compounds (VOCs) (Wixtrom and Brown, 1992; Backer, 1997; Kinawy, 2009). A vast number of studies have reported increased

levels of these VOCs in the ambient air of petrochemical and related occupational sites (Chakroun *et al.*, 2002; Georgieva *et al.*, 2002; Melikian *et al.*, 2002; Chanvaivit *et al.*, 2007; de Oliveira *et al.*, 2007; Hoet *et al.*, 2009). Similar findings were obtained following air monitoring in breathing zones of individuals at different sources of exposure such as gasoline filling stations and heavy traffic areas among other settings (Inoue *et al.*, 2001; Waidyanatha *et al.*, 2001; Fustinoni *et al.*, 2005; Qu *et al.*, 2005; Manini *et al.*, 2006; Chanvaivit *et al.*, 2007). In keeping with this, individuals exposed to gasoline have been found to have elevated levels of the VOCs in their blood (Carere and Crebelli, 1997; Chanvaivit *et al.*, 2007; Pandey *et al.*, 2008; Hoet *et al.*, 2009).

Fuel octane number is a standard measure of the performance of a motor or aviation fuel. It is a measure of its resistance to knock. The higher the octane number, the more compression the fuel can withstand before detonating. Efforts to replace antiknock lead derivatives in gasoline made the United States and other countries to enact legislations in order to help find non toxic additives and to minimise CO, O_3 and volatile organic compounds. Some substances may be added to commercial gasoline in order to increase its octane number and they include methanol, ethanol, Methyl tertiary butyl ether (MTBE), ethyl tertiary butyl ether (ETBE), tertiary butyl alcohol (TBA), tertiary amyl methyl ether (TAME), *tertiary*-amyl ethyl ether (TAEE), and diisopropyl ether (DIPE) (Schuetzle *et al.*, 1994; Ahmed, 2001). Fuel is said to be oxygenated when it contains these additives (Costantini, 1993).

2.2.2. Toxicity of gasoline and its individual constituents/additives

A number of studies have been conducted to examine the toxicity of gasoline separately as well as its common constituents. Exposure to gasoline was found to cause reproductive toxicity which manifested in form of reduced serum level of estradiol and progesterone in female rats; elevated level of testosterone (at shorter duration of exposure), and reduced testosterone level (with longer duration) as well as a concomitant distortion of testicular tissues in male rats (Uboh *et al.*, 2007; Uboh *et al.*, 2010c). Exposure to gasoline was also found to be associated with nephrotoxicity, hepatotoxicity, and haematotoxicity in rats. It was demonstrated that such exposure caused impairment of kidney functions marked by increased levels of serum creatinine, urea, blood urea nitrogen, uric acid, and potassium ion as well as decrease in the levels of serum sodium and chloride ions (Uboh *et al.*, 2008a; Uboh *et al.*, 2010a). The liver toxicity observed in experimental rats exposed to gasoline manifested as increase in activities of its enzymes such as alanine tansaminase (ALT), aspartate transaminase (AST), gamma-glutamyltransferase (GGT), and alkaline phosphatase (ALP), as well as elevated serum levels of bilirubin with an accompanying hepatomegaly (Uboh *et al.*, 2005; Uboh *et al.*, 2008; Uboh *et al.*, 2009b). The haematotoxicity manifested as decreased levels of Hb, PCV and RBC, and a concomitant increase in WBC count (Uboh *et al.*, 2008b). Gasoline exposure is also associated with central nervous system toxicity. Kinawy reported that rats exposed to gasoline had disturbances in physiological levels of monoamine neurotransmitters with a concurrent reduction in Na-, K-ATpase and total protein concentration in the brain of experimental rats; and this effect was accompanied with a higher possibility of developing aggressive behaviour (Kinawy, 2009). Molecular evidences have also indicated that inhalation exposure to gasoline caused increased

frequency of mironucleates in exfoliated buccal cells of gasoline station workers which are believed to increase the risk of cytogenetic damage among the exposed subjects (Çelik *et al.*, 2003). A recent study has further corroborated these findings by demonstrating significant DNA damage amongst gasoline station attendants in service stations (Pandey *et al.*, 2008 Rekhadevi et al., 2010). An increased risk of developing cancers of the lung and kidney as well as nasal carcinomas has been reported to be associated with long term exposure to gasoline (Lynge *et al.*, 1997). Occupational exposure to benzene is associated with occurrence of certain types of leukaemias such as acute myeloid leukaemias (Hayes *et al.*, 1997; Rinsky *et al.*, 2002). Occupational exposures to benzene are also associated with increased frequency of aneuploid sperm (Xing *et al.*, 2010) and decreased levels of antibodies and leukocytes which can impair both humoral and cellular components of immune system (Aksoy *et al.*, 1987; Yin *et al.*, 1987).

Toluene and Xylene are naturally occurring compounds found in small concentration in a standard gasoline formulation and when inhaled in significant amount can cause central nervous system dysfunction in both rats and mice (Saito and Wada, 1993; Tegeris and Balster, 1994).

One of the major additives to gasoline, methyl-tertiary-butyl ether (MTBE), is widely used as fuel oxygenate owing to its ability in reducing harmful emissions caused by gasoline combustion and its use in the US started in the late 70s as an octane enhancer at the time of lead phase-out (Costantini, 1993; Hong *et al.*, 1997). This additive is however, not without toxicity as has been reported to be associated with rise in incidences of leydig cell tumours, leukaemias, and kidney tumours in

experimental rat models, as well as liver tumours in mice (Belpoggi *et al.*, 1997; Swenberg and Lehman-McKeeman, 1999; Borghoff and Williams, 2000; Zhou *et al.*, 2000; McGregor, 2006). MTBE has been recently confirmed to be an animal carcinogen with yet to established carcinogenetic relevance to humans (ACGIH, 2005). It has been suggested that inhalation of 800 and 1600 mg/day of MTBE is capable of inducing reproductive toxicity with subsequent impairment of secretion of testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) in male rats (Li *et al.*, 2008). A related in vitro study has reported that 5000 µM of MTBE caused significant reduction in the viability of spermatogenic cells, enhanced plasma membrane damage, as well as increased proportion of necrotic cells compared with a control (Li *et al.*, 2006). MTBE and tertiary-amyl-methyl ether (TAME) both cause significant but transient central nervous system depression with the later causing slightly more severe depression than the former (White *et al.*, 1995; Lington *et al.*, 1997; Daughtrey *et al.*, 1998).

Alcohols such as methanol and ethanol are used as fuel either alone or as gasoline oxygenates. Methanol is easily absorbed in humans after exposure; it causes mild central nervous system depression associated with headache, vertigo, and vomiting. Ethanol on the other hand was reported to cause increased levels of norepinephrine and metenkephalin, as well as decreased levels of serotonin in the central nervous system of both paternally and maternally exposed rat offspring (Nelson *et al.*, 1988).

2.3. Oxidants and antioxidant defence system

Under physiological conditions exists a protective mechanism called antioxidant defence system which operates at cellular, tissue, and organ level as well as in body fluids (Niture *et al.*, 2010). The major role of this important system is to build and preserve a steady-state balance between the liberation and degradation of free radicals (Lushchak, 2011). As originally defined, an antioxidant is "any substance that delays, averts, or eliminates oxidative damage to a target molecule" (Halliwell and Gutteridge, 2007). In general, antioxidants can be classified into enzymatic and non-enzymatic. Enzymatic antioxidants are enzymes which scavenge or remove reactive species released during biological or oxidative process (Lushchak, 2011). The major enzymatic antioxidants are Superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidise (GPx), glutathione reductase (GR) (Lieberman *et al.*, 2012), and glutathione-S-transferases (GSTs) (Hayes *et al.*, 2005). The non-enzymatic antioxidants comprised of glutathione, Vitamin E (α-tocopherol), vitamin C (ascorbic acid), flavonoids (Lieberman *et al.*, 2012), and metal binding proteins such as transferrin and ceruloplasmin (Halliwell and Gutteridge, 1990). In general, the enzymatic antioxidants work together to protect cells against oxidative damage by converting ROS or free radicals into non-toxic products whereas the non-enzymatic antioxidants terminate free radical chain reactions or avert the generation of free radicals (Halliwell and Gutteridge, 2007; Lieberman *et al.*, 2012).

2.3.1 SOD

SOD is an enzyme that converts $O_2^{\cdot-}$ to H_2O_2 and O_2. It is found both extracellularly and intracellularly in the cytoplasm and mitochondria. Hence, SOD helps to keep the $O_2^{\cdot-}$ concentrations in the cells within a physiological range (Halliwell and