RESEARCH ARTICLE

# Inhibitory Effects of Tualang Honey on Experimental Breast Cancer in Rats: A Preliminary Study

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## Abstract

**The study was conducted to determine the effect of Malaysian jungle Tualang Honey (TH) on development of breast cancer induced by the carcinogen 7,12-dimethylbenz(α)anthracene (DMBA) in rats. Forty nulliparous female Sprague-Dawley rats were given 80 mg/kg DMBA then randomly divided into four groups: Group 1 served as a Control while Groups 2, 3 and 4 received 0.2, 1.0 or 2.0 g/kg bodyweight/day of TH, respectively, for 150 days. Results showed that breast cancers in the TH-treated groups had slower size increment and smaller mean tumor size (≤2cm$^3$) compared to Controls (≤8cm$^3$). The number of cancers developing in TH-treated groups was also significantly fewer (P<0.05). Histological grading showed majority of TH–treated group cancers to be of grade 1 and 2 compared to grade 3 in controls. There was an increasing trend of apoptotic index (AI) seen in TH-treated groups with increasing dosage of Tualang Honey, however, the mean AI values of all TH-treated groups were not significantly different from the Control value (p>0.05). In conclusion, Tualang Honey exerted positive modulation effects on DMBA-induced breast cancers in rats in this preliminary study.**

**Keywords:** Honey - induced breast cancer - inhibitory effects - rat model

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## Introduction

The global incidence of breast cancer in women is alarmingly high and still increasing in both developing and developed countries. Carcinogenesis could be simplified into three main stages; initiation, promotion and progression (Barrett, 1993) and each step is believed to happen over a long period of time. As such there is a possibility that multiple steps involved in carcinogenesis could be intervened or modulated with chemo-preventive agents. For a common cancer prevention study, potential agents which could be used are given either before or shortly after the carcinogen exposure (Mehta, 2000).

Honey is known for centuries for its medicinal and health promoting properties. Honey is produced from complex enzymatic process of nectar and saccharine exudates collected from various kinds of floral sources (Ball, 2007). It contains various kinds of phytochemicals with high phenolic and flavonoids content which contribute to its high antioxidant activity (Yao et al., 2003; Iurlina et al., 2009; Pyrzynska and Biesaga, 2009). Agent that has strong antioxidant property may have the potential to prevent development of cancer (Valko et al., 2007). Phytochemicals available in honey can be narrowed down into phenolic acids and polyphenols. Variants of polyphenols in honey are reported to have antiproliferative property against several types of cancer (Jaganathan and Mahitosh, 2009).

Tualang Honey (TH) is a wild jungle honey produced by Apis dorsata bees which make hives on tall Kompassia excelsa (Tualang) trees of tropical rainforests (Tan et al., 2009; Nasir et al., 2010). TH is reported to have antiproliferative effect against oral cancer osteosarcoma cell lines (Ghashm et al., 2010). The aim of this study was to evaluate the effects of Malaysian jungle Tualang Honey (AgroMas™) in modulating the development of breast cancer induced by 7,12-dimethylbenz(α)anthracene (DMBA) in rats. This is a preliminary *in vivo* study of the effect of honey on induced breast cancer in rats.

## Materials and Methods

*Animals*

Forty female Sprague-Dawley rats aged between 45 to 48 days old were used. The animals were purchased from Laboratory Animal Research Unit USM (LARUSM) and were acclimatized for a week prior to induction. Animals were housed individually in a polycarbonate cage and maintained on a 12 hour light/dark cycle at 24±2°C. Animals were provided with food pellets and reverse osmosis water ad libitum.

*Chemicals and honey*

All chemicals used in this study were of analytical

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grade. DMBA was purchased from Sigma-Aldrich Co., St. Louis, MO. Tualang Honey (AgroMas™) was provided by FAMA (Federal Agricultural Marketing Authority), Ministry of Agriculture and Agro-based Industry, Malaysia. The honey samples were filtrated, evaporated at 40$^0$C (to achieve 20% water content) and were subjected to gamma irradiation at 25 kGy for sterilization by STERILE GAMA™ , Selangor.

*Study design*

The research protocol of the study was approved by Universiti Sains Malaysia Animal Research Ethic Committee [USM/Animal Ethics Approval/2008/(33)(111)]. After an approximately 18 hours of fasting, rats were given 80 mg/kg DMBA via oral gavaging. Rats were randomized into four groups with ten animals per group (n=10). Animals in Group 1 were given only distilled water (vehicle) and used as Control, whereas Group 2, 3 and 4 were administered with 0.2, 1.0 and 2.0 g/kg Tualang Honey diluted in 0.5 ml distilled water respectively given via oral gavage daily for 150 days.

The rat breast tissue areas were palpated twice weekly to detect the appearance of breast tumour masses and for monitoring their progression. Once a mass in the breast pad was detected, its size and location were recorded. After 150 days of treatment, animals were sacrificed after intraperitoneal injection of 70-100 mg/kg ketamine. Tumor masses were examined in vivo then excised, rinsed in phosphate buffered saline (PBS), individually weighed and measurement taken as per published protocol (Lai and Singh, 2006). One half of each cancer was fixed in neutral buffered formalin for histological analysis while the other half was kept at -80°C for vascular endothelial growth factor (VEGF) assay.

*Histopathological examination of the breast cancers*

The breast tumour specimens were fixed in buffered formalin. Once fixed, they were blocked, sections and stained with hematoxylin and eosin using the standard method. The stained sections were examined under light microscope at 100x, 200x and 400x magnification using Olympus BX41 microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The tissue sections were examined by a pathologist (NHO, corresponding author) who was blind to the treatment received by the rats. The cancers were graded as per human cancers grading system using the modified Bloom and Richardson as stated by published study (Mukhopadhyay et al., 2006).

*Detection of apoptotic cells by TUNEL assay*

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was carried out using Roche© In Situ Cell Death Detection Kit, POD (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Sections were examined under Olympus BX41 microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The apoptotic cells were counted from ten randomly-selected fields under 400x magnification. Apoptotic index (AI) was determined by calculating the amount of TUNEL positive cells over total number of cells counted.

*VEGF level analysis*

Breast cancers were weighed, minced and homogenized in 5 volume of ice-cold extraction buffer of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF and EDTA-free protease inhibitor cocktail tablets by using Ultra-Turrax T25 basic (IKA®, Staufen, Germany) disperser. The homogenates were centrifuged at 10,000 g for 15 minutes at 4°C. Supernatants were collected and used for protein quantification and ELISA. Total protein concentration was measured using BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The amount of VEGF in the supernatant was measured using rat VEGF ELISA kit for tissue lysate (RayBiotech, Inc., Norcross, Georgia, USA) according to manufacturer's instructions. All tissue lysate were diluted 1:40 in sample diluents. Mean absorbance was obtained to quantify mean VEGF concentration of the Control and Test (treatment) samples.

*Statistical analysis*

Comparisons between mean values of Control and Test (treatment) groups were analyzed using one-way ANOVA with post-hoc test of Tukey Honest Significance Differences (Tukey's HSD). Comparisons of median values between several groups were done using Kruskal-Wallis and between two groups by Mann-Whitney U test. Data was considered significantly different when p value was less than 0.05 ($p<0.05$).

## Results

*The morphology of the cancers in general*

Of the 40 rats which were recruited, a total of 31 rats completed the study. The 9 rats which did not complete the study died due to various causes and were excluded from analysis. All rats in the Tualang honey treatment group had significant less body weight gain. There were a total of 43 tumour nodules noted in these 31 rats (a few rats had more than one tumour nodule). The rats in the Control group had earlier tumour development compared with TH-treated rats and showed significant increment of tumour size over shorter period of time with the largest size reaching 8 cm$^3$ (Figure 1). Tumour masses in TH-treated groups had slower size increment and had lesser mean size (≤2 cm$^3$) (Table 1). In terms of the number of tumour masses developed, Control rats had more number of tumour nodules (tumor multiplicity) (Table 1). The medians weights (g) and volumes (cm$^3$) of the tumour

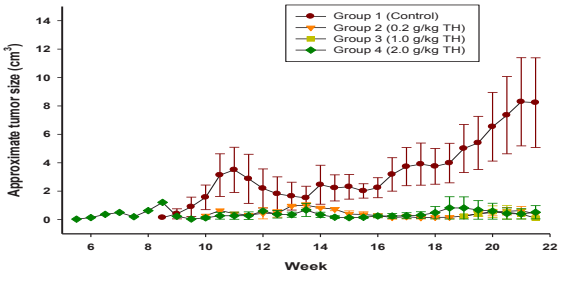


**Figure 1. The Size of Cancer (in cm$^3$) Measured Against Time Taken (in weeks) after DMBA Induction.** Data is presented as mean±SEM. TH=Tualang Honey

masses in all TH-treated groups were significantly lower ($p<0.05$) compared to Control (Table 1).

The vasculature around the tumour nodules was more prominent in the Control group compared with honey treated groups (Figure 2A). The tumours were also larger in size, firmer in consistency, more solid while in honey-treated groups were generally smaller, softer and paler with spots of necrosis (Figure 2B) and in some cases resembled normal structure of breast tissue.

*The histology of the tumour masses*

Five of the 43 tumour nodules showed benign histology and were not included in the analysis. The rest of the nodules showed invasive ductal adenocarcinoma with varying degree of Ductal Carcinoma in situ (DCIS) component. The cancers in the Control group showed more pleomorphic cells with more prominent nuclei. The mitotic figures were numerous and scattered throughout the tissue sections. In areas the cells were vacuolated and showed degenerative changes (Figure 2C). In contrast, cancer cells from the TH-treated groups were more uniformed in size and shape and had denser nuclei (Figure 2C). There were also presence of moderate to high number of inflammatory cells, mainly lymphocytes, plasma cells and eosinophils (Figure 2C-G3).

Majority of the cancers in the Control group showed high grade while cancers in honey-treated groups were of medium or low grade (Table 2). Ductal cancer in situ (DCIS) was observed both in Control and in TH-treated groups (less than 5%).

*Apoptotic index*

There was an increasing trend of apoptotic index (AI) seen in TH-treated groups with increasing dosage of Tualang Honey (Figure 3), however, the mean AI values of all TH-treated groups were not significantly different from the Control ($p>0.05$).

*VEGF level as determinant for angiogenesis*

Median values of VEGF protein expression level in all TH-treated groups were seen lower than Control group (32.81, IqR 33.65 pg/mg protein). The difference between the groups was not statistically significant ($p>0.05$) (Table 3).

**Table 1. The Characteristics of the Breast Cancers in Rats after DMBA Administration in Control and Tualang Honey (TH) Treated Groups**

| | Group 1 (Control) | Group 2 (0.2g/kgTH) | Group 3 (1.0g/kgTH) | Group 4 (2.0g/kgTH) |
|---|---|---|---|---|
| No. of rats | 9.00 | 8.00 | 7.00 | 7.00 |
| Day when first palpable tumor was detected# | 106.00±9 | 127.00±8 | 140.00±4 | 101.00±18 |
| Average % of rat body weight gain** | 126.36±8.24 | 88.11±8.12* | 91.58±7.04* | 97.24±10.50* |
| No of cancers developed | 14.00 | 12.00 | 5.00* | 7.00* |
| Weight of cancer mass (g) | 1.07 (5.15) | 0.18 (0.28)* | 0.12 (1.12)* | 0.16 (0.24)* |
| Volume of cancer mass ($cm^3$) | 1.23 (6.26) | 0.19 (0.45)* | 0.09 (1.08)* | 0.06 (0.16)* |

*Statistically significant against Control ($p<0.05$). **Inclusive of weight of cancerous mass. #Data is presented as mean±SEM

**Table 2. The Grading of Breast Cancers Developed after DMBA Administration in Control and Tualang Honey (TH) Treated Groups**

| Groups | No. of Cancers | Tumor grade* | | |
|---|---|---|---|---|
| | | 1 (%) | 2 (%) | 3 (%) |
| 1 (Control) | 14 | 1(7.1) | 4(28.6) | 9(64.3) |
| 2 (0.2 g/kg TH) | 12 | 5(41.6) | 6(50.0) | 1(8.3) |
| 3 (1.0 g/kg TH) | 5 | 1(20.0) | 2(40.0) | 2(40.0) |
| 4 (2.0 g/kg TH) | 7 | 1(14.3) | 6(85.7) | 0(0) |

*Based on modified Bloom and Richardson grading system

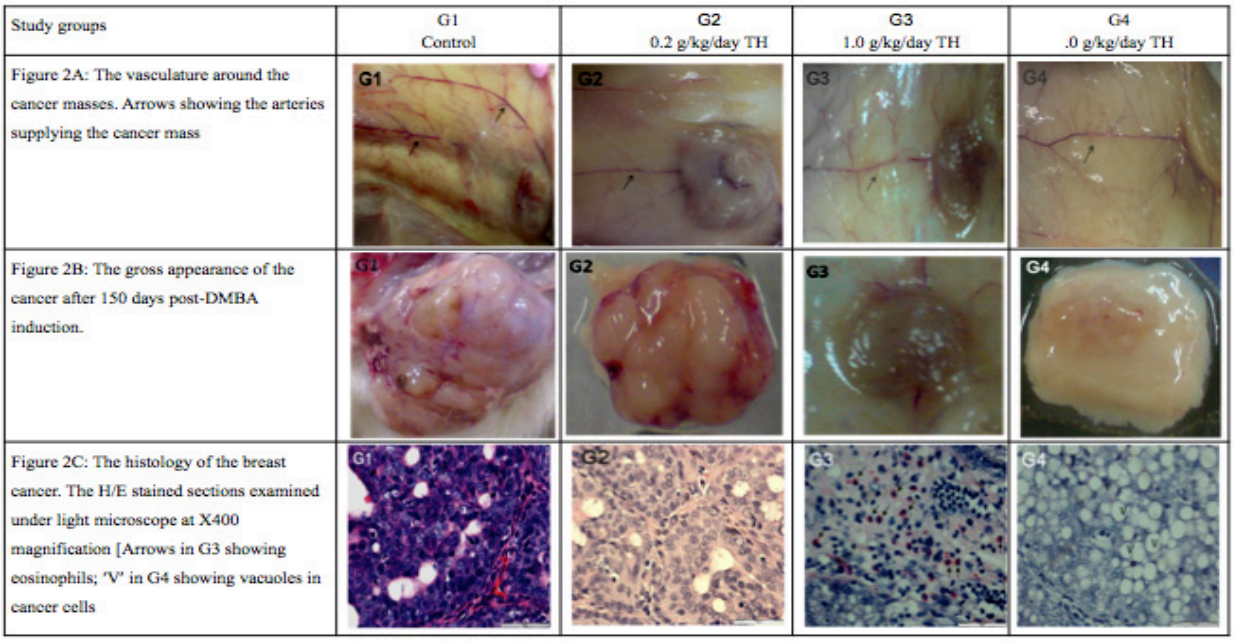


**Figure 2. Grid Showing the Gross Morphology and the Histology of the Breast Cancers in Rats Developed after DMBA Administration in Control and Tualang Honey (TH) Treated Groups**

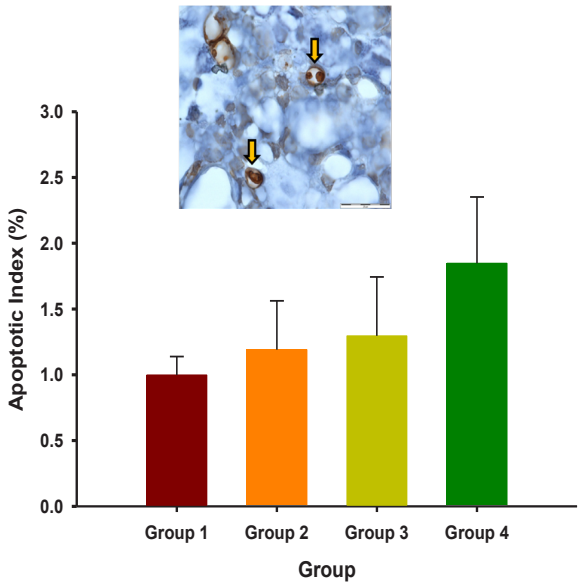


**Figure 3. Percentage of Apoptotic Cells Per Total Number of Cells Counted (Apoptotic index, AI) (Group 1=Control, Group 2=0.2 g/kg/day TH, Group 3=1.0 g/kg/day TH, Group 4=2.0 g/kg/day TH).** TH=Tualang Honey. Top Insert- arrows showing apoptotic cells

**Table 3. The Concentration of VEGF Protein (pg/mg total protein) of Breast Cancers Developed after DMBA Administration in Control and Tualang Honey (TH) Treated Groups**

| Groups | | | | P value |
|---|---|---|---|---|
| 1 (Control) | 2 (0.2**) | 3 (1.0**) | 4 (2.0**) | |
| VEGF level (pg/mg protein) | | | | |
| 32.81 (33.65) | 10.18 (38.28) | 17.61 (0) | 5.16 (0) | 0.704 |

*Data is presented as median and interquartile range (IqR). **g/kg TH

## Discussion

This preliminary study shows honey had some effect in modulating breast cancer development induced by DMBA. The cancers in the Control rats had significant rapid growth and attained bigger size compared to those in the honey-treated groups (Figure 1). Histology showed majority of the cancer cells in the Control group were of higher grade, had more mitotic activities and were more pleomorphic in nuclear size. The vasculature of the cancers in the Control was also more prominent than those in honey-treated cancers. This study supports the findings of previous study where Tualang honey was shown to have anti-proliferative effect when tested against oral squamous cell cancers and human osteosarcoma cell lines (Ghashm et al., 2010).

TH seems to have the ability to modulate carcinogenic effect of DMBA induction as we noted the tumour nodules developed later than seen in Control rats. The nodules were smaller in size and in some rats became impalpable with time. All the benign breast lesions we observed were in the honey-treated groups. We also noted lesser number of tumour nodules developed in these rats. This is an interesting observation implying TH has the potential to be used as an agent to modulate cancer development in persons who are exposed to carcinogens. TH has been shown to have significant anticancer effect against human breast and cervical cancer cell lines due to induction of apoptosis (Fauzi et al., 2010). The lesser number of cancers could also be explained due to ability of honey in controlling metastasis (Gribel and Pashinskii, 1990). This could be related to presence of caffeic acid phenyl ester (CAPE) that can be largely found in honey. CAPE is a strong antimetastatic agent specialized in inhibition of invasive potential of malignant cells (Hwang et al., 2006).

The anti-promoting activity might be related to the strong antioxidant activity in TH as shown by the high total phenolic content and total antioxidant activity from ferric reducing ability of plasma (FRAP) assay of Tualang honey (Mohamed et al., 2010). Various polyphenols constituents in honey can also be connected to the anticancer property. Among wide range of polyphenolic compounds found in honey, only several compounds were identified for the antiproliferative property such as caffeic acid, caffeic acid phenyl ester, chrysin, galangin, quercetin, acacetin, kaempferol, pinocembrin, pinobanksin and apigenin (Jaganathan and Mahitosh, 2009). Quercetin was proven to inhibit breast cancer growth in mice by exerting antiproliferative effect against MCF-7 breast cancer cell line (Indap et al., 2006) and apigenin had antiproliferative effect against HER2/neu-overexpressing cells (Way et al., 2004).

Skeptics will argue on the beneficial effect of honey as anti-cancer modulating agent as honey is essentially rich in carbohydrates. It also has small amount of lipids (Ball, 2007). Carbohydrates in honey are in concentrated form which could yield huge amount of calories per small quantity of honey. High calorie diet could initiate breast cancer formation and promotes cancer development (Kritchevsky et al., 1984; Welsh, 1994). However, the high caloric amount in TH did not cause major influence on the bodyweight of the animals in our study. We are currently investigating on why the sugars in honey are not carcinogenic.

Application of histological grading in assessing cancer of the breast is crucial for prognosis and predicting the post-treatment survival (Harvey et al., 1995; Dalton et al., 2000). The cancers in TH-treated groups were of better grade than in Control indicating the possibility of honey in controlling the behavior of the tumor at cellular level. Of the three criteria used in histological grading, mitotic count is considered the most accurate and significant in predicting the clinical outcome (Volpi et al., 2004).

DCIS is a form of early cancer and it is often detected at mammography (Silverstein, 1998). DCIS is claimed to be the precursor of invasive breast cancer as both type shares the same biological characteristics (Burstein et al., 2004). We noted DCIS was present in both honey and Control groups. Such observation was also made on previous studies on DMBA-induced breast cancer model (Dias et al., 1999; Russo and Russo, 2000; Costa et al., 2002).

We observed the apoptotic index is high in TH-treated cancers (Figure 3). Several polyphenols constituents in honey have been studied for the apoptotic property. Chrysin is able to induce apoptosis through activation of caspase-3 and Akt signal pathway in U937 cancer cell lines

(Woo et al., 2004). Flavonoid type acacetin is capable of inducing apoptosis in non-small cell lung cancer and liver cancer cell lines (Hsu et al., 2004a; 2004b). We however did not determine which polyphenols are responsible for apoptosis in our study.

In rats, angiogenesis is usually found in neoplastic epithelium rather than in normal epithelium (Maiorana and Gullino, 1978). Pro-angiogenic VEGF is the most significant tumor-derived factor as it modulates activation, migration and proliferation of endothelial cells (Makrilia et al., 2009). In human with primary breast cancer, it is claimed that VEGF is produced in the cytoplasm of the tumor cells (Toi et al., 1996). As such it is more accurate to measure angiogenic activity directly from the cancer tissue rather than measuring the circulating VEGF from the serum. VEGF is also capable to determine the aggressiveness of DCIS since DCIS can elicit new vascularization (Tosetti et al., 2002). We found the concentration of VEGF protein measured from tumor tissue lysate in the Control rats was higher compared to TH-treated cancers affirming its role in promoting angiogenesis as depicted by increased tumor vasculature.

Necrotic spots seen in cancers are due to inadequate supply of oxygen as cancer cells grow more rapidly than normal cells. Hypoxia can trigger the release of multiple angiogenic factors including VEGF, platelet-derived growth factor (PDGF) and tumor necrosis factor-α (TNF-α) (Makrilia et al., 2009). This would further increase the amount of VEGF thus neovascularization activity. Regressing size of tumors in DMBA-induced rats has been shown to have lower capacity of angiogenic response (Maiorana and Gullino, 1978).

The limitation of our study is in the number of rats which completed the study period of 150 days; only 31 of the 40 rats induced by DMBA as 9 rats died of various causes. The number of tumour nodules developed (43) somewhat compensated for this limitation. We are currently working on a larger number of rats per group using different method of breast cancer induction.

In conclusions, daily Tualang honey consumption continuously for 150 days starting a day after DMBA administration was positively modulating the progression of DMBA-induced breast cancer in rats. Cancers of honey-treated rats were much less in number, volume and weight with better histological grade and morphology compared to non-honey treated rats. Apoptotic activity was also higher with increasing honey concentration and reduced level of angiogenesis was observed. The results suggest that Tualang honey might have the potential to be used as a prophylactic or therapeutic agent to combat growth of breast cancer or as adjuvant to conventional chemotherapy.

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