Original Article

Phytochemical compound and non-cytotoxicity effect of sting bee and stingless bee honey against normal human gingival cell lines.

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Abstract:

Objective: Both honeybees (*Apis* spp.) and stingless bees (*Trigona* spp.) produce honeys which normally taken orally, have high nutritional and therapeutics value. Until recently, phytochemical comparison of both honey is still scarce and elucidating cytotoxicity effects on human gingival fibroblast cells (HGF) in oral cavity is of interest.

***Materials and Methods*:** Kelulut honey (KH), acquired from the stingless bees and acacia honey (AH) from the sting bees honey samples were underwent GC-MS analysis to ascertain their composition. HGF were exposed to various concentrations of KH and AH from the lowest 0.015% to the highest 5% by MTT assay for 24h, 48h and 72h.

Results: GC-MS analysis determined various beneficial compounds such as flavonoids, furans, pyrans, levoglucosan and hydroxymethylfurfural from both of honey samples. MTT assay showed that the HGF cells demonstrated good viability up to percentages (v/v) as high as almost 2% in both honeys. The IC_{50} values for both honey for all time frames fall at above 2%.

Conclusion: Both honey showed good survivability of HGF cells up to 2% of concentration.

Keywords: Phytochemical; Honey; Cytotoxicity; Human gingival fibroblast.

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Introduction

Synthetic drugs are the most common form of therapeutics to be used for various medical conditions[1]. For oral conditions, they play a major role in the promotion of good oral health and wellness, from treatment of various oral diseases to good daily oral hygiene maintenance[2]. Despite the benefits from medication, there is a risk of the inherent side effects from prolonged usage. It is prudent to search for more natural alternatives to ensure a reduced risk of side effects.

Honey is one of the more popular of the bio-alternatives as it has a history of use since ancient times. KH is harvested by a species of stingless bee called *Trigona sp*. These bees produce honey from variety of multifloral and stored in their nest as small resin pots. AH, product of a sting bee variety is known for its pale-yellow colour, herbaceous and delicate flavour[3]. These both honey varieties have shown in various studies to have medicinal potential[4,5,6,7]. Nowadays, it is being actively investigated to confirm its medicinal effects and uses in various parameters of medicine. Due to the importance role of honey in the use of traditional medicine, numerous investigations were performed by different researchers throughout several decades culminating to its place in modern medicine[8].Until now, studies have been conducted to ascertain the properties of honey from different parts of the world as an antibacterial[9,10,11,12,13,14,15,16]. capability to overcome gastrointestinal[17], cardiovascular[18] and, liver problems[19], possess properties within its natural composition that

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prevents bacterial growth and therefore promotes healing[20,21], capability to stimulate immune responses and exhibit anti-inflammatory activity in a wound[22,23], and anticarcinogenic effects[24,25,26]. Many studies which have been conducted for honey, greatly favour its use in medicine[27]. Although it has been claim by some herbal remedies that the use of plants as herbal medicine is safe due to the fact that it anything natural is synonymous with being safe, health care practitioners of modern medicine seldom recommend their use because of ill equipped database of their safety and potency[28]. Some herbal medicines are known to have resulted in severe side-effects after ingestion, which may be due to the toxic properties of the herbs or plants used, while the interactions of the plants or herbal medicine with other drugs being used by the patient can also lead to adverse effects[29,30]. Despite of massive usefulness of honey in the medical field as a source of alternative biomedicine, there is little focus on whether these raw honeys are safe in an oral cell *in vitro* to reflex the effect of the honey to the oral periodontium cells when taken orally.

Materials and Methods:

Preparation of honey

Both honey samples were obtained in raw from Syamille Agro Farms, Kati Kuala Kangsar, Perak. For analysis, initially the raw honey samples of kelulut and acacia were subjected to sterilization by γ-irradiation at a dose of 25 kGy. There is no significant loss of antibacterial activity of honey by this dose of radiation[31]. The samples were sent to Agensi Nuklear Malaysia, MINTec Singama for the sterilization process.

Gas Chromatography-Mass Spectrometry (GC-MS)

A Hewlett Packard 6890 Gas Chromatograph with a 5973N Mass Selective Detector was used to carry out the GC-MS. The column was fused silica capillary, HP-5 column (30 m x 0.25 mm i.d x 0.25 µm lm thickness) (Agilent Technologies, USA). The carrier gas was helium with a ow rate of 1.0 ml/ min with the oven temperature programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 20°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively. A 1-ml sample was injected in splitless mode and was analysed in MS full scan mode (*m/z* 40-650). The electron ionisation was set at 70eV. Acquisition of data was performed using the Chemsation software.

Identification of Phytochemical Compounds

The mass spectrum of the GC-MS was interpreted based on the database of the National Institute of Standards and Technology (NIST02) and Wiley275 libraries with matches of ≥80 % to identify the phytochemical compounds.

Cell Viability Analysis via MTT Assay.

MTT (3-(4,5-di methylthiazol -2-yl)-2,5-diphenyltetrazolium bromide salt) cell viability assay was used to assess the cytotoxicity effects of the honey on HGF cell lines. A total of 3-5 X 10^3 cells per well were seeded into 96-well plates and were allowed to adhere for 24h. The cells were then treated with 0.015%, 0.031%, 0.062%, 0.125%, 0.250%, 0.5%, 1%, 2%, 3%, 4% and 5% of KH and AH. Each concentration/assay was performed three times for 24h, 48h and 72h. The well plates were incubated at 37°C in the presence of 5% CO_2. After the incubation period, 10 µL of MTT solution (prepared with phosphate-buffered saline to a concentration of 5 mg/ mL and filtered) was added into each well and further incubated for 4 hrs. Then the medium with excess MTT was removed from the wells, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the dark blue formazan crystals formed by viable cells. To ensure that all crystals were dissolved, the plate was further incubated for 1 hr. and shaken for 3 min[32].

The measurement of absorbance was taken using the Tecan Sunrise© ELISA (enzyme linked immunosorbent assay) plate reader at a wavelength of 570 nm with 600 nm as reference. The average of the triplicates from the control, blank and treatment wells was calculated, and applied in the following formula to determine cell viability[33]. The average of the triplicate of each time trial was considered as the result.

$$\frac{Avg.Sample - Avg.Blank}{Avg.Control - Avg.Blank} \times 100$$

IC_{50} calculation

To find the value of IC_{50}, the percentage concentration of honey was transformed in log10 and analyzed with GraphPad Prism 7 software. The inhibition curve was fitted with nonlinear regression (variable slope).

Statistical Analysis.

All the data were reported as means ± mean standard deviation (SD) of three independent experiments. The

nonlinear regression was determined by GraphPad Prism 5 (GraphPad Software, San Diego, CA).

Ethical clearance

The study involved cell lines *in vitro*. No ethical clearance needed.

Results

GC-MS analysis of KH and AH

KH was identified with furfural compounds which are furan derivatives, hemiterpenoids, levoglucosan, flavonoids and naturally occurring ketones. About 12 major compounds were identified in KH samples as shown in Table 1. Two larger percentage within these compounds were 2-Furancarboxaldehyde (hydroxymethyl) (30.87%) and levoglucosan (Beta. -D-Glucopyranose,1,6-anhydro) (10.03%). AH was identified with a total of seven prominent compounds as shown in Table 2. Furan derivatives like 2-Furancarboxaldehyde, 5 (hydroxymethyl) (32.81%) was found in a larger percentage.

Table 1: Compounds in KH

Compounds	Group	% of presence in honey
Furfural	Furan derivative (flavonoid)	5.55%
Furfuryl Alcohol	Furan derivative (flavonoid)	0.22%
2-Hydroxy-2-cyclopenten-1-one	Diterpene	1.21%
Methyl succinic anhydride	Terpenoid	2.81%
2-Furancarboxaldehyde, 5-methyl	Furan derivative (flavonoid)	0.93%
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	Furan derivative (flavonoid)	0.73%
furan-2,5-dicarboxaldehyde	Furan derivative (flavonoid)	5.01%
4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	flavonoid	4.88%
4H-Pyran-4-one,3,5-dihydroxy-2-methyl	flavonoid	0.90%
5-Formyl-2-furfurylmethanoate	Furan derivative (flavonoid)	0.66%
2-Furancarboxaldehyde,5-(hydroxymethyl) (furan derivative)	Furan derivative (flavonoid)	30.87%
beta. -D-Glucopyranose,1,6-anhydro	Levoglucosan	10.03%

Table2: Compound in AH

Compounds	Group	% of presence in honey
Furfuryl Alcohol	Furan derivative (flavonoid)	2.29%
2-Hydroxy-2-cyclopenten-1-one	Diterpene	2.66%
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (furan	Furan derivative (flavonoid)	0.50%
4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	flavonoid	3.88%
2-Furancarboxaldehyde, 5	Hydroxy methyl (Furan derivative) (VOC)	32.81%
Decycltetraglycol	Glycol	0.38%
Tetra ethylene glycol monododecyl ether	Glycol	0.16%

Cytotoxicity effect of KH

Figure 1 shows the viability of HGF cells treated with KH decreased highly significant at 4% for all time frame compare to control at 0 % of KH concentration. Meanwhile Figure 2 shows that KH had an inhibitory effect on HGF cells with an IC_{50} value of 4.257 (R^2 0.91) at 24 hrs, IC_{50} value of 3.974 (R^2 0.88) at 48 hrs and, IC_{50} value of 3.990 (R^2 0.89) at 72 hrs.

Cytotoxicity effect of AH

Figure 3 shows the viability of HGF cells treated with KH decreased significantly at 3%, 4% and 5% for 24hrs, 48hrs, and, 72 hrs respectively compare to control at 0 % of AH concentration. Meanwhile Figure 2 shows that AH had an inhibitory effect on HGF cells with an IC_{50} value of 4.257 (R^2 0.91) at 24 hrs, IC_{50} value of 3.974 (R^2 0.88) at 48 hrs and, IC_{50} value of 3.990 (R^2 0.89) at 72 hrs.

Discussion:

It has been reported that biological activities in the selected plants were exhibited by different class of phytochemicals[34]. Same as honey in the world, their composition varies depending on its floral, geographical and entomological sources[35]. As phytochemicals often play an important role in plant defence against prey, microorganism, stress as well as interspecies protections, these plant components have been used as drugs for millennia[36]. Hence, chemicals screening serves as the initial step in predicting the types of potential active compounds from honey.

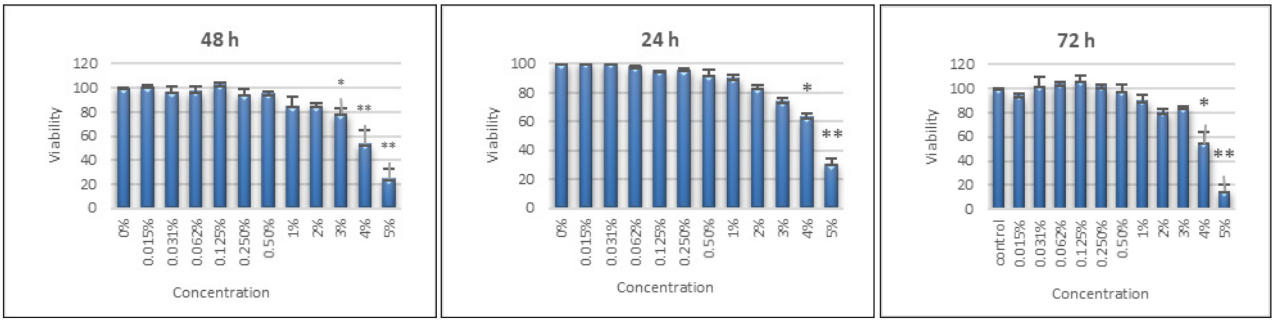

Figure 1: Cell viability for HGF cells treated with Kelulut honey (KH) for 24h, 48h and 72 h by MTT assay. The viability is described as ± mean standard deviation (SD). *$P<0.05$ with control; ** highly significant

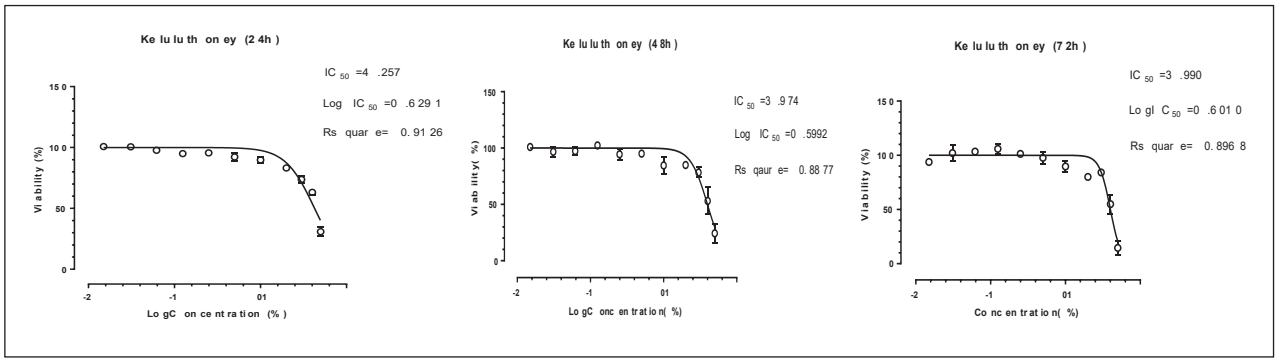

Figure 2. The inhibition curve of KH in the HGF cell line. Dose-response inhibition data points represent the mean value of three independent experiments using graph pad prism 7. The results are expressed in %. The bars represent the standard deviation.

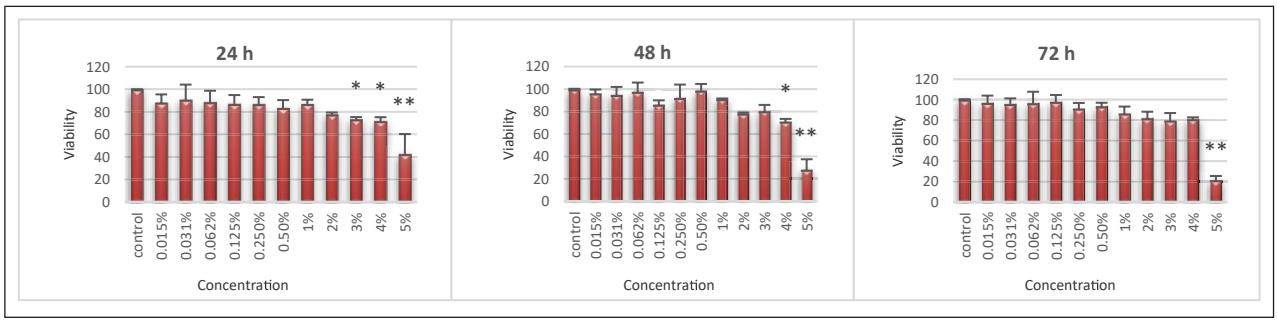

Figure 3. Cell viability for HGF cells treated with Acacia honey (AH) for 24h, 48h and 72 hrs. after MTT assay. The viability is described as ± mean standard deviation (SD).*$P<0.05$ with control; ** highly significant

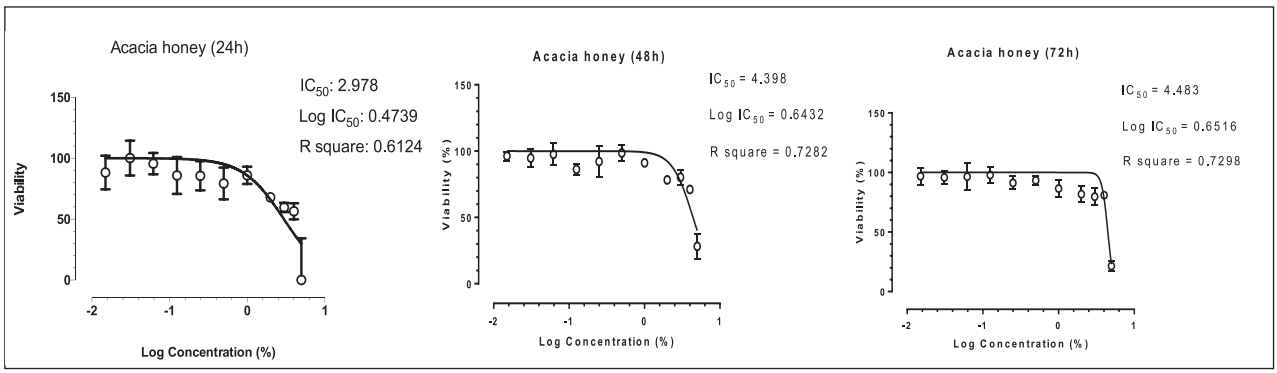

Figure 4. The inhibition curve of AH in the HGF cell line. Dose-response inhibition data point represent the mean value of three independent experiments using graph pad prism 7. The results are expressed in %. The bars represent the standard deviation.

The presences of common compounds such as flavonoids, terpenoid and hydroxy methyl furfuran are found in KH and AH as have been reported by other types of honey[37,38]. The bioactive chemical of 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl from both AH and KH were found to exhibit antifungal activity[39]. The major difference of biological active compound in our stingless bee honey and sting bee honey compound found is terpenoid in KH. Terpenoid are known to be active against a wide range of micro-organisms including Gram negative and positive bacteria, viruses as well as fungi[40,41]. Compounds found in our sample of KH and AH majority consisted of furfural from flavonoid group, which was also found in other honey[42]. The percentage found to be safe as Codex Alimentarius Standard commission has set the maximum limit for HMF in honey at 40 mg/kg (with a higher limit of 80 mg/kg for honeys originating from tropical regions) to ensure that the product has not undergone extensive heating during processing and is safe for consumption[43]. In another study conducted by Hazirah *et al.*, (2019), analysis of stingless bee honey showed that the flavonoid and phenolic components in KH may be the active compounds that contribute to the oxidative damage protection of lymphoblastoid cell line. The health benefits of KH were also highlighted in a study by Rashid *et al.* (2019) where KH consumption for 30 days had no effect on the fasting lipid profiles, fasting blood glucose and other metabolic parameters in patients with impaired fasting glucose.

In order to determine the cytotoxic effects of each honey, a number of concentrations were tested on HGF cell lines, and their cytotoxic effects determined using MTT assays. It was found that there are no cytotoxic effects of AH and KH on HGF cell lines were observed for concentration of less than 2% used. The highest concentration of 2% the honey might be used on cells in order to avoid any cytotoxic effects.

Earlier studies with other honey species showed that these honeys have rather a great significance for their traditional use in the treatment of other pathologies[46]. The IC50 value of these honey could be used as a guideline value limit to other cytotoxicity studies from these honeys. It is important to mention that to the best of our knowledge, this study represents the first report on cytotoxic, evaluation for chemical compound of raw commercialised KH and AH. The obtained results support to some extent the safe traditional uses of these honeys for the treatment of some poverty related diseases in folk medicine especially taken orally. Isolation, purification, and structure elucidation of constituents from these honeys are important to support discovery of new chemical entities for biological activities.

Conclusion

The phytochemical compound found could be attributed to its biological activity. The results obtained for cytotoxicity assays indicated that both AH and KH may be suitable for use as medicinal agent as the extract tested did not show high cytotoxicity potential. The assays used were regulatory preclinical toxicity testing assays and the proof of non-cytotoxicity is an indication of proof of safety and indicator for a potential his selection for pharmacological activities to improve traditional phytomedicine.

Source of Fund

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Author Contributions

Siti Lailatul Akmar (SLA), Zurairah Berahim (ZB), Wan Nazatul Shima Shahidan (WNSS) designed the study. Moeez Ansari (MA), performed the experiment and collected data for the study. WNS and ZB validated the experimental data of study. WNSS writing the first draft of the manuscript, which was critically revised by SLA and ZB. All authors approved the final version of the paper, and agree to be accountable for all aspects of the work.

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