

Phytochemical, antioxidant and anticancer properties of honey and black seed mixture on MCF-7 cell lines

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Abstract

Breast cancer is a leading cause of death of women in Malaysia, accounting for 17.7% of all cancer cases reported and 31.1% of all female cases. Chemotherapy drugs are effective in breast cancer treatment but may cause physiological and psychological distress to the patient. Therefore, an alternative way to provide better anticancer treatment with less side effects is important. Honey and black seed have been reported to show strong anticancer and antioxidant properties. Thus, in the present study, methanolic extracted honey and black seed mixture (ME), aqueous extracted honey and black seed mixture (AE) and mixture of methanolic extracted honey and aqueous extracted black seed (ME+AE) were evaluated for their anticancer and antioxidant properties. Antioxidant properties of the mixtures were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and the half maximal effective concentration (EC₅₀) were determined. Meanwhile, anticancer properties of the mixtures were evaluated on MCF-7 breast cancer cell line. Results revealed, in the antioxidant assays, the EC₅₀ values of AE, ME and ME+AE mixture were 4.15 mg/mL, 2.47 mg/mL and 4.17 mg/mL, respectively. In the anticancer study, there was no significant difference ($p > 0.05$) between extraction methods on the cytotoxicity of MCF-7 cell line. The IC₅₀ values obtained from different extraction method range between 13.27 µg/mL to 16.45 µg/mL. The presence of bioactive compounds such as alkaloid, flavonoid, phenol, tannin and saponin in honey and black seed mixture might contribute to its high cytotoxic activity. Therefore, the use of honey and black seed mixture as a health supplement for its of anticancer and antioxidant benefits should be considered.

Keywords: MCF-7, Black seed, Honey, Anticancer, Antioxidant, Phytochemical properties

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Introduction

Cancer is a term referring to genetic diseases characterized by the continuous growth of abnormal cells beyond their usual boundaries that can then invade nearby tissue or spread in the body of any organ (National Cancer Institute, 2015). There were 18,343 cases of breast cancer diagnosed in year 2007 to 2011 and reported to NCR (National Cancer Registry Report 2007-2011, 2016). The growing of incidence is due to increase life expectancy, urbanization and adoption of western lifestyles (WHO, 2015). Currently, chemotherapy is the main choice of treatment and plays a major role in controlling and cures the tumour. However, drug resistance and dose-limiting toxicity of cytotoxic drugs prevent chemotherapy to destroy all the cancer cells in a tumour's patient (Ozben, 2006). Thus, low doses of chemotherapy drugs or other natural food sources without drug resistance is required in breast cancer treatment.

Honey and black seed as a plant-derived dietary antioxidant with high anticancer properties is believed able to lower the risk of cancer. Honey is known to have many bioactivities such as anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, antibiotic and wound healing activities to immune-stimulatory properties (Mandal and Mandal, 2011; Waykar and Alqadhi, 2016; Oryan et al., 2016). In a previous study, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay (antioxidant test) showed 28–55% radical scavenging. In addition, another studies demonstrated the antiproliferative effects of honey on ovarian cancer, oral squamous cell carcinomas and human osteosarcoma in dose and time dependent manner (Vit et al., 2012; Ghashm et al., 2010). Besides, honey is popular with its presence and high amount of total phenolic content and total flavonoid content (Moniruzzaman et al., 2013; Islam et al., 2012; Jantakee and Tragoolpua, 2015).

Black seed, scientifically known as *Nigella sativa*, has been used as nutritional flavouring agent and natural remedy for many ailments in many countries. Bioactive compounds such as thymohydroquinone, thymoquinone and thymol with anticancer, antioxidant, antimicrobial, anti-inflammation and other properties have been identified in black seed (Randhawa and Alghamdi, 2011). A previous study reported that low concentrations of aqueous extract of black seed have a hormetic (growth stimulation) effect rather than cytotoxic effect, with a lethal concentration

(LC₅₀) as high as 50 mg/mL, while lipid extract black seed have an effect on human breast cancer (MCF-7) at 2.72 mg/mL (Mahmoud and Torchilin, 2013). However, a high dosage of aqueous extract black seed of 180 mg/mL was identified to be the best anticancer agent against MCF-7 cell lines compared to other solvent tests, with doxorubicin and cisplatin act as standard reference (Reddy et al., 2015). In addition, phytochemical test on black seed confirmed the presence of alkaloid, flavonoid, phenol and tannin (Yessuf, 2015).

The lipophilic antioxidant capacity in honey, black seed and mixture of black seed and honey, expressed as Trolox equivalent, were reported to be at 7.18, 9.67 and 29.94 µg/mg, respectively (Mohamad, 2013). This shows that lipid-soluble antioxidant in black seed and honey mixture had higher antioxidant capacity as compared to black seed or honey alone. It is possible that there is some chemical reaction between these two samples which might also produce higher anticancer properties. However, to date no study on honey and black seed mixture has been performed in the context of breast cancer study. Besides, the anticancer properties of honey and black seed were also documented individually but not on its mixture. It is postulated that the link between antioxidant and anticancer activity might due to the structural features of flavones, the arrangement of hydroxyl group which determines their activity especially anti-proliferative and kinase inhibiting effects (Kanaswami et al., 2005). Lastly, the contributors to anticancer properties of honey and black seed mixture still unknown which it might be associated by its antioxidant activity or due to the presence of bioactive compound and this yet still need to be explored.

Thus, this study aimed to determine the phytochemical properties of honey and black seed mixture that been extracted using methanol and water. In addition the synergistic effects of honey and black seed mixtures also evaluated their ability as natural antioxidant and anticancer against the MCF-7 breast cancer cell line.

Material and Methods

Material

Human breast carcinoma (MCF-7) cells lines were obtained from the Institute of Marine Biotechnology (IMB) Laboratory, Universiti Malaysia Terengganu (UMT), from Roswell Park Memorial Institute medium (RPMI) 1640, Fetal bovine serum (FBS), MTT (2-(4,5-dimethylthiazol-2-yl)-2, 5-



diphenyltetrazolium bromide, a tetrazole), antibiotic (penicillin and streptomycin), sodium pyruvate and non-essential amino acids were purchased from Life Technologies and Nacalai Tesque.

Sample preparation and extractions

Black seeds were obtained from local supplier in Kuala Terengganu, originated from Syria. The seeds were washed to remove foreign substances and roasted in a convection oven at 100°C for one hour, then grounded into fine powder. The grounded seeds was mixed with honey at a ratio of 1:1 (wt/wt) to form a paste. This preparation was adapted from the procedure done by Mohamad et al. (2015).

Honey and black seed were extracted using methanol and aqueous with three variations. First, 25 g of honey and black seed mixture was extracted using 50 mL of 100% methanol. The sample was then filtered using a polytetrafluoroethylene (PTFE) membrane (0.45 µm) to obtain a clear solution. After that, the sample was concentrated using rotary evaporator to remove the solvent used at 40°C for 30 minutes. The extracted samples were kept in freezer (-80°C) for 24 hours and further dried in a vacuum freeze drier. The dried samples were quickly reconstituted with dimethyl sulfoxide (DMSO) and stored in a freezer (-40°C) until later use. This extraction method was adapted from the procedure done by Syazana et al. (2011). Second was the aqueous extraction, whereby 25 g of honey and black seed mixture were diluted in 100 mL distilled water. Then, each sample was centrifuged at 3000 rpm for 15 minutes. The supernatant was filtered using filter paper and saturated using rotary evaporator at 50°C for 2 hours. Next, the extracted samples were kept in a freezer (-80°C) for 24 hours and further dried in a vacuum freeze drier. The dried samples were quickly reconstituted with DMSO and stored in freezer (-40°C) until later use. This extraction method was from Hosseinzedah et al. (2013). Third was a mixture of methanolic extract of honey and aqueous extract of black seed. In this procedure, 25 g of honey was mixed with 50 mL of pure methanol, followed by anhydrous sodium sulfate to remove residual water, while 25 g of black seed was boiled gently in 100 mL of distilled water and centrifuged at 3000 rpm for 15 minutes. The extracted honey and supernatant of black seed were then filtered using filter paper and saturated using a rotary evaporator. Both extracted honey and black seed were kept in a freezer (-80°C) for 24 hours and further dried in freeze drier, then mixed at a ratio of

1:1. The dried mixture was quickly reconstituted with DMSO and kept in a freezer (-40°C) until later use.

MTT cytotoxic assays

MTT assay was used to study the cytotoxicity effect of honey and black seed mixture. MCF-7 cells were counted and seeded at 8.0×10^4 cells per well per 100 µL complete culture medium (1% penicillin, 1% non-essential amino acid, 1% sodium pyruvate and 10% FBS into RPMI 1640 medium) in 96 well plate and incubated at 37°C in 5% CO₂ incubator for 24 hours. Then, 100 µL prepared concentrations of samples were replaced in the original culture medium and further incubated at 37°C in 5% CO₂ incubator for 72 hours. After that, 20 µL of the MTT solution was then added into each well and further incubated for another 4 hours. The formazon crystals formed were proportional to the number of existing viable cells. After 4 hours, 120 µL of the medium was removed and replaced with 100 µL of DMSO to solubilise the formazon crystals. Optical density (OD) of the samples was measured by using a spectrophotometer at 570 nm. The method was adapted from a procedure done by Akbari and Javar (2013). The percentage of cell inhibition were obtained by using the following formula:

$$\frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

The cytotoxicity effects of honey and black seed mixture on MCF-7 cell lines was determined by measuring the cytotoxic dose (inhibition concentration) that kill 50% (IC₅₀) of the cell population compared to untreated control. The IC₅₀ was determined from a graph plotting % inhibition vs concentration of honey and black seed mixture.

The method from Vijayarathna and Sasidharan (2012) was applied in order to study the cell morphology. The treated cell line for IC₅₀ was incubated in an incubator for 72 hours. The morphology of treated cells was observed under inverted microscope and picture of the cell was captured every 24 hours for three days.

Cell apoptosis assay

To study the apoptosis of MCF-7 cells, Acridine Orange (AO) and Propidium iodide (Pi) staining was carried out according to method described by Meiyanto et al. (2007). Cells (1×10^4 cells/well) in RPMI 1640 medium containing 10% FBS were seeded into 96-well plates. The medium was removed after 24



hours incubation. After that, 15 μ L of AO and Pi mixture at ratio 1:1 was added to each well. The cells were visualised immediately using high content screening machine with FITC-Texas Red filter and images were captured.

DPPH radical scavenging activity

To evaluate the antioxidant activity of honey and black seed mixture, the DPPH radical scavenging activity was measured using modified method by Ramli et al. (2008). A 2, 2-diphenyl-1-picrylhydrazyl (DPPH) stock solution was prepared by dissolving 2.37 mg of DPPH in 80 mL of 100% of methanol. A potent synthetic antioxidant compound quercetin was used as a positive control by dissolving 1 mg of quercetin powder with 1 mL DMSO. All samples were prepared with DMSO to the concentration of 10 mg/mL, respectively and approximately 20 μ L of each sample was loaded in 96 well plate. Then, 200 μ L of DPPH stock solution was loaded into each well and incubated in the dark for 30 minutes. The absorbance was measured at 517 nm using ELISA reader. The percentage of DPPH scavenging activity was calculated using the following formula:

$$A = [(A_0 - A_e)/A_0] \times 100$$

Where, A_0 = Absorbance value for DPPH
 A_e = Absorbance value for samples

Phytochemical content analysis

Phytochemical tests were carried out to determine alkaloid, flavonoid, phenol, tannin and saponin in fresh honey and black seed mixture. The methods to carry out each test are described below:

Alkaloid detection (Wagner's test): 50 mg of methanol extracted honey and black seed mixture was added with 2 mL of 1% HCl and filtered using filter paper. Then, 1 mL of solution was added with 1 mL of Wagner's reagent. A brownish red formation indicated the presence of an alkaloid (Chanda et al., 2016).

Flavonoid detection (Ferric Chloride's test): To 1 mL of methanol extracted honey and black seed mixture, few drops of ferric chloride was added and an intense green colour indicated the presence of flavonoids (Yessuf, 2015).

Phenol detection (Lead Acetate's test): 5 mg of raw honey and black seed mixture was diluted in 1 mL

water and added with 10% lead acetate solution. A bulky white precipitate indicated the presence of phenolic compound (Asokan and Jayanthi, 2017).

Tannin detection (Ferric Chloride's test): A volume of 2 mL of aqueous extracted honey and black seed mixture was added with few drops of 1% ferric chloride. The formation of a brownish-green or blue-black colour indicated the presence of tannin (Ezeonu and Ejikeme, 2016).

Saponin detection (Emulsion test): A volume of 5 mL of aqueous extracted honey and black seed mixture was shaken vigorously with 5 mL of distilled water and warmed. Formation of 1 cm stable foam indicated presence of saponin (Bargah, 2015).

Statistical analysis

Data was expressed as mean and standard deviation, of triplicate determinations performed using black seed and honey mixtures derived from different concentrations with Minitab software version 18.0. One-way ANOVA was conducted and multiple comparison analysis was carried out using Tukey post-hoc test. Data are considered significantly difference at $p < 0.05$.

Results and Discussion

Table 1 shows the percentage of MCF-7 cells inhibition following treatment with different concentration of different extraction method of honey and black seed mixture.

Results indicated that cytotoxicity activity of honey and black seed was seen with concentration more than 25 μ g/mL. The significant difference between concentrations is in parallel with a previous study showing that anti-proliferation effect of honey is in a dose dependent manner (Ahmed and Othman, 2013). Black seed has a hormetic effect rather than cytotoxic effect in low concentrations. A high dose of aqueous extracted black seed of 180 mg/mL was identified to be an anticancer agent against MCF-7 cell lines (Mahmoud and Torchilin, 2013; Reddy et al., 2015). However, honey and black seed mixture in the present study was able to demonstrate a cytotoxicity effect rather than a hormetic effect on MCF-7 cell line at a low concentration of 25 μ g/mL.



Table 1. Inhibition of MCF-7 cells after treatment with different concentration of different extraction method of honey and black seed mixture in MTT assay

Concentration ($\mu\text{g/mL}$)	% of MTT Inhibition			
	ME	AE	ME+AE	H ₂ O ₂
0	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a
0.390625	20.47(11.85) ^{ab}	11.38(6.80) ^a	13.99(5.39) ^a	86.28(3.20) ^b
0.78125	19.77(7.87) ^{ab}	11.29(2.76) ^a	11.24(3.89) ^a	86.30(3.29) ^b
1.5625	19.25(4.00) ^{ab}	7.71(3.01) ^a	11.45(3.94) ^a	86.75(2.52) ^b
3.125	23.75(6.31) ^{ab}	10.88(2.66) ^a	17.82(11.39) ^a	86.96(2.37) ^b
6.25	18.99(12.54) ^{ab}	22.29(11.97) ^{ab}	20.65(14.24) ^a	87.29(0.89) ^b
12.5	41.26(15.26) ^b	37.57(17.98) ^b	49.62(6.93) ^b	86.72(1.44) ^b
25	70.70(14.11) ^c	69.48(7.34) ^c	73.89(2.38) ^c	87.19(1.13) ^b
50	89.95(0.99) ^c	82.94(4.82) ^c	84.72(2.15) ^c	86.54(1.56) ^b
100	89.61(2.79) ^c	84.01(5.47) ^c	87.62(0.60) ^c	87.32(0.20) ^b

Result are presented as mean \pm S.E.M of triplicate. Within the same column, different letters (a, b and c) indicate significant different ($p < 0.05$) by one-way ANOVA and Tukey post-hoc test. Note: ME=Methanolic extraction of honey and black seed mixture; AE=Aqueous extraction of honey and black seed mixture; ME+AE=Mixture of methanolic Extraction of honey and aqueous extraction of black seed; H₂O₂=Hydrogen Peroxide.

Figure 1 shows that different extraction methods and positive control able to inhibit growth of MCF-7 cell line up to 80% after 72 hours. However, a higher dose of honey and black seed mixture is needed compared to hydrogen peroxide. Hydrogen peroxide is a reactive oxygen species that enhances cell apoptosis and is efficient in cancer therapy (Liu et al., 2015). The present study revealed no significant difference between different extractions methods, but a significant difference between extraction methods and positive control individually. Thus, it is indicated that extraction methods do not affect the anticancer properties of honey and black seed mixture. This result is in parallel with a study by Farah and Begum (2003) which showed that both methanolic and aqueous extracted black seed were found effective *in vitro* to inactivate MCF-7 cell lines.

Moreover, the results of cytotoxicity of honey and black seed mixture are also presented in half maximal inhibitory concentration (IC₅₀), as shown in Table 2. A mixture of methanolic extracted honey and aqueous extracted black seed showed the lowest IC₅₀ indicating the highest cytotoxicity activity towards the MCF-7 cell lines.

Although there were no significant differences between extraction methods on IC₅₀, the mixture of methanolic extraction of honey and aqueous extraction of black seed showed the lowest IC₅₀. This might be due to the thermal properties of black seed. Black seed heated to 50°C, 100°C or 150°C had a stronger ability in inhibiting tumour cell growth, while no heating or

heating to 25°C had a mild antiproliferative effect. Heating to 200°C or 250°C had no effect (Agbaria et al., 2015). In the present experiment, aqueous extraction of black seed was heated to 100°C during the extraction process, while aqueous extracted honey and black seed mixture do not apply any heat treatment.

Table 2. Summary IC₅₀ value honey and black seed mixture against human breast cancer (MCF-7) cell line

Extraction Method	IC ₅₀ concentration ($\mu\text{g/mL}$)
ME	15.76 (5.45) ^a
AE	16.45 (4.84) ^a
ME+AE	13.27 (2.18) ^a
H ₂ O ₂	0.23 (<0.01) ^b

Result are presented as mean (SEM) of triplicate. In the same column, different letters (a and b) indicate significant different ($p < 0.05$) by one-way ANOVA and Tukey post-hoc test. Note: ME=Methanolic extraction of honey and black seed mixture; AE=Aqueous extraction of honey and black seed mixture; ME+AE=Mixture of methanolic Extraction of honey and aqueous extraction of black seed; H₂O₂=Hydrogen Peroxide.

According to the guidelines of the National Cancer Institute (2015) plant screening program, plants with IC₅₀ less than 20 $\mu\text{g/mL}$ would be considered as active



cytotoxicity activity and act as an ideal anticancer agent (Boik, 2001). All extraction methods of honey and black seed mixture had IC₅₀ less than 20 µg/mL, as shown in Table 2. In addition, previous study reported a strong correlation between bioactive compound (alkaloid, flavonoid, phenol, tannin and saponin) with anticancer properties (Mujeeb et al., 2014). Thus, the anticancer properties of honey and black seed mixture might be due to the presence of bioactive compound in honey and black seed mixture. Thus, we can conclude that honey and black seed mixture has a potential of natural chemopreventive agent for breast cancer.

Honey and black seed mixture showed stimulation of growth of MCF-7 at lower concentration and inhibition of growth of MCF-7 at higher concentration. This might be due to high contents of phenolic compound present in honey since phenolic compound are phytoestrogens which exert dual actions of both inhibitory and stimulatory effect (Erejuwa et al., 2014). Phytoestrogens have a similar structure with estrogen and can bind with estrogen receptors, and are thus able to elicit estrogenic or antiestrogenic effect depending on certain factors including concentration (Erejuwa et al., 2014). Honey also contain sugar and sugar also known as energy source for cancer cell (Fadaka et al., 2017).

AO/Pi staining was performed in order to study the membrane integrity of MCF-7 cell after treated with different extraction method of honey and black seed mixture at concentration of IC₅₀. AO and Pi are nuclei acid binding dye which give strong fluorescent when bind with DNA where AO is permeable to both live and dead cell while Pi only permeable to dead cell (Foglieni et al., 2001). Cells stained with red indicated apoptotic or necrotic cell which had already lost membrane integrity, while green indicated viable cells (Ismail et al., 2016).

Figure 1 shows the cell morphology of negative control, positive control and cells treated with difference extraction methods of honey and black seed at concentration of IC₅₀. The MCF-7 cells were stained with green colour with round and green nuclei because Pi impermeable to intact membrane of viable cells. However, cells of positive control were stained red and the cells have lost its shape because small round shape showed that the cells treated with hydrogen peroxide were dead within 24 hours due to its strong anticancer properties (Liu et al., 2015). Results found that all extraction methods showed cells stained with both red and green colours. The nuclei stained with red colour

for different extraction method still have the shape of cell, indicating that it was at phase of late apoptosis but not dead yet (Ismail et al., 2016). This implied that membrane integrity of MCF-7 cell lost after treated with honey and black seed mixture at concentration IC₅₀. In short, honey and black seed mixture-induced apoptosis indicate that a mixture has a cytotoxicity effect against MCF-7 (Han et al., 2016).

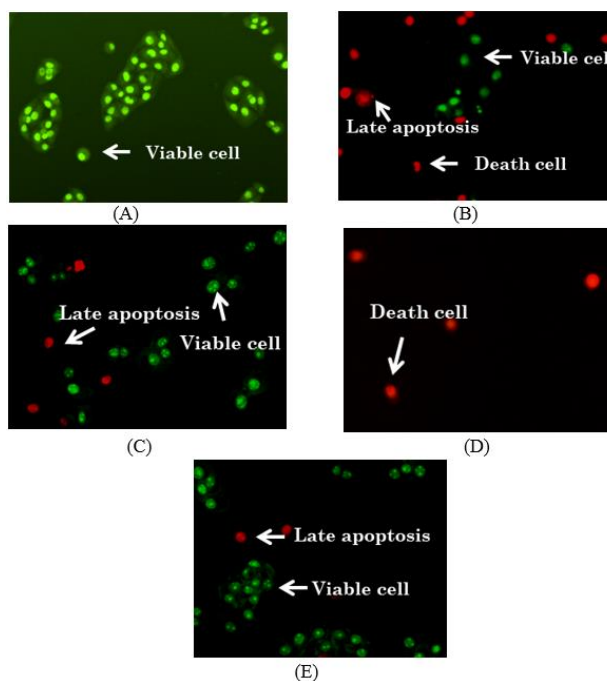


Fig. 1. AO/Pi staining of MCF-7 cell

- (A) Untreated MCF-7 Cell Line (Negative Control)
- (B) MCF-7 Cell Line Treated with Hydrogen Peroxide (Positive Control)
- (C) MCF-7 Cell Line Treated with IC₅₀ of Methanolic Extracted Honey and Black Seed Mixture
- (D) MCF-7 Cell Line Treated with IC₅₀ of Aqueous Extracted Honey and Black Seed Mixture
- (E) MCF-7 Cell Line Treated with IC₅₀ of Mixture of Methanolic Extracted Honey and Aqueous Extracted Black Seed Mixture

Table 3 shows significant differences for most concentrations among all extraction methods of honey and black seed mixture. This demonstrates that the antioxidant levels of honey and black seed mixtures were very dose dependent and differed significantly, even at low concentrations. However, in term of extraction method of honey and black seed mixture, no significant ($p > 0.05$) difference between extraction method but a significant difference between extraction methods and positive control individually.

Table 3. Percentage of DPPH inhibition by different extraction method of honey and black seed mixture in antioxidant assay

Concentration (mg/mL)	% of DPPH Inhibition			
	ME	AE	ME+AE	Quercetin
0	0 ^a	0 ^a	0 ^a	0 ^a
0.156	12.77(3.56) ^b	12.16(2.29) ^b	7.73(1.85) ^b	57.07(1.82) ^b
0.313	22.55(3.76) ^c	24.92(2.53) ^c	11.30(2.76) ^{bc}	73.45(2.94) ^c
0.625	24.64(3.14) ^c	39.66(1.56) ^d	15.59(3.08) ^c	75.65(5.63) ^{cd}
1.25	26.02(0.65) ^c	44.68(3.75) ^{de}	23.22(2.21) ^d	79.87(3.24) ^{cd}
2.5	38.17(0.79) ^d	50.84(3.69) ^e	35.84(1.97) ^e	84.60(3.08) ^d
5	53.65(2.50) ^e	60.43(2.58) ^f	57.69(1.14) ^f	84.64(3.08) ^d
10	68.43(1.81) ^f	70.76(2.19) ^g	74.22(3.31) ^g	84.75(3.33) ^d

Result are mean (SEM) of triplicate. In the same column, different letters (a, b, c, d, e, f and g) indicate significant different ($p < 0.05$) by one-way ANOVA and tukey post-hoc comparison. Note: ME=Methanolic extraction of honey and black seed mixture; AE=Aqueous extraction of honey and black seed mixture; ME+AE=Mixture of methanolic extraction of honey and aqueous extraction of black seed; Q=Quercetin.

This might be due to the solvents used were methanol and aqueous. Nandhakumar and Indumathi (2013) stated that both methanol and aqueous are best solvents for extraction of antioxidant compound. All extraction methods were able to reduce the stable, purple colour radical DPPH into yellow colour DPPH by 50% reduction. The results demonstrated an increase of antioxidant properties with increase concentration of sample.

The EC₅₀ value of result indicated that aqueous extracted honey and black seed mixture exhibit the best result of antioxidant properties of honey and black seed. This might be due to the higher temperature and longer time during saturated process using rotary evaporator. Due to water has higher boiling point (100°C) as compared to methanol (65°C), so water is more difficult to vaporize (PubChem, 2004). Thus, the temperature and time for methanolic extraction of honey and black seed mixture and aqueous extraction method of honey and black seed mixture were not similar, 40°C for 30 minutes and 50°C for 2 hours, respectively. The variation shortens the saturation period of aqueous extraction of honey and black seed mixture. This variation was indirectly increase the antioxidant activity of aqueous extraction honey and black seed mixture as supported by previous study which showed that total phenolic content from sample was the highest when extracted at 49°C for 2.8 hours (Juhaimi and Ghafoor, 2013). Another study reported a strong correlation between total phenolic content and DPPH assay of antioxidant activity (Schlesier et al., 2014). Thus, the results of the present study are in agreement with a previous study showing that heating

does enhance antioxidant activity in fruit and vegetables due to the development of the antioxidant properties of naturally occurring compounds or the formation of new compounds, such as Mailard reaction products which have antioxidant activity (Sharma et al., 2015).

There was only one previous study on antioxidant properties of honey and black seed mixture (Mohamad, 2013). The study reported the lipid soluble antioxidant capacity by photochemiluminescence (PCL) assay of honey and black seed mixture (29.94 µg/mL) had more than total lipid soluble antioxidant capacity of honey (7.18 µg/mL) and black seed (9.67 µg/mL), respectively. PCL assay is used to determine the antioxidant activity of sample extracts against superoxide anion radical (O₂⁻) generated from luminol, a photosensitizer, under exposure to UV light (Przygodzka et al., 2013), whereas DPPH is used to determine the ability to reduce the radical cation (Schlesier et al., 2014). Both PCL and DPPH use organic radical producers, but PCL assay is more sensitive since it can analyse antioxidant activity in the nanomolar range while DPPH assay only can determine antioxidant activity in the micromolar range (Schlesier et al., 2014). However, both PCL assay and DPPH assay are fully applicable to determine the antioxidant capacity of lipophilic fraction samples and both assay are highly correlated ($r=0.91$) (Sielicka et al., 2014). Thus, the antioxidant activity results obtained from both assay are comparable. In the PCL assay, the higher the antioxidant capacity indicated the higher antioxidant activity where the lower the EC₅₀ value in the DPPH assay indicated the higher



antioxidant activity. From PCL assay done by Mohamad et al. (2015), the antioxidant activity of honey and black seed mixture is more than total antioxidant capacity of total honey and black seed, indicating some unidentified chemical reaction occurred between the mixtures. This might be due to unknown properties of honey because there is another study done by Mohamed et al. (2016) also showed a similar trend on his sample of *S. dubium* seeds and honey mixture.

In the present study, the trend was observed in which the EC₅₀ of honey and black seed mixture is lower than half of total black seed and honey. By comparing to Poh (2016), the EC₅₀ of methanolic extracted honey was not achieved and aqueous extracted black seed was 9.39 mg/mL using DPPH assay. The lower values of EC₅₀ indicate that lower concentrations are needed to inhibit 50% of oxidation. Other than black seed and honey, a study by Mohamed et al. (2016) using *S. dubium* seed and honey as sample also showed higher antioxidant activity of honey and seed combination as compared to honey and seed separately. From the study, we can conclude that the higher antioxidant activity of honey and black seed mixture might be due to antioxidant properties of honey, since antioxidants from a combination of different seeds with honey exhibit similar trends.

In addition, the combination of honey and black seed mixture at ratio 1:1 has been used in the formulation of proposed for blood lipid modulation. High antioxidant activity in honey might play an important role in control of lipid metabolism (Nemoseck et al., 2011). It has been shown that honey and black seed mixture showed significant cardio protective effect based on lipid profile modulation (Mohamad et al., 2015). In the present study, phytochemical screening test was carried out to qualitatively confirm the presence of five bioactive compounds in honey and black seed mixture using standard method. Bioactive compounds are also known as secondary plant metabolites that contain biological properties of antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property (Saxena et al., 2013). Results indicated the presence of alkaloid, flavonoid, phenol, tannin and saponin in honey and black seed mixture.

It has been reported that methanolic extract of honey and black seed mixture becomes turbid brown after reacting with Wagner's reagent, indicating the

presence of alkaloids (Solihah et al., 2012). In addition to protective functions in plants, alkaloid have protective properties in human such as antiproliferation and antimetastasis effects on various type of cancers both in vivo and in vitro (Lu et al., 2012). These compounds are the most important active ingredients in natural herbs and have been successfully developed into chemotherapeutic drugs such as camptothecin (CPT), a famous topoisomerase I (TopI) and vinblastine, which interacts with tubulin to kill cancerous cells (Huang et al., 2007).

The development of intense green colour after the reaction between methanolic extract honey and black seed mixture and ferric chloride indicated the presence of flavonoid (Yessuf, 2015). Many flavonoids exhibit antioxidant activity, free radical scavenging capacity, coronary heart disease prevention, hepatoprotective, anti-inflammatory and anticancer activities, while some flavonoids show antiviral activity (Kumar and Pandey, 2013). Furthermore, flavonoids are vital in nutraceutical, pharmaceutical, medical and cosmetic applications (Panche et al., 2016).

Phenols are active in nutrient uptake, protein synthesis, enzyme activity, photosynthesis; structural components and allelopathy in herbs. Phenols also have vital function in inhibitors of procarcinogen activation, inducers of drug binding of carcinogens and inhibitors of tumour genesis which showed the anticancer properties (Saxena et al., 2013). In addition, phenols also play an important role in the prevention and curing of various diseases related to free radicals due to their strong antioxidant properties (Singh et al., 2015).

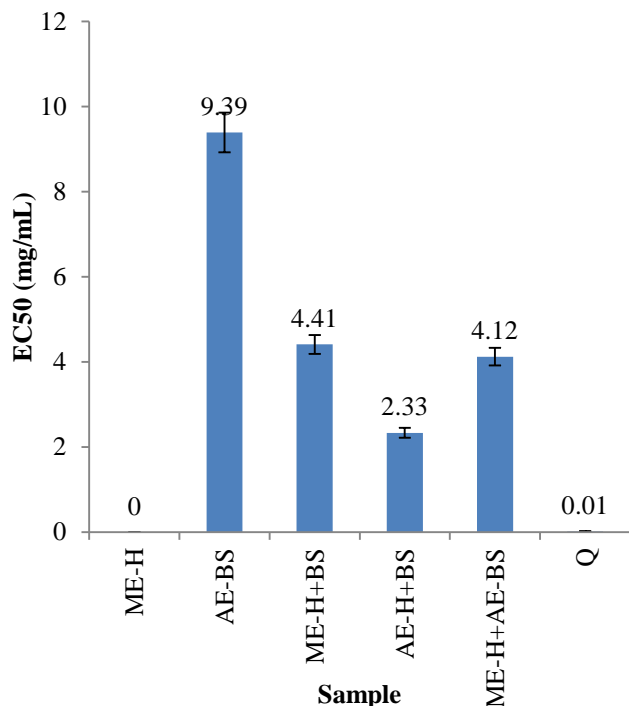
Tannins as a phenolic compound are considered as primary antioxidants or free radical scavengers (Hossain et al., 2013). Tannin-containing plant are used as medicine in Asian (Japanese and Chinese) as a astringents used to against diarrhea, diuretics, stomach and duodenal tumours, as anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Saxena et al., 2013). This compound also shown antitumor activity against HL-60 cells, anticancer effects against human tumorigenic (colon, HCT-116; breast, MCF-7) and inhibition of the proliferation of M220 pancreatic cancer cells and MCF-7/HER2 and JIMT-1 breast cancer cells (Yildirim and Kultu, 2015).

Finally in plants, saponins act as a defence system. In animals, saponins extracted from plants showed biological and pharmacological activities such as anti-inflammatory, anti-hepatotoxic, wound healing,



veinotonic, expectorant, spasmolytic, hypoglycemic, antimicrobial and antiviral properties (Visweswari et al., 2017). In addition, most saponins have been reported to have cytotoxic effect on various cancer lines (Singab et al., 2015).

Fig. 2. EC₅₀ of honey, black seed and different extraction method of honey and black seed mixture.



Note: ME-H=Methanolic extraction of honey; AE-BS=Aqueous extraction of black seed; ME-H+BS=Methanolic extraction of honey and black seed mixture; AE-H+BS=Aqueous extraction of honey and black seed mixture; ME-H+AE-BS=Mixture of methanolic extraction of honey and aqueous extraction of black seed; Q=Quercetin

A study by Mohamed et al. (2016) reported that the some bioactive compounds in *S.dubium* seed and honey mixture are lower than honey or seed separately. This phytochemical screening is used to identify the presence of bioactive compounds in a mixture, because it might be possible that some bioactive compound could decrease or lost in the mixture of honey and black seed that caused by unknown reaction. In the present study, a simple and quick phytochemical screening test was carried out to confirm alkaloid, flavonoid, phenol, tannin and saponin levels in the honey and black seed mixture. This can simplify the process for further study. Since these bioactive compounds are confirmed to be present

in honey and black seed mixture, quantitative test of each bioactive compound are suggested to be carried out in future studies.

Although many plants have these bioactive compounds, honey and black seed mixture with unknown reaction is intriguing as it might show higher quantities of bioactive compounds in mixture than found in honey and black seed separately. This can be indirectly proven by the strong antioxidant properties of honey and black seed mixture as compared to honey and black seed alone, since there is strong correlation between antioxidants and bioactive compounds (Przygodzka et al., 2013).

Conclusion

Methanolic extracted honey and black seed, aqueous extracted honey and black seed and mixture of methanolic extracted honey and aqueous extracted black seed have all shown potential as anticancer agent, since they have active cytotoxicity activity with IC₅₀ less than 20 µg/mL. Additionally, honey and black seed mixtures also showed a cytotoxic effect on MCF-7 cell line by causing changes to cell morphology and membrane integrity. In addition, honey and black seed mixtures possess high antioxidant activity than either alone as compared to a previous study using the same sources of honey and black seed. In the present study, the results have indicated no significant difference between all extraction methods, since methanol and aqueous are both equal solvents in terms of antioxidant determination and cytotoxic effect on MCF-7. On the other hand, alkaloid, flavonoid, phenol, tannin and saponin are all bioactive compounds that play important roles in anticancer, antioxidant, anti-inflammation, and antimicrobial properties, as shown in the present study.

Contribution of Authors

Ping KM: Conducted research, analysed the data and drafted the manuscript
 Islamiah M: Supervised research experiment
 Hadi N: Read and rearranged the manuscript
 Yusof HM: Planned the experimental design and supervised the experiment

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