Antioxidant potential and low-density lipoprotein cholesterol (LDL-c) uptake of the black seed and honey mixture on human hepatocellular carcinoma (HepG2) cells

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Abstract

High lipid levels especially low-density-lipoprotein cholesterol (LDL-c) are associated with increased risk of cardiovascular (CVD) and coronary heart disease (CHD). Both black seed (Nigella sativa L.) and honey are well-known in the hypolipidemic potential and have CVD protective effects. In the present study, LDL-c uptake of the black seed and honey mixture was tested on HepG2 cells. Antioxidant activities of black seed and honey mixtures were determined through the 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay. The anticancer potential of black seed and honey mixtures in HepG2 cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. Black seed possessed the highest antioxidant activities with EC<sub>50</sub> value 6.54 mg/mL as compared to honey with EC<sub>50</sub> value 9.56 mg/mL while the black seed and honey mixture have EC<sub>50</sub> between black seed and honey. From the results obtained, no synergistic effect was observed in the mixtures as the EC<sub>50</sub> values were within the range of black seed and honey. Furthermore, no significant difference (p>0.05) among ratios (1:1, 2:1 and 1:2). However, the decrease in cell proliferation was the highest in black seed and honey mixture at 1:1 ratio (p<0.05) than individually treated black seed and honey. Thus, the black seed and honey mixture at ratio 1:1 was the most potent anticancer agent with an IC<sub>50</sub> value of 7.44 μg/mL. The present study illustrated that black seed and honey mixtures possess a lipid-lowering effect via LDL-c uptake in HepG2 cells (p<0.05). The highest LDL-c uptake was observed at 15 μg/mL with the treatment of black seed and honey mixture at 1:2 ratio which was 294.4%. Further studies should be conducted on primary human liver cells to further justify the correlation between the antioxidant level and LDL-c uptake mechanism of black seed and honey mixtures.

1. Introduction

Hyperlipidaemia is a condition characterized by an abnormal elevation in one or more of the serum lipids such as total cholesterol, low-density lipoprotein cholesterol (LDL-c) and triglycerides (Shinde et al., 2013). Hypercholesterolemia is the main risk factor for cardiovascular diseases such as ischaemic heart disease and ischemic stroke. Elevated LDL-c is a risk factor for the development of cardiovascular disease. Recently, the role of antioxidants in plant-derived foods were studied in relation to human health. A previous study reported the beneficial effect of fruits and vegetables rich diets with a protective effect against chronic diseases including CVD (Wang et al., 2014) due to the medicinal compound in the plant (Saman et al., 2010).

There are several forms of cholesterol-lowering drugs used to treat hypercholesterolemia. These drugs can be associated with side effects. One of the most popular cholesterol-lowering drugs, statin, has been shown to contribute to symptoms of intolerance or even adverse side effects such as myopathy, hepatotoxicity, neurological effects and diabetes mellitus (Wilkinson et al., 2010).

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In addition, cancer chemotherapy is associated with fatigue, pain, diarrhoea, nausea and vomiting, sores in the mouth and throat, loss of appetite, hair loss and several others (Lorusso et al., 2017). As a result, many people today prefer natural herbs or plants to replace their medication since herbal therapies have fewer side effects as compared to prescription drugs.

Previous studies reported that both black seed and honey are effective in lowering the serum LDL-c, possess high antioxidant activity and are effective against cancer cells (Majid et al., 2013; Shafiq et al., 2014; Sahebkar et al., 2016). Nigella sativa, from the family Ranunculaceae, which is also known as black seed consists of several bioactive compounds such as thymoquinone, α-hederin, alkaloids, flavonoids, antioxidants, fatty acids and many others (Sultan et al., 2015). There is evidence that shows that consumption of black seed can significantly decrease LDL-c in the blood (Asgary et al., 2013; Sahebkar et al., 2016) apart from total cholesterol and triglycerides lowering. Recently, Rashidmayaev et al. (2019) reported that N.sativa seed oil supplements exhibited benefits on serum liver enzymes and lipid profile in non-alcoholic fatty liver disease (NAFLD) patients. The hypocholesterolemic effect of black seed is due to the presence of bioactive compounds that can help to lower serum cholesterol such as phytochemicals, antioxidants and unsaturated fatty acids (Sahebkar et al., 2016) including monounsaturated oleic acid, polyunsaturated omega-6 linoleic acid and omega-3 linolenic acid. Besides, the major bioactive compound thymoquinone and its dimer dithymoquinone, thymohydroquinone, and thymol in black seed also have an anti-cancer effect (Shafiq et al., 2014). These compounds possess cytotoxicity activity against a few human cancer cell lines such as HepG2 cells, and also colon, breast and lung cancers (Shafiq et al., 2014). Honey, on the other hand, has beneficial therapeutic components that are also related to its antioxidant properties. Polyphenol compounds such as phenolic acid and flavonoids, rather than enzymes (peroxidase, catalase), vitamins and mineral trace elements are known to be responsible for this action (Gheldof et al., 2002).

Studies proved that consumption of honey can reduce LDL-c in serum (Majid et al., 2013; Wan Ghazali and Mohamed, 2015). Extensive studies have shown that honey is effective against cancer cells including HepG2 cells. A study reported that honey exhibits its cytotoxic effects against HepG2 cells through reduction of expression of the B-cell lymphoma 2 (Bcl-2) gene which leads to apoptosis (Sadeghi-Aliabadi et al., 2015). The anti-cancer properties of honey are contributed by its flavonoids and phenolic content (Erejuwa et al., 2014).

One of the functions of the liver is to maintain an individual cholesterol balance by controlling the concentration of LDL-c in the plasma. The concentration of cholesterol can affect the function of the low-density lipoprotein receptor (LDLR) in the liver. LDLR is responsible for mediating the intake of LDL plasma by endocytosis regulated by the receptor and cholesterol in cells used (Goldstein and Brown, 2009). Cancer refers to the uncontrolled growth of cells that display malignant conduct. The abnormal cells expand quickly outside their natural limits and then spread to neighbouring body parts and other organs. Some of the common death from cancer are lung, liver, stomach, colorectal, breast and oesophagus (Stewart and Wild, 2015). Hepatocellular carcinoma (HCC) is also known as primary hepatic cancer. It’s one of the world’s most common cancer. Persistent Hepatitis B (HBV) or Hepatitis C (HCV) infections are the major risk factors for HCC. Other risk factors include aflatoxin exposure from Aspergillus fungi, alcohol, smoking, overweight, diabetes, family factors as well as dietary factors such as high intake of meat and high glycemic load (Bosetti et al., 2004). HepG2 is one of the common HCC forms.

Since previous studies reported that both black seed and honey are effective in lowering the serum LDL-c, possess high antioxidant activity and are effective against cancer cells, it is, therefore, important to discover and develop a natural product from foods that has multiple effects in cholesterol-lowering through the increase of LDL-c uptake in liver and antioxidant activities. However, the exact mechanisms responsible for cholesterol-lowering are not established because there are a few mechanisms responsible for cholesterol-lowering such as LDL-c uptake, reduction of LDL-c synthesis and reduction of LDL-c absorption. In addition, several studies have documented that the combination of black seed and honey may improve health problems such as functional dyspepsia and asthma (Mohtashami et al., 2015; Koshak et al., 2017). Furthermore, according to Hassan et al. (2012), both black seed and honey are effective against HepG2 cells but further studies should be performed on the impact of their mixtures on HepG2 cells. Besides, HepG2 cells are chosen in this study because they still exhibit multiple hepatic roles, including those linked to cholesterol and triglyceride metabolism (Donato et al., 2015). Thus, in the present study, the black seed and honey mixtures will be used to determine their effects on lowering LDL-c, antioxidant activity and its anti-cancer properties.

2. Materials and methods

2.1 Samples

Human Hepatocellular Carcinoma (HepG2) cells
were obtained from the Institute of Marine Biotechnology (IMB) Laboratory, Universiti Malaysia Terengganu (UMT). Eagle’s minimal essential medium (EMEM) supplement and lipid-depleted fetal bovine serum (FBS) was from ThermoFisher Scientific. Trypan blue stain (0.4%) for the usage together with a hemocytometer, trypsin-EDTA solution, cholesterol (>99%), FBS, streptomycin/pensetrip, phosphate buffer solution, thiazolyl blue tetrazolium bromide (MTT) were all from Sigma Aldrich, Unites States of America. 25-hydroxycholesterol was from Cayman Chemical, US and BODIPY® FL LDL was from Invitrogen, US.

2.2 Assay of 2, 2'-diphenyl-1-picrylhydrazyl (DPPH)

Antioxidant activity was evaluated using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) as described by Ramli et al. (2008), with some modifications. DPPH stock solution was prepared by dissolving 2.37 mg of DPPH powder in 100 mL absolute (100%) methanol. Black seed and honey mixtures stock solution were prepared with dimethyl sulfoxide (DMSO) to the concentration of 10 mM, respectively together with quercetin as the positive control. The solutions were loaded into a 96-well plate with performed serial dilution. Negative control was purely DMSO without test material. DPPH stock solution of 200 μL was applied to each treated well and incubated for 30 minutes in dark. The absorbance of the mixture was read at 517 nm using an ELISA microplate reader. The percentage of the DPPH free radical activity was calculated using the following equation:

\[ \text{DPPH scavenging effect (\%)} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]

Where \( A_0 \) was the absorbance of the control and \( A_1 \) was the absorbance in the presence of the extracts. The \( EC_{50} \) (concentration providing 50% inhibition) values were calculated using the dose inhibition curve in the linear range by plotting the extract concentration versus the corresponding scavenging effect.

2.3 Cell viability study

Cell viability was examined using MTT cell assay. The cell density of \( 1.0 \times 10^4 \) per well was seeded in a 96 well plate and grown to confluence. Ratio concentrations of black seed and honey mixture 1:0, 0:1, 1:1, 2:1 and 1:2 were prepared and administered to cells. Cells were incubated for 16 hrs at 37°C with 5% CO₂.

2.4 Low-density lipoprotein- cholesterol intake (LDL-c)

Confluent cultures were seeded after applying trypsin for 4 mins. Cells were then seeded at \( 1.0 \times 10^4 \) cells per well per 100 μL culture medium in 96 well plates and allowed to grow until confluent. To study the effect of black seed and honey mixture on the LDL-c uptake in HepG2 cells, HepG2 cells were treated with EMEM complete medium, 0.05% lipid depleted FBS, 10 μg/mL cholesterol, 1 μg/mL 25-hydroxycholesterol with different ratios of black seed: honey (1:0, 0:1, 1:1, 1:2, 2:1).

To study the effect of black seed and honey mixture combinations at different ratios of (1:0, 0:1, 1:1, 2:1, 1:2) and incubated for 16 hrs. All groups of cells were in quadruplets. Varioskan fluorescent plate reader was used to read the plate at 490 and 515 nm (Ishikawa et al., 2015).

2.5 Statistical analysis

Data were expressed as mean and standard deviation, of multiple determinations performed using black seed and honey mixtures derived from different concentrations with Minitab software version 14.0. One-way ANOVA was conducted and multiple comparison analysis was carried out using Fisher’s LSD test. In all analyses, if \( p < 0.05 \), the difference was considered significant.

3. Results and discussion

In the present study, the antioxidant of black seed and honey extracts mixtures with different ratios were performed to determine the best free scavenging activity through DPPH reduction. Table 1 shows the trends indicated with the increased sample concentration subsequently increased the scavenging effects on DPPH radical. The efficiency concentration of black seed and honey mixtures to inhibit 50% DPPH radical (\( EC_{50} \)) is shown in Table 2. The \( EC_{50} \) is a parameter used to quantify antioxidant activity, with a lower value suggesting higher antioxidant potency. Among the treatments, the sample with black seed extract showed the highest DPPH reduction percentage while the sample with honey extract alone showed the lowest DPPH reduction percentage (Table 1) as shown with their \( EC_{50} \) value in Table 2. Thus, it can be observed that the antioxidant properties in the mixtures were mostly due to the antioxidant activity in black seed extract and can be suggested that there might be no synergistic effect between the black seed extract and honey extract samples.

Quercetin is a polyphenolic flavonoid compound that is commonly present in plants and plant food resources. It is a good natural antioxidant because of its ability to scavenge free radicals and bind transition metal ions (Baghel et al., 2012). Thus, in the present study, quercetin was selected as a positive control for the antioxidant assay. Mohamad et al. (2014) reported that
Table 1. DPPH Inhibition by black seed and honey mixture

| Concentration (mg/mL) | B   | H   | BH  | BBH | BHH | Q   |
|-----------------------|-----|-----|-----|-----|-----|-----|
| 0.156                 | 2.19(0.69)c | 1.15 (0.52)c | 1.31(0.27)c | 7.32(1.00)b   | 2.56(1.02)c | 15.25(4.59)a |
| 0.313                 | 4.15 (0.84)c | 2.09(0.28)c   | 3.47(1.43)c | 7.71(0.60)b   | 7.02(0.81)b | 30.05(1.66)a |
| 0.625                 | 13.43(4.29)b | 4.39 (0.36)c   | 6.75(1.66)c | 15.79(1.90)b | 9.04(1.02)c | 68.62(8.22)a |
| 1.25                  | 16.00(3.83)c | 8.10 (0.54)d  | 15.97(3.04)d | 21.37(2.65)b | 12.53(1.40)c | 96.81(0.61)d |
| 2.5                   | 34.24(5.06)c | 12.42(1.42)d   | 23.75(4.83)c | 25.91(3.04)c | 14.13(1.35)d | 97.93(0.56)d |
| 5                     | 40.25(1.85)c | 28.68(2.00)f  | 38.80(2.77)f | 45.04(5.10)b | 31.31(4.29)d | 97.98(0.58)d |
| 10                    | 72.13(3.65)c | 51.38(1.87)d | 57.02(2.97)f | 59.28(1.87)c | 56.79(3.16)c | 98.42(0.48)f |

Values are presented as mean(SD) from quadruplicate. Values with different superscript within the same row indicate significant difference (p<0.05) by one-way ANOVA.

Table 2. EC\textsubscript{50} of black seed and honey mixtures

| Treatment | EC\textsubscript{50} (mg/mL) |
|-----------|-----------------------------|
| B         | 6.54                        |
| H         | 9.56                        |
| BH        | 7.97                        |
| BBH       | 7.34                        |
| BHH       | 8.67                        |
| Q         | 0.76                        |

B = Black seed, H = Honey, BH = mixture of black seed and honey with the ratio 1:1, BBH = mixture of black seed and honey with the ratio 2:1, BHH = mixture of black seed and honey with the ratio 1:2, Q = Quercetin

Lower antioxidant activity can be seen in the present study due to the aqueous extraction of black seed used in this study, which may lead to lower phenolic and flavonoid content. Besides, Islam et al. (2012) reported that black seed extract had an IC\textsubscript{50} value of 0.16 to 1.03 mg/mL in DPPH radical scavenging activity using different extraction methods, including essential oil, methanol extract, hexane extract, chloroform extract and ethyl acetate extract. The black seed essential oil displayed the lowest IC\textsubscript{50} value at 12.76 μg/mL compared to another method of extraction. This is because 24 constituents have been identified in the black seed oil, although only 16 components have been identified in the extracts. It was confirmed that the black seed oil had the highest and the strongest radical scavenging activity compared to the extracts. Meanwhile, Haron et al. (2014) determined the antioxidant activity of black seeds of different origins by DPPH inhibition. They reported that black seed from Iran had the lowest IC\textsubscript{50} of 1.49 mg/mL followed by black seed from Kelantan with IC\textsubscript{50} of 1.79 mg/mL. The IC\textsubscript{50} obtained in the present study was higher than the previous research suggesting a lower antioxidant activity in the samples used. Differences may be due to the method of extraction used in which the black seed was extracted by aqueous extraction in the present study, which may lead to lower phenolic and flavonoid contents of the extract, whereas the methanol extract and black seed oil were used in the previous study. The difference in antioxidant activity values may also be attributable to the different origins of the black seed. As shown in the previous report, local black seed (Kelantan black seed) has lower antioxidant activity than foreign black seed (Iran) as a proportion of its total phenolic content. The extraction method can also affect the antioxidant activity by which oil extraction has the highest antioxidant activity compared to other extraction solvents (chloroform and ethyl acetate) whereas the aqueous extract showed the lowest (Haron et al., 2014). The low antioxidant activity of the aqueous black seed extract was also proven in the present study.

Based on Table 3, it was observed that all ratios
lowered the HepG2 cell viability on a dose-dependent pattern. Among all treatments, the mixture of black seed and honey with a 1:1 ratio showed the significantly (p<0.05) lowest percentage of cell viability (3.45%) at the highest concentration, 100 μg/mL. The lowest cell viability might be due to the synergistic effect of both black seed extract and honey extract at 1:1 ratio, but not at 2:1 or 1:2 ratio mixture. However, the effect of the black seed and honey mixture of 1:1 ratio was most probably contributed by the potential of black seed which was shown by the lowest percentage of cell viability of black seed extract at 50 μg/mL.

Table 4 shows that black seed extracts possessed the most potent anticancer activity against HepG2 cells (IC$_{50}$ value 7.62 μg/mL) while honey extracts possessed the lowest IC$_{50}$ value 31.10 μg/mL. All treatments were ideal anti-cancer agents with IC$_{50}$ values of less than 20 μg/mL except for the treatment with honey extract alone with the moderate cytotoxicity of IC$_{50}$ values between 20 to 100 μg/mL. (Sajjadi et al., 2015). In general, the anticancer effect of black seed and honey mixtures could be due to the anti-cancer properties of black seed instead of honey, particularly at a lower concentration. In general, the anticancer effect of black seed and honey mixtures could be due to the anti-cancer properties of black seed instead of honey, particularly at lower concentrations. However, there was a possibility that black seed and honey mixture 1:1 ratio to show the synergistic effect at a higher concentration since the mixture showed a significant reduction of cell viability starting from 12.5 μg/mL.

According to Hassan et al. (2012), treatment of HepG2 cells with the black seed at the highest concentration of 5000 μg/mL reduced the survival rate of HepG2 cells by 37.8% after 72 hrs of treatment which had a lower effect as compared to the present study. In the present study, black seed showed a higher reduction of cell viability at a lower concentration (100 μg/mL) of extracts and a shorter treatment period (24 hrs). This is due to the different types of black seed as the black seed used in the previous study was from Egypt. For honey, a previous study showed that Gelam honey exerted high cytotoxic on HepG2 cells and human hepatic cells (WRL-68). Gelam honey reduced the cell viability of HepG2 less effectively by showing IC$_{50}$ at 25% w/v of 31.10 μg/mL which is equivalent to 0.0031% w/v. This proved that Gelam honey exhibited growth inhibition at higher concentrations and was less sensitive in HepG2 cells as compared to the current honey sample.
immunomodulatory activity, cyclooxygenase-2 (COX-2) modulation and tumour necrosis factor (TNF) modulation (Ahmed and Othman, 2013).

Figure 1 shows that the highest LDL uptake was exhibited by the black seed and honey mixture at ratio 1:2 with 294.4% at 15 μg/mL as compared to the positive control. This suggests that the black seed and honey mixture might gave a synergistic effect.

Figure 1. Effect of black seed and honey extracts mixtures on LDL-c uptake in HepG2 cells. Values are presented as mean (SD) from quadruplicate. Bars with the different notation within the same group indicate significant difference (p<0.05) by one-way ANOVA.

A previous study showed that black seed and honey mixture had significant benefits in hypercholesterolemic subjects as compared to healthy subjects. For hypercholesterolemic subjects, their total cholesterol, triglycerides, and the ratio of TC: HDL-c decreased significantly while HDL-c increased significantly; while there was also a decrease in LDL-c but not significantly (Mohamad et al., 2014). Thus, it shows that the result was correlated with the present study whereby the black seed and honey mixture showed a synergistic effect on cholesterol-lowering. El-Kholy et al. (2007) reported that rats with the treatment of black seed and honey mixture showed significant improvements in total lipids, total cholesterol, triglycerides, HDL-c and LDL-c as compared to the treatment of black seed and honey alone.

In the present study, black seed extract showed high LDL uptake (246.0%) at 20 μg/mL while honey extract had LDL-c uptake by 248.0% at 15 μg/mL. Previously, Ibrahim et al. (2014) found that menopausal women that were orally given one gram of black seed capsules daily for two months demonstrated significant improvement in total cholesterol, triglycerides, LDL-c, HDL-c and blood glucose although there were no significant changes in body weight. On the other hand, young healthy Pakistani males who received 70 g of honey every day for 4 weeks showed a significant decrease in total cholesterol and LDL level while HDL increases significantly. However, there were no significant changes in their blood glucose and triglyceride (Majid et al., 2013).

A thymoquinone-rich fraction from black seed was observed to induce LDL-c uptake through the upregulation of LDL receptor (LDLR) gene and inhibition of cholesterol synthesis through suppression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) gene in HepG2 cells more than thymoquinone. This suggests that the effect of the thymoquinone-rich fraction may be due to the combination of rich components in black seed (Al-Naqeep et al., 2009). Besides, quercetin in honey increases the clearance of circulating LDL-c levels from the blood through up-regulation of LDLR gene expression at both mRNA and protein levels in HepG2 cells (Moon et al., 2012). Based on this study, it can be observed the lower antioxidant activity was due to the aqueous extraction method of black seed used which may contribute to lower phenolic and flavonoid content. However, since the LDL expression in the black seed and honey mixture was not parallel to antioxidant activity, there are possibilities that other mechanisms may contribute to LDL-c uptake in HepG2 cells. LDL-c uptake may be due to the down-regulation of proprotein convertase subtilisin/kexin type 9 (PCSK9) gene which was responsible for the degradation of LDLR in lysozyme and other lipoproteins metabolism-related gene expression includes sterol regulatory element-binding protein 2 (SREBP2) (Lagace, 2014).

4. Conclusion

The present study was the first research investigating the potential effect of black seed and honey extracts mixtures on lipid uptake in HepG2 cells. The black seed and honey mixture with a ratio of 1:2 was observed with the highest LDL-c uptake (294.4%) at 15 μg/mL. On the other hand, there was no possible synergistic effect on the antioxidant properties of the black seed and honey mixtures. This is proved by the highest EC_{50} 6.54 mg/mL in black seed extract as compared to other treatments. Since cancer cells, HepG2 cells were used in the present study for the determination of LDL-c uptake and antioxidant, the anticancer potential of black seed and honey mixtures were observed too. Black seed and honey mixtures with a ratio of 1:1 exhibited the strongest anticancer agent with an IC_{50} value of 7.44 μg/mL as compared to other treatments. In conclusion, high EC_{50} in antioxidants is not necessarily linked to high LDL-c uptake and high reduction of cell viability (IC_{50}).
Therefore, it was suggested that there may be other mechanisms that lead to LDL uptake and reduction of cancer cell viability. The present study has demonstrated evidence on the hypocholesterolemic, antioxidant and anticancer potential of black seed and honey extracts mixtures.

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