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Original Research Article

Changes of Thymoquinone, Thymol, and Malondialdehyde Content of Black Cumin (*Nigella sativa* L.) in Response to Indonesia Tropical Altitude Variation

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A B S T R A C T

Black cumin cultivated in many subtropical regions in the world, including Asia, Middle East, and North Africa. The most active constituent of black cumin is thymoquinone representing 18.4%–24% of the volatile oil and thymol. Data about thymoquinone and thymol came from the country of origin, but no data from tropical region. This study aimed to analyze the production of chlorophyll, thymoquinone, thymol, and malondialdehyde from black cumin cultivated at three altitudes of Indonesian tropical region. The result showed that Kuwait accession cultivated at middle altitude contains the highest levels of thymoquinone (2940.43 mg/kg), and the highest levels of thymol were found in India accession cultivated at high altitude (141.46 mg/kg). Data showed that the level of malondialdehyde at low (220 meter above sea level [masl]) and middle (560 masl) altitudes is higher than high (1.280 masl) altitude.

1. Introduction

Black cumin (*Nigella sativa* L.) has been used since ancient times. Presently, it is cultivated in many subtropical regions in the world, including Asia, the Middle East, and North Africa (Rabbani *et al.* 2011). It has been used for many conditions related to respiratory health, stomach and intestinal complaints, kidney and liver function, circulatory and immune system support, rheumatism, and associated inflammatory disease (Ramadan 2007). The most active constituent of black cumin is thymoquinone, representing 18.4%–24% of the volatile oil (El-Dakhakhny 1963) and thymol (Al-Saleh *et al.* 2006). There were a number of studies demonstrating the pharmacological effect of thymoquinone and thymol as the crude source of drugs in indigenous medicine system, as well as for pharmaceutical industry. To meet the increasing demand, these medicinal plants should maintain the quality and cultivated continuously for the raw material and its finished product.

The environment, especially microclimate factor has a significant role in plant growth and development. It will affect the quality of herbal ingredients even when it is produced in the same country. This condition may produce major variations in the bioactive ingredients present in the plants. The bioactive ingredients of many herbs vary with region altitude distribution. On *Fagopyrum esculentum*, it has been proved that rutin concentration at middle altitude (600 meter above sea level [masl]) is higher than high altitude (1150 masl) (Lumingkewas *et al.* 2015), asiaticoside concentration of *Centella asiatica* at high altitude (1500 masl) is higher than low altitude (250 masl) (Martono 2011), and reserpine concentration of *Rauwolfia serpentina* at low altitude (409 masl) is higher than middle and high altitudes (640 and 1066 masl, respectively)(Kumar *et al.* 2010). Al-Saleh *et al.* (2006) reported that thymoquinone and thymol content of black cumin (from Ethiopia, India, Saudi Arabia, Syria, and Sudan) were 3098.5 and 230.6; 2362.8 and 201.16; 2250.6 and 133.88; 1371.9 and 120.4; and 1274.6 and 113.40 mg/kg, respectively.

In developing black cumin cultivation in tropical region, climate is the limiting factor, particularly air temperature. This climate factor cannot be controlled, but it can be modified to be close to environment condition where black cumin plant originated. Modifying climate factor in tropical region could be done by replacing latitude position with altitude or elevation. According to Ahren (2009), the spreading of earth's surface air temperature is

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influenced by several factors including the amount of radiation received by the earth's surface, the influence of land and sea, and also the elevation and the slope of the region. Ahmad *et al.* (2010) stated that the air temperature is one of the climatic factors that influence the rate of growth and metabolic processes of plants. The influences can be seen at the development rate of plants including the germination, leaf formation, and initiation of reproductive organs. At temperatures higher than the optimum cardinal temperature, the physiological activities decline as a consequence of inactivation of enzymes and other proteins.

Exposures to high temperature induce reactive oxygen species (ROS) accumulation in plants, and causing an oxidative stress (Wormuth *et al.* 2007). ROS are toxic and reactive, and can lead to the oxidative destruction of cells. It has been known that ROS produced under stress cause lipid peroxidation and enzyme inactivation (Blokhina *et al.* 2003). Lipid peroxidation is a natural metabolic process in normal conditions, and its activation is one of the possible results of a rapid response to stress. It was defined as a reaction between polyunsaturated fatty acids, and lipid peroxide is then decomposed into several molecules, including malondialdehyde (MDA; Alessio 2000). MDA was investigated as one of the lipid peroxidation products, and its contents are often used as indicator of lipid peroxidation resulting from oxidative stress (Malancic *et al.* 2004; Krishnamurthy & Wadhwani 2012). Jaafar *et al.* (2012) showed that the MDA production has established significant positive correlations with total phenolics and total flavonoids, indicating that an increase in MDA might be involved in the upregulation of the secondary metabolite production under high water stress in *Labisia pumila.* Differences in air temperature of black cumin environment may lead to positive or negative correlations between its secondary metabolite (thymoquinone and thymol) and with its MDA production.

Most studies were using thymoquinone and thymol in black cumin seed oil. Data about thymoquinone and thymol content in black cumin seed from the country of origin (Ethiopia, India, Saudi Arabia, Syria, and Sudan) have been reported by Al-Saleh *et al.* (2006), but no data were found about its content from tropical region like Indonesia. The aim of this study is to analyze the production of chlorophyll, thymoquinone and thymol, as bioactive ingredients, and MDA content evaluation for black cumin cultivated at three altitudes of Indonesian tropical region.

2. Materials and Methods

2.1. Plant material and experimental conditions

Leaves and seeds as plant material harvested from the experiment was conducted from June to October 2015 at Bogor Agricultural University research station in Leuwikopo, Dramaga (220 masl); Sukamantri, Ciapus (560 masl); and Sari Alam Medicinal Plant Garden in Ciwidey, Bandung (1280 masl). This experiment followed 2×3 factorial arrangement: two black cumin accessions, three altitudes, with four replicates. The black cumin seeds from the country of origin, i.e. Kuwait and India were planted in polybags filled with the mixed of soil and cow manure (1:1) (v/v) and agricultural lime ($CaCO_3$) 2 ton/ha as the planting media. Seeds were hydroprimed for 12 hours before planting. After the draining process, three seeds were planted in a polybag and an "ultraviolet" plastics were also used as a shading for covering the plants from the sun hit. Urea and SP-36 in dose given at 120 kg N/ha and 157 kg P_2O_5/ha at 5 weeks after planting (Suryadi 2014). The parameters studied were chlorophyll content (Sims & Gamon 2002), MDA of leaf and seed content (Peixoto *et al.* 1999), and thymoquinone and thymol content (Al-Saleh *et al.* 2006). Those parameters were analyzed at Laboratory of Molecular Marker and Spectrophotometry UV-VIS, Department of Agronomy and Horticulture, Faculty of Agriculture; Laboratory of Biochemical and Laboratory of Chemical, Department of Food and Science Technology, Faculty of Agriculture Technology, Bogor Agricultural University, Indonesia.

Supporting data collected from sites location were daily, maximum, minimum air temperature, and air relative humidity using thermohygrometer on each location; data of rainfall and solar radiation were collected from Meteorology, Climatology, and Geophysics Council. Informations about sites of general condition are given in Table 1.

Reference standard of thymoquinone and thymol was purchased from Sigma Chemical Company (Sigma, MO, USA). All the solvents and chemicals used for extraction and analysis (trichloroacetic acid [TCA] and thiobarbituric acid [TBA]) were of high purity and quality.

2.2. Measurement of chlorophyll (Sims and Gamon 2002)

Fresh fully expanded leaves of the second leaves on initial flower stage were taken as samples for measuring the content of chlorophyll, anthocyanin, and carotenoid. About 0.02 g fresh leaf was placed in a mortar and grinded into powder. The powder was then added with 2 mL of the extraction solvent, 85% acetone, and 15% Tris stock buffer (1% w/v Tris final concentration; adjusted to pH 8 with HCl). The extract was centrifuged at 12,000 rpm for 3 min. A defined quantity of supernatant (1 mL) was removed and diluted to 3 mL then vortex. Absorbance was measured at 537, 663, 647 and 470 nm by using spectrophotometer UV-Vis. The pigment content was then calculated according to Equations (1–4).

$$\text{Anthocyanin} = (0.08173^*A_{537}) - (0.00697^*A_{647}) - (0.002228^*A_{663}); \quad (1)$$

$$\text{Chl a} = (0.01373^*A_{663} - 0.000897^*A_{537})\,(0.003046^*A_{647}); \quad (2)$$

$$\text{Chl b} = (0.02405^*A_{647}) - (0.004305^*A_{537}) - (0.005507^*A_{663}); \quad (3)$$

$$\text{Carotenoids} = (A_{470}\,[17.1^*\{CHl_a + CHl_b\} - 9.479^*\mathit{Anthocyanin}])/119.26. \quad (4)$$

2.3. Measurement of thymoquinone and thymol content (Al-Saleh *et al.* 2006)

Ground of black cumin seed sample of 0.01 g was extracted with 1 mL methanol, vortexed for 1 min and sonicated for 20 min. After that, it was left overnight in constant rotamix, vortexed for 1 min and centrifuged for 25 min at 1400 rpm. The supernatant was aspirated and an aliquot of 20 μL was injected into high-performance liquid chromatography with UV detector at a wavelength of 275 nm. The mobile phase used was methanol:water (75:25) at 1.0 mL/min flow rate. Calibration curves of peak area versus the concentration 20, 40, 80, and 160 μg/mL for thymoquinone and 2, 4, 8, and 16 μg/mL for thymol were constructed.

2.4. Measurement of MDA content (Peixoto *et al.* 1999 with modification)

2.4.1. Sampling procedure

Leaves were obtained from the plant on the stage of 30 days after flowering (Moghadam *et al.* 2013). Samples were collected and placed immediately into a coolbox. Then weighed and quickly frozen in liquid nitrogen followed by storage in a deep freezer (−30°C).

Table 1. Research site general conditions

Specification	Low altitude	Middle altitude	High altitude
Location	Leuwikopo*, West Java	Sukamantri*, West Java	Ciwidey†, West Java
Altitude (masl)	220	560	1280
Latitude	6° 56′ S	6° 61′ S	7° 09′ S
Longitude	106° 73′ E	106° 78′ E	107° 50′ E
Temperature (°C)			
Daily (Jun–Sep 2015)‡	30.30	30.20	26.70
Maximum (Jun–Sep 2015)‡	39.34	38.70	33.30
Minimum (Jun–Sep 2015)‡	19.75	19.50	18.00
RH (Jun–Sep 2015)‡ (%)	69	61	53
Rainfall (Jun–Sep 2015)§ (mm/d)	0.8	0.8	1.0
Solar radiation (Jun–Sep 2015)§ (h/d)	7.0	7.0	7.9

masl = meter above sea level; RH = relative humidity.
* Bogor Agricultural University Research Station.;
† Sari Alam Medicinal Station.;
‡ Use hygrometer.;
§ Meteorology, Climatology, and Geophysics Council (BMKG)—Indonesia..

2.4.2. Sampling analysis

Sample of 0.2 g fresh leaves or dry seeds were homogenized in 4 mL of 1% (w/v) TCA solutions. The homogenate was centrifuged at 3000 rpm for 15 min. One mL aliquots of the supernatants were added to 3 mL of 0.5% (w/v) TBA in 20% (w/v) TCA and tubes were incubated in a water bath at 95°C for 2 h. The reaction was stopped by placing the reaction tubes into ice bath. The tubes were subsequently centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 and 660 nm (Cakmak & Horst 1991) on a spectrophotometer and the concentration of the MDA-TBA complex produced was calculated using the molar extinction coefficient of 155 mM/cm (Health & Packer 1968).

2.5. Statistical analysis

Data are presented by taking into consideration the standard deviation. Results were analyzed by one-way analysis of variance to identify significant differences between the groups, and compare means test analyzed by the Tukey test at 5% level.

3. Results

3.1. Chlorophyll content

The production of total chlorophyll and carotenoid was affected by the accession and altitude ($p < 0.05$). Total chlorophyll and carotenoid on India accession was higher than Kuwait accession. The highest value of total chlorophyll was found on India accession cultivated at low and middle altitudes and the lowest value on Kuwait accession cultivated at high altitude. This pattern was similar with carotenoid content at three altitudes. Data of anthocyanin were not statistically significant affected by the treatment, although have higher tendency at high altitude for both accessions. (See Table 2).

3.2. Thymoquinone and thymol seed content

Seeds from Kuwait accession cultivated at middle altitude (560 masl) contained the highest thymoquinone (2940.43 mg/kg), followed by Kuwait accession cultivated at high altitude and India accession cultivated at middle altitude, respectively, 16.4% and 30.8% lower than Kuwait accession cultivated at middle altitude. The lowest thymoquinone was found on India accession cultivated at high altitude (64.6% lower than Kuwait accession cultivated at middle altitude; Table 3). Meanwhile, the highest content for thymol was found on India accession cultivated at high altitude (141.46 mg/kg), followed by Kuwait accession cultivated at middle altitude and Kuwait accession cultivated at high altitude, respectively, 8.5% and 33.4% lower than India accession cultivated at high altitude. The lowest level of thymol was found on Kuwait accession at low altitude (88.6% lower than India accession cultivated at high altitude; Table 3).

3.3. MDA of leaf and seed content

The leaf and seed of black cumin cultivated at low and middle altitudes contain higher MDA than high altitude for both accessions. The highest level of leaf MDA was found on India accession cultivated at middle altitude (4.839 μmol/g), followed by India accession cultivated at low altitude and Kuwait accession cultivated at middle altitude, respectively, 4.5% and 15.3% lower than India accession cultivated at middle altitude. The lowest level was found on Kuwait accession cultivated at high altitude (35.6% lower than India accession cultivated at middle altitude; Table 4). The level of seed MDA as well as leaf MDA was found highest on India accession cultivated at middle altitude (1.957 μmol/g), followed by India accession cultivated at low altitude and Kuwait accession cultivated at middle altitude, respectively, 10.4% and 17.6% lower than India accession cultivated at middle altitude. The lowest level was found on Kuwait accession cultivated at high altitude (45.6% lower than India accession cultivated at middle altitude; Table 4).

3.4. Thymoquinone, thymol, and MDA content in seed from the country of origin

All biochemical parameters in seed from the country of origin were observed in this research (thymoquinone, thymol, and MDA)

Table 2. Total chlorophyll, carotenoid, and anthocyanin content in black cumin cultivated at three altitudes

Altitude	Accession	Total chlorophyll (mg/g)	Carotenoid (mg/g)	Anthocyanin (mg/100 g)
Low	Kuwait	1.993 ab ± 0.24	0.425 ab ± 0.04	0.065 a ± 0.03
Low	India	2.173 a ± 0.43	0.482 a ± 0.08	0.066 a ± 0.02
Middle	Kuwait	2.029 a ± 0.27	0.443 ab ± 0.05	0.043 a ± 0.01
Middle	India	2.188 a ± 0.56	0.486 a ± 0.14	0.048 a ± 0.02
High	Kuwait	1.343 b ± 0.26	0.326 b ± 0.05	0.081 a ± 0.03
High	India	1.591 ab ± 0.21	0.360 ab ± 0.03	0.066 a ± 0.03

Values are mean ($n = 3$) ± SD. Means in the same column followed by the same letters are not significantly different at 5% by the Tukey HSD test.
SD = standard deviation.

Table 3. Seed water, thymoquinone, and thymol content in black cumin seed cultivated at three altitudes

Altitude	Accession	Seed water content (%)	Thymoquinone (mg/kg seed)	Thymol (mg/kg seed)
Low	Kuwait	10.319 a ± 0.828	1,539.14 bcd ± 460.52	16.18 d ± 14.76
Low	India	10.200 a ± 0.058	1,497.15 cd ± 820.79	50.81 cd ± 32.43
Middle	Kuwait	10.031 a ± 0.112	2,940.43 a ± 201.96	129.45 ab ± 54.04
Middle	India	10.567 a ± 0.074	2,033.67 abc ± 460.39	76.30 bc ± 19.99
High	Kuwait	10.394 a ± 0.235	2,459.56 ab ± 438.67	94.26 abc ± 18.87
High	India	10.258 a ± 0.087	1,039,85 d ± 217.56	141.46 a ± 7.69

Values are mean ($n = 3$) ± SD. Means in the same column followed by the same letters are not significantly different at 5% by the Tukey HSD test.
SD = standard deviation.

Table 4. Malondialdehyde content in black cumin cultivated at three altitudes

Altitude	Accession	MDA of leaf (μmol/g Fresh Weight (FW))	MDA of seed (μmol/g seed)
Low	Kuwait	3.914 c ± 0.233	1.505 c ± 0.130
Low	India	4.623 ab ± 0.149	1.753 ab ± 0.197
Middle	Kuwait	4.097 bc ± 0.318	1.613 bc ± 0.032
Middle	India	4.839 a ± 0.551	1.957 a ± 0.081
High	Kuwait	3.118 d ± 0.130	1.065 d ± 0.032
High	India	3.183 d ± 1.019	1.559 bc ± 0.049

Values are mean ($n = 3$) ± SD. Means in the same column followed by the same letters are not significantly different at 5% by the Tukey HSD test.
MDA = malondialdehyde; SD = standard deviation.

on India accession higher than Kuwait accession. Thymoquinone on India accession was higher (6.1%), thymol and MDA were almost 300% and 100%, respectively, higher than Kuwait accession (Table 5). This information needed as a data comparison for this research.

4. Discussion

Combination treatments were analyzed and found rich in thymoquinone and thymol, especially compared with seed from the country of origin (Figure), but thymol content of both accessions cultivated at low altitude was lower than seed from the country of origin, 29.67% and 56.52% on Kuwait and India accessions, respectively. Thymoquinone content resulted from this study was higher than seed from the country of origin, between 18.11% and 125.64% on Kuwait accession and 8.30% and 47.11% on India accession, respectively, except India accession at high altitude was lower, 24.78%, than seed from the country of origin (Figure).

MDA as a biomarker for oxidative stress, and this research was having same pattern on both accessions at three altitudes. All values of MDA seed content were found lower than seed from the country of origin at range 24.62%–61.33% (Figure).

Chemical composition was influenced by geochemical and climatological conditions, and it could reflect on its chemical value, particularly as medicinal plant. Chlorophyll, one of the biochemical analyzed on this research gave variation result. Chlorophyll content on both accessions was at high altitude lower than middle and low altitudes. Daily temperature difference between middle and high altitudes was 3.5°C, between low and high altitudes was 3.6°C, decrease chlorophyll content between 0.582 and 0.597 mg/g on India accession, and between 0.590 and 0.686 mg/g on Kuwait accession. Hendriyani and Setiari (2009) stated that synthesis of chlorophyll was affected by radiation, carbohydrate, water, temperature, and genetic factor.

Leaf and seed used in this study were harvested from three altitudes of Indonesian tropical region. Air temperatures were diverse from the three altitudes on high, middle, and low altitudes; the daily temperatures were 26.70°C, 30.20°C, and 30.30°C; the maximum temperatures were 33.30°C, 38.70°C, and 39.34°C; and the minimum temperatures were 18.00°C, 19.50°C, and 19.75°C, respectively (Table 1); meanwhile, Mediterranean regions have low temperature (under 20°C) (Tuncturk *et al.* 2010; Khoulenjani & Salamati 2011). Daily temperature difference between middle and low altitudes was only 0.1°C, but between middle and high altitudes was 3.5°C, and between low and high altitudes was 3.6°C. Wahid *et al.* (2007) showed that daily temperature can be used as indicator of plant response on heat temperature stress. MDA content was affected by this condition. On this research, MDA content at high altitude on both accessions was lower than two other altitudes. It means that plant cultivated at high altitude was

Table 5. Thymoquinone and malondialdehyde content in black cumin seed from the country of origin

Accession	Thymoquinone (mg/kg seed)	Thymol (mg/kg seed)	MDA of seed (μmol/g seed)
Kuwait	1303.16	22.94	2.140
India	1382.45	63.21	4.032

MDA = malondialdehyde.

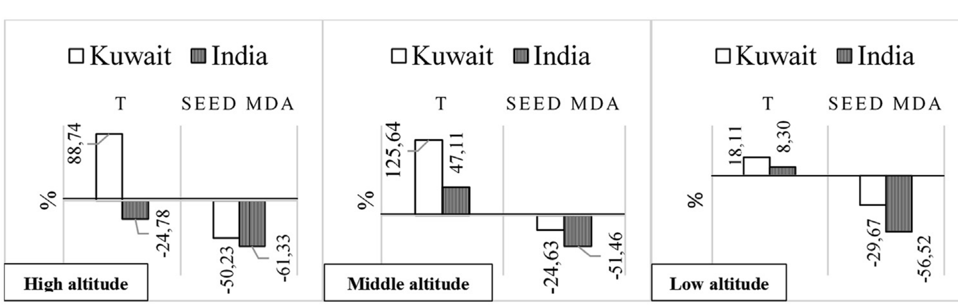

Figure. Percentage of THQ, THY, and MDA S compared with seed from the country of origin in three altitudes. MDA S = seed malondialdehyde; THQ = thymoquinone; THY = thymol.

less stressful than lower altitude. This fact was also supported by data ratio total chlorophyll and carotenoid at high altitude lower than low and middle altitudes at 2.0% and 1.8% on India accession, and 12.2% and 3.5% on Kuwait accession, respectively. Wahid *et al.* (2007) stated that decrease on the ratio of chlorophyll and carotenoid was related with plant thermotolerance on high temperature stress condition.

Exposures to high temperature induces ROS accumulation in plants, causing an oxidative stress (Wormuth *et al.* 2007), and accumulation of lipid peroxidation (Savicka & Skute 2010). Diverse environmental stress differently affects plant process that leads to a loss of cellular homeostasis accompanying by the formation of ROS, which causes oxidative damage to membranes, lipids, proteins, and nucleic acids (Srivalli *et al.* 2003). ROS produced under stress that causes lipid peroxidation and enzyme inactivation (Blokhina *et al.* 2003). Lipid peroxidation is a natural metabolic process in normal conditions and its activation is one of the possible results of a rapid response to stress. MDA was investigated as one of the lipid peroxidation products and its content is often used as indicator of lipid peroxidation resulting from oxidative stress (Malancic *et al.* 2004). Zubair *et al.* (2013) stated that thymoquinone and thymol exert its biological functions by modulating the physiological and biochemical processes involved in ROS generation, where it acts as antioxidant and pro-oxidant, respectively.

The ROS-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level (Jain *et al.* 2001). An enhancement of lipid peroxidation, as indicated by MDA content, was observed in leaf and seed in response to tropical altitude variation that have higher temperature (daily range 26.70°C–30.30°C) compared with black cumin cultivated in subtropical regions (under 20°C). The influence of high temperature from tropical altitude variation on MDA content differs not only depending on the altitudes, but also on black cumin accessions.

In conclusion, cultivating black cumin in different altitudes of Indonesian tropical region produced different thymoquinone, thymol, and MDA values that caused by high temperature. The highest thymoquinone contents 2940.43 and 2459.56 mg/kg were obtained from cultivating Kuwait accession at 560 masl and 1280 masl, respectively; and the highest thymol content was found in India accession cultivated at high altitude. Thymoquinone content from this study was higher than seed from the country of origin between 18.11% and 125.64% in Kuwait accession and 8.30% and 47.11% in India accession, except India accession at high altitude was lower, 24.78, than seed from the country of origin. Thymol content was higher than seed from the country of origin, except at low altitude lower than seed from the country of origin, 29.67% and 56.52% on Kuwait and India accession, respectively. Oxidative stress under its new tropical growth environment was showed by leaf and seed MDA. MDA leaf and seed at low and middle altitudes were higher than high altitude.

Conflicts of interest

The authors declare that there is no conflict of interest on this research.

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