

The Effect of Black Cumin (*Nigella Sativa*) Extract to Tensile Strength through Decreasing Oxidative Stress (MDA) on Artificial Achilles Tendon of Diabetes Mellitus Type 2 Wistar Rat

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Achilles tendon rupture is a condition when the Achilles tendon is injured or torn. It has been reported that the incidence of Achilles tendon rupture in patients with diabetes mellitus was 2.35 per 1000 population per year. This study aimed to determine the role of black cumin (*Nigella sativa*) as an antioxidant in the healing of Achilles tendon rupture in Wistar rats model with type 2 DM, with an indicator of a decrease in malondialdehyde and an increase in tensile strength of the Achilles tendon. The research design used in this study was the true laboratories experimental method with post test only control group, the samples were type 2DM and non-DM Wistar rats (*Rattus norvegicus*). Tenotomy and tendon repair were carried out in rats. After that, they were divided into 4 groups, namely: non-DM rats that were not treated with black cumin, non-DM rats treated with black cumin, DM rats that were not treated with black cumin and DM rats treated with black cumin. After three weeks, tendon sampling was carried out to measure malondialdehyde levels and tensile strength. The results showed that the highest average malondialdehyde value in the DM group that was not treated with black cumin was 493.6, and the lowest average malondialdehyde value in the non-DM group treated with black cumin was 332.0. In addition, it can be seen that the highest average tensile strength value in the non DM group treated with black cumin was 36.9, and the lowest average tensile strength value in the DM group that was not treated with black cumin was 11.8. From these data, it can be seen that the DM group treated with black cumin had lower malondialdehyde levels and higher tensile strength than the DM group who was not treated with black cumin. Based on the regression test, it was found that black cumin had a significant effect on malondialdehyde levels and tensile strength. Based on the Pearson correlation test, it was found that there was a significant relationship between malondialdehyde and tensile strength values. It can be concluded that the groups of rats that underwent artificial Achilles tendon rupture and was treated with black cumin extract had stronger tensile strength and lower malondialdehyde levels than those who were not treated with black cumin extract. In addition, there was a significant relationship between malondialdehyde levels and tensile strength. It is suggested for further studies to perform an investigation on tendon rupture in humans with type 2 DM complications, so that black cumin can be considered as adjuvant therapy for tendon rupture.

Keywords: Black Cumin, Malondialdehyde, Tensile Strength

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I. Introduction

Achilles tendon rupture is a condition when Achilles tendon is injured or torn (Movin, 2005; Sharma *et al.*, 2005; Michael *et al.*, 2015). The incidence of Achilles tendon rupture varies, it has been reported that there were 6 cases per 100,000 population in Scotland and 37 cases per 100,000 population in Denmark. It was also reported that the incidence of Achilles tendon rupture was 6.5-18% in runners, 9% in dancers, 5% in gymnasts, 2% in tennis players, and less than 1% in American football players. It is estimated that Achilles tendon rupture occurs in at least 1 million athletes per year (Saglimbeni, 2005; Michael *et al.*, 2015). Achilles Tendon has least vascularization, so that if degeneration and rupture occur, the healing process will be slower than in the areas with most vascularization (Sharma *et al.*, 2005; Sharma *et al.*, 2006). Healing process disruption of Achilles tendon will be worsened if the patient has diabetes mellitus (Rosen *et al.*, 2001; Schulze *et al.*, 2004; Schreml *et al.*, 2010). Oxidative stress may occur in diabetes mellitus, especially the uncontrolled one which can disrupt extracellular matrix synthesis, so that tissue repair including Achilles tendon repair can be disrupted. As a result, tensile strength of Achilles tendon decreases ((Schulze *et al.*, 2004; Schafer *et al.*, 2008; Schreml *et al.*, 2010). Achilles tendinopathy commonly affects athletes, long-distance runners have a risk of 52%. It has been reported that the incidence of Achilles tendon rupture in patients with diabetes mellitus was 2.35 per 1000 population per year (Payne, 2016). This condition can be prevented by administering antioxidants (Roy *et al.*, 2006; Schafer *et al.*, 2008; Wagener *et al.*, 2013). Exogenous antioxidants can be obtained from plants such as turmeric,

blackberry, grape seed extract and black cumin extract (Zarka, 1996; Basir, 1998). The contents in black cumin playing a role in the antioxidant process are *thymoquinone*, *carvacrol*, *t-anethole* and *4-terpineol* (Zarka, 1996; Basir, 1998; Burits and Bucar, 2000). To date, there are no studies using black cumin in the treatment of Achilles tendon rupture in diabetes mellitus, where tendon healing is more difficult in this condition due to increased Txnip inhibiting ROS-scavenging function of thioredoxin, so that cellular redox balance is disrupted (Schulze *et al.*, 2004). In addition, diabetes mellitus forms AGEs producing free radicals (Singh *et al.*, 2014). As a result, oxidative stress occurs which inhibits healing and causes tensile strength of the tendon to decrease. The administration of black cumin in the treatment of Achilles tendon rupture in diabetes is expected to control excessive ROS production by removing thymoquinone acting as a superoxide anion scavenger so that with reduced ROS, it is expected that oxidative stress also decreases marked by reduced levels of malondialdehyde and can cause better healing by improving the structure and increasing the quantity of formed collagen tissue. This is also expected to have an effect on strengthening biomechanics so that mobilization or early rehabilitation can be carried out (Zarka, 1996; Schulze *et al.*, 2004; Sonejo *et al.*, 2005).

II. Material And Method

The research design was true laboratories experimental method with post test only control group. This research used four treatment groups, namely: diabetic rat treated with black cumin, non-diabetic rats treated with black cumin, diabetic rat treated without black cumin and non-diabetic rats treated without black cumin groups, each group consists of 6 rats. If the number of samples has been fulfilled, the rats were put in a cage for 1 week for adaptation to the environment and food. The experimental animals were not fed for 3 hours before surgery, prophylactic antibiotics were given in the form of cefazolin of 50 mg/kg body weight in 30 minutes before surgery. Premedication was performed by administering 10 mg/kg diazepam and sedation was performed by administering 40 mg/kg ketamine hydrochloride (ketalar). Operating field was sterilized by shaving, then it was cleaned by a solution of savlon, 70% alcohol, and providone iodine, and closed with doek sterile.

After being sedated, the rats underwent a surgery with the following techniques:

- a. Incise of the rat Achilles tendon area.
- b. Perform proximal 5 mm tenotomy of the calcaneus insertion.
- c. Perform Achilles tendon repair with modified kessler technique with monofilament 6.0.
- d. Suture and cover the wound with tulle and sterile gauze followed by cast application.

Post-Treatment of Model

The experimental animals were put into a cage and were fed every day according to the habits. Rats received anti-pain medication in the form of 100 mg/kg aspirin, if there were signs of pain in the form of lethargy, eating difficulty, and shivering. On the first day, 1.3cc/kgbw/day black cumin extract was administered to post-operative test group once a day for 3 weeks. Black cumin extract was administered using feeding tube.

Research Sample Examination

Sampling was carried out after 3 weeks of treatment, the rats were killed by performing disarticulation of rat neck. After that, the tendon sample was taken by breaking between the tendon and muscle in the proximal part, while ankle dysarticulation was carried out in the distal part, where the tendon was retained to remain attached to the calcaneus. The Achilles-calcaneus tendon complex was then released from the surrounding tissue. The measurement of tensile strength using tensiometer was performed immediately after the tendon was taken. In addition, tendon samples were taken for examination of malondialdehyde levels. The attached tendon to the calcaneus was fixed in two hooks to make it close to physiological condition as when tendon were pulled vertically. The tendon was then pulled at a constant velocity of 0.45 mm/s until it broke, then the peak of tensile strength was measured.

Data Analysis Method

The data obtained were tabulated, and the control group and the treatment group were compared. Data analysis in this study used Kruskal-Wallis statistical test. Mann-Whitney test was performed to determine the difference between the groups. The statistical calculation was carried out using SPSS 18 software.

III. Result

From the research conducted, it was obtained the following results:

Table 1 Result of MDA levels and tensile strength measurement in rats by the treatment group

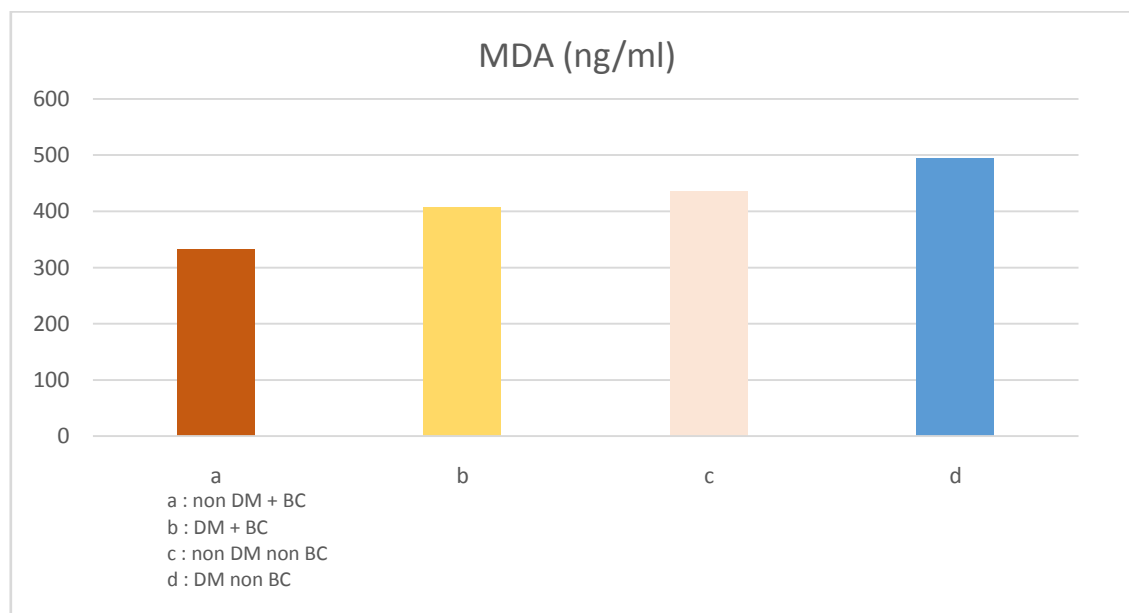
Group	n	Malondialdehyde levels (ng/ml)	Tensile strength (N/m ²)
Non-DM + non-BC	6	435.5 ±18.625	19.0 ±0.764
Non-DM + BC	6	332.0±7.510	36.9± 5.638
DM + non BC	6	493.6±3.611	11.8 ±1.845
DM + BC	6	406.2±11.830	28.6 ±2.411

Based on the table above, it can be seen that the highest average malondialdehyde value is in DM non-BC group of 493.6, and the lowest average malondialdehyde value is in non DM + BC of 332.0. From these data, it can be seen that DM group treated with black cumin has lower Malondialdehyde levels than the DM group which are not treated with black cumin.

Table 2 Result of Kruskal-Wallis Test on Malondialdehyde

Chi-square count	Significance	Chi-square table	Conclusion
21.156	0.000	7.815	Significant

Based on the result listed in the table above, it can be concluded that there is at least one group that has a significant difference of Malondialdehyde level compared to other groups.



Graph 1 Average Malondialdehyde value in each treatment

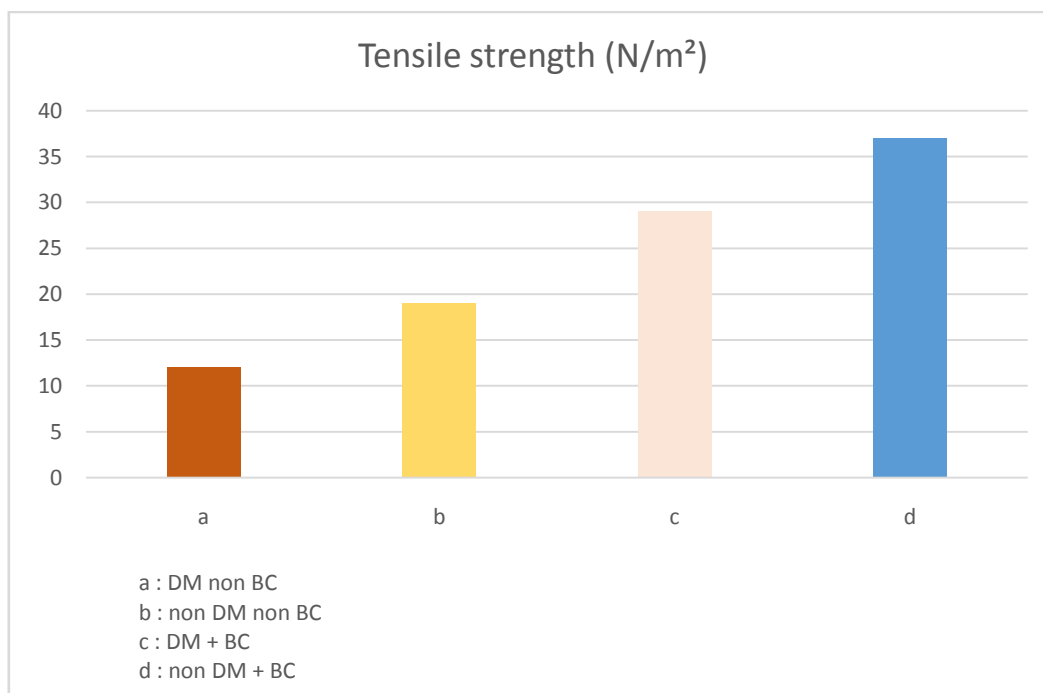
The figure above shows that treatment groups have a significant difference of Malondialdehyde levels from one another. This can be seen from the notation obtained between different treatments. Thus, it can be concluded that the DM groups treated with black cumin have significantly lower Malondialdehyde level compared to the DM groups which are not treated with black cumin.

Based on table 1, it can also be seen that the highest average tensile strength value is in the non DM + BC group of 36.9, and the lowest average tensile strength value is in the non-DM + BC group of 11.8. From these data, it can be seen that the DM group treated with black cumin have higher tensile strength than the DM groups that are not treated with black cumin.

Table 3. Result of Kruskal-Wallis Test on Tensile Strength

Chi-square count	Significance	Chi-square table	Conclusion
20.756	0.000	7.815	Significant

Based on the result listed in Table 3, it can be concluded that at least one group has significantly different tensile strength than the other groups



Graph 2 Average tensile strength in each treatment

Figure above shows that the treatment groups have different and significant tensile strength from one another. This can be seen from the notation obtained between different treatments. Thus, it can be concluded that DM groups treated with black cumin have a significantly higher tensile strength compared to the DM groups which are not treated with black cumin. Furthermore, regression analysis was carried out to obtain the effect of the independent variable (X (Black Cumin)) on the variable Y (Malondialdehyde).

Table 4 Regression Test of Black Cumin Dummy Variable on Malondialdehyde

Variable	B	t _{count}	Significance	Description
Constant	437.722			
X (Black Cumin)	-105.722	-6.655	0.000	Significant
A		= 0.050		
Coefficient of Determination (R ²)		= 0.668		
t-table (t _{22,0.05})		= 2.074		

From the table above, it can be concluded that the variable X (Black Cumin) has a significant effect on the variable Y (Malondialdehyde). The variable X (Black Cumin) has a negative effect with a coefficient of -105.722 and is significant to the variable Y, the negative coefficient indicates that the variable X (Black Cumin) can reduce the variable Y (Malondialdehyde).

Based on the calculation result in Table 1, the contribution of the independent variable to the dependent variable according to the coefficient of determination (R Square) is 0.668. The result explains that the contribution of the independent variable (X (Black Cumin)) to the variable Y (Malondialdehyde) in the regression equation is 66.8%, while the remaining 33.2% is contributed by other variables that are not included in this equation. Furthermore, regression analysis was performed to obtain the effect of the independent variable (X (Black Cumin)) on the variable Y (Tensile Strength).

Table 5 Regression Test of Black Cumin Dummy Variable on Tensile Strength

Variable	B	t _{count}	Significance	Description
Constant	19.794			
X (Black Cumin)	17.122	5.228	0.000	Significant
A		= 0.050		
Coefficient of Determination (R ²)		= 0.554		
t-table (t _{22,0.05})		= 2.074		

Based on the table above, it can be concluded that variable X (Black Cumin) has a significant effect on variable Y (Tensile Strength). Variable X (Black Cumin) has a positive effect with a coefficient of 17.122 and is significant to variable Y, the positive coefficient indicates that variable X (Black Cumin) can increase variable Y (Tensile Strength). Based on the calculation result in table 5, the contribution of the independent variable to the dependent variable according to the coefficient of determination (R Square) value is 0.554.

The result explains that the contribution of the independent variables (X (Black Cumin) to the variable Y (Tensile Strength) in the regression model is 55.4%, the remaining 44.6% is contributed by other variables not included in this equation.

In addition to the above test, Pearson correlation test was performed to determine the relationship between malondialdehyde and tensile strength.

Table 6 Pearson Correlation Test between Malondialdehyde and Tensile Strength

1.1	Correlation Coefficient	1.3	Significance	1.4	Decision
1.2	(r)	1.6	0.000	1.7	Significant Relationship
1.5	-0.889				

Based on table 6, it can be concluded that there is a significant relationship between Malondialdehyde and tensile strength values. Pearson correlation value indicates a negative or inverse correlation. If the Malondialdehyde value increases, then the tensile strength value decreases. The correlation coefficient is 0.889, meaning that the relationship between malondialdehyde and tensile strength values belongs to the category of high correlation.

IV. Discussion

Achilles tendon rupture is a condition when the Achilles tendon is injured or torn (Movin, 2005; Sharma *et al.*, 2005; Michael *et al.*, 2015). The disruption of healing process of Achilles tendon rupture will be worsened if the patient has diabetes mellitus (Rosen *et al.*, 2001; Schulze *et al.*, 2004; Schreml *et al.*, 2010), where tendon healing is more difficult in this condition due to increased Txnip inhibiting ROS-scavenging function of thioredoxin so that cellular redox balance is disrupted (Schulze *et al.*, 2004). In addition, diabetes mellitus can form AGEs producing free radicals (Singh *et al.*, 2014). As a result, oxidative stress occurs inhibiting healing process and causes tensile strength of the tendon to decrease.

ROS produced by oxidative stress plays an important role in the clotting process, because ROS is involved in inducing tissue factor (TF)-mRNA. ROS is also involved in increasing platelet recruitment and collagen-induced platelet activation. Platelet activation and aggregation are important for forming clots and also for removing various kinds of growth factors and cytokines that play a role in initiating the healing process. Various types of growth factors produced by platelets, fibroblasts and leukocytes play a role in recruitment and activation of neutrophils and monocytes in the wound, which initiate wound re-epithelialization and angiogenesis. TGF produced by fibroblasts and leukocytes can induce these cells in an autocrine manner to produce other cytokines such as TNF- α , IL-1 β and PDGF which eventually increase the inflammatory response. Thus, it can be seen that oxidative stress plays an important role in wound healing. However, excessive oxidative stress can prolong the inflammatory phase due to the stimulation of neutrophils and macrophages by ROS so that the next phase in the wound healing process is inhibited. In addition, the prolonged inflammatory phase can cause the protease enzymes produced by neutrophils and macrophages to increase. The high level of protease causes increased destruction of the extracellular matrix, so that it interferes with the wound healing process (Sonejo *et al.*, 2005).

The administration of black cumin in the treatment of Achilles tendon rupture in diabetes is expected to control excessive ROS production by removing thymoquinone acting as superoxide anion scavenger so that with reduced ROS, it is expected to reduce oxidative stress characterized by reduced malondialdehyde levels and can lead to better healing by improving the structure and increasing the quantity of formed collagen tissue. This condition is also expected to have an effect on strengthening biomechanics so that mobilization or early rehabilitation can be carried out (Zarka, 1996; Schulze *et al.*, 2004; Sonejo *et al.*, 2005).

Based on the research, it can be seen that the highest malondialdehyde levels were found in the DM group which were not treated with black cumin. This was because the high levels of free radicals in this group caused by diabetes mellitus were not neutralized by administering antioxidants, in this case in the form of black cumin. This causes high malondialdehyde levels as an indicator of free radicals.

In diabetes mellitus especially uncontrolled one, hyperglycemia can occur which induces Thioredoxin-interacting protein (Txnip) mediated by p38 MAPK. Txnip has been shown to inhibit ROS-scavenging function of thioredoxin (Schulze *et al.*, 2004). In addition, diabetes mellitus gives rise AGEs producing free radicals (Singh *et al.*, 2014). As a result, redox balance disturbance occurs where the amount of free radicals is higher than the amount of antioxidants. This causes oxidative stress (Schulze *et al.*, 2004).

Malondialdehyde is a metabolite that results from lipid peroxidation by free radicals (Asni, 2009). Malondialdehyde can be formed when hydroxyl free radicals such as Reactive Oxygen Species (ROS) react with the components of fatty acids from the cell membrane resulting in a chain reaction known as lipid peroxidation. Malondialdehyde is an indicator often used as an indication of lipid peroxidation (Nielsen, 1997). Malondialdehyde is a compound that can describe the activity of free radicals in cells so that it is used as an indication of oxidative stress caused by free radicals (Rahardjani, 2010).

The lowest Malondialdehyde levels were found in the non-DM group treated with black cumin. This is because there was no hyperglycemia in this group which induced the formation of free radicals (Schulze *et al.*, 2004; Singh *et al.*, 2014). In addition, the administration of black cumin as an antioxidant has been shown to neutralize existing free radicals, so that the amount of free radicals decreased. As a result, the amount of malondialdehyde which was a metabolite resulting from lipid peroxidation by free radicals as an indication for oxidative stress decreased. The effect of black cumin antioxidant was obtained due to the presence of thymoquinone found in black cumin extract. Thymoquinone has been shown to have a scavenger effect on free radicals, especially in radicals with OH Thymoquinone group which also has the potential as a powerful antioxidant against superoxide ($O_2^{\cdot-}$), where the thymoquinone binding $O_2^{\cdot-}$ forms an unreactive bond (Al-jassir, 1992; Zarka, 1996; Burits and Bucar, 2000; Badary and Gamel, 2001).

In this study, it can be seen that the DM group treated with black cumin had significantly lower malondialdehyde levels compared to the DM groups which were not treated with black cumin. This was due to the antioxidant effect of thymoquinone found in black cumin extract, so that the levels of free radicals in the DM group treated with black cumin were lower than the DM group that were not treated with black cumin. As a result, the amount of malondialdehyde which was a metabolite resulting from lipid peroxidation by free radicals as an indication for oxidative stress decreased. Thymoquinone has been shown to have a scavenger effect on free radicals, especially in radicals with OH Thymoquinone group which also has the potential as a powerful antioxidant against superoxide ($O_2^{\cdot-}$), where the thymoquinone binding $O_2^{\cdot-}$ forms an unreactive bond (Al-jassir, 1992; Zarka, 1996; Burits and Bucar, 2000; Badary and Gamel, 2001).

The highest tensile strength value was found in the non-DM group treated with black cumin. This was because there was no hyperglycemia in the group that induced the formation of free radicals (Schulze *et al.*, 2004; Singh *et al.*, 2014). In addition, the administration of black cumin can control the production of free radicals by removing thymoquinone, so that with reduced free radicals, stress oxidative decreased and caused better healing by improving the structure and increasing the quantity of formed collagen tissue. This caused tensile strength to be better (Zarka, 1996; Schulze *et al.*, 2004; Sonejo *et al.*, 2005).

The lowest tensile strength value was found in the DM group that was not treated with black cumin. This was because the group had high levels of free radicals caused by non-neutralized diabetes due to the administration of antioxidants, in this case in the form of black cumin. As a result, redox balance disturbance occurred where the amount of free radicals was higher than the amount of antioxidants, this caused oxidative stress (Schulze *et al.*, 2004). In a state of oxidative stress, disruptions of migration, proliferation and synthesis of extra cellular matrix molecules occurred such as collagen (Schafer and Wermer, 2008; Sen and Roy, 2008; Schreml *et al.*, 2010). In addition, oxidative stress caused a decrease in the production of keratinocytes and fibroblasts. This disrupted healing of wounds, including Achilles tendon rupture. As a result, tensile strength of Achilles tendon decreased (Schafer and Wermer, 2008; Sen and Roy, 2008; Schreml *et al.*, 2010; Sen, 2009; Kurahashi and Fujii, 2015).

From the research, it can be seen that DM group treated with black cumin had a significantly higher tensile strength compared to the DM group which was not treated with black cumin. This was because the administration of black cumin can control the production of free radicals by removing thymoquinone so that with reduced free radicals, stress oxidative decreased and led to better healing by improving structure and increasing the quantity of formed collagen tissue. This caused tensile strength to be better (Zarka, 1996; Schulze *et al.*, 2004; Sonejo *et al.*, 2005). This explained why the result of regression analysis showed that black cumin had a significant effect on tensile strength.

V. Conclusion

1. Tensile strength of rats underwent artificial Achilles tendon rupture treated with black cumin extract was stronger than those which were not treated with black cumin extract.
2. Malondialdehyde levels of group of rats underwent artificial Achilles tendon rupture treated with black cumin extract were lower than those which were not treated with black cumin extract.
3. It was found that there was a significant relationship between Malondialdehyde levels and tensile strength.

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