DOI: https://dx.doi.org/10.4314/jaaps.v11i1.5

J. Afr. Ass. Physiol. Sci. 11(1): 45-54, 2023

Journal of African Association of Physiological Sciences

Official Publication of the African Association of Physiological Sciences

https://www.ajol.info/index.php/jaaps

Research Article

Modulatory Role of Dose-dependent Quercetin Supplemented Diet on Behavioral and Anti-oxidant system in *Drosophila melanogaster* model

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Keywords:
Quercetin; Dose-dependent; Modulation; Anti-oxidant; Behavior; *D. Melanogaster*

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Received: 8 December 2022
Revised: 28 June 2023
Accepted: 3 August 2023

ABSTRACT

Background: Quercetin is an abundant bio-flavonoid in foods having several biological activities including anti-oxidant properties. However, the toxicity of quercetin may limit its use. Therefore, screening a graded dose of quercetin may be an important step in its use as a dietary supplement. Hence, the effects of graded doses of quercetin on the survival, locomotive behavior and oxidant-antioxidant system in *D. Melanogaster* was investigated.

Methods: A Survival assay was conducted by feeding 50 fruit flies per vial with 50-400 mg/g quercetin-diet for 14 days. For behavioral and biochemical studies, flies were divided into 4 vials (50 flies each) 0, 100, 200 and 300 mg/g quercetin-diet. Flies were treated with vehicle or quercetin for 7 days. Behavioral assessment of negative geotaxis was determined from the climbing activities of flies. Finally, flies were killed, homogenized and centrifuged for supernatant for the assay of Catalase (CAT), Superoxide dismutase (SOD), Glutathione (GSH), Malondialdehyde (MDA) and Total thiol.

Results: Quercetin treated groups showed a dose-dependent significant decrease ($p<0.001$) in survival rate. Climbing activity was unaltered at 100 mg but significantly ($p<0.05$) reduced at 200 and 300mg quercetin. Total thiol was not significantly altered ($p>0.05$) across the groups, MDA was reduced ($p<0.05$) at 300 mg compared to control, CAT activity was significantly increased ($p<0.05$) at 100-200 mg compared to control while GSH and SOD at 100-200 mg of quercetin increased.

Conclusion: Quercetin showed better antioxidant activity at lower doses and adversely affected the climbing behavior and survival rate at higher doses. Therefore, quercetin use should be dose modulated and used with caution.

1. Introduction

Drosophila Melanogaster has been used as a model for different aspects of biological research like genetics, microbial pathogenesis and drug screening. There is an increased use of this non-mammalian model to evaluate the effects of different substances on various disease processes. *D. melanogaster* has several advantages compared to rodents due to its simple nervous system, short lifespan, fast regeneration time as well as ease of genetic manipulation (Zhanga *et al.,* 2019).

Quercetin is one of the most abundant bio-flavonoids in food with a wide range of biological activities (Perez-Vizcaino *et al.,* 2009). It is ubiquitous in the human diet and the average person can unknowingly consume more than 1g of Quercetin per day (Dostal and Modriansky, 2019). Quercetin has gained scientific interest for its anti-oxidative (Lu *et al.,* 2009; Nabayi *et al.,* 2012), anti-apoptotic, anti-angiogenesis, anticancer and anti-inflammatory properties (Kobori *et al.,* 2015; Zhao *et al.,* 2016) by scavenging free radicals. Free radicals can activate transcription factors that generate pro-inflammatory cytokines, which are often found elevated in patients that suffer from chronic inflammatory diseases (Boots *et al.,* 2008). Quercetin has also been found to inhibit lipid peroxidation, platelet aggregation and capillary permeability (Li *et al.,* 2016).

An increasing amount of evidence shows that oxidative stress is connected to either the primary or secondary pathophysiological mechanisms of multiple acute and chronic human diseases (Espinosa-Diez *et al.,* 2015). Oxidative stress is the result of a disequilibrium in oxidant-antioxidant status leading to continuous increase in Reactive Oxygen Species (ROS). It is a deleterious process and an important mediator of damage to cell structures leading to severely compromised cell health and viability or induce a variety of cellular responses which could lead to cell death (Valko *et al.,* 2007; Flora *et al.,* 2009).

Products of lipid peroxidation have commonly been used as biomarkers of oxidative stress damage. Lipid peroxidation generates a variety of relatively stable decomposition end products, including malondialdehyde (MDA), which is an indirect index of oxidative stress. Antioxidants can be endogenous or obtained exogenously as part of a diet or as dietary supplements. Endogenous antioxidants play a critical role in keeping optimal cellular functions. However, under oxidative stress, endogenous antioxidants may not be sufficient, and dietary antioxidants may be required for optimal cellular functions. The most efficient enzymatic antioxidants include glutathione peroxidase, catalase and superoxide dismutase. Non-enzymatic antioxidants include Vitamin E and C, thiol antioxidants (glutathione) and others (Rahman, 2007; Valko *et al.,* 2007). Antioxidant supplementation has become an increasingly popular practice to maintain optimal body function. However, some antioxidants exhibit pro-oxidant activity depending on the specific set of conditions. Of particular importance are their dosage and redox conditions in the cell (Kurutas, 2016).

There are previous reports on the potential toxic effects of quercetin: its pro-oxidant activity, mitochondrial toxicity, inhibition of key enzymes involved in hormone metabolism which may delimit its use (Okamoto, 2005). Early studies suggested a relatively low oral LD50 value for quercetin, indicating potential toxicity at higher doses. However, subsequent longer-term animal toxicity studies have demonstrated that quercetin is generally well-tolerated at oral dose levels exceeding the initially reported LD50 value (Harwood, 2007). Such reports have necessitated the need for further investigations on the anti-oxidant profile of Quercetin. Therefore, screening a graded dose of quercetin may be an important step in its use as supplemented anti-oxidants. Hence, in this study, we investigated the graded-dose effects of quercetin in *D. melanogaster* viz-a-viz survival, locomotory behavior and antioxidant system.

2. Materials and Methods

2.1. Chemicals

All the chemicals and reagents used were of analytical grade. Quercetin and cold pressed sesame oil were products of Sigma-Aldrich, USA while phosphate buffer saline (PBS) was prepared in Eureka Drosophila Laboratory, Department of Anatomy, Babcock, Nigeria.

2.2. Experimental procedure

Both male and female *drosophila melanogaster – D. melanogaster* (Harwich strain) were obtained from the Drosophila Laboratory, Department of Biochemistry, University of Ibadan, Ibadan, Nigeria. The flies were reared and maintained at 22-25°C (room temperature) in Eureka Drosophila Laboratory, Department of Anatomy, Babcock University, Ilishan-Remo, Nigeria. They were fed on a diet containing cornmeal-60g, brewer's yeast – 7 g, Nipagin (antifungal agent) – 0.7 g and Agar (thickener)-7.5 g (Oyetayo *et al.*, 2020).

A Survival assay was conducted by exposing 50 fruit flies per vial to a dose regimen of 50-400 mg/g quercetin-diet for 14 days, the outcome of which informed the doses used in subsequent studies. For behavioral and biochemical studies, 50 flies per vial were administered with vehicle or 100, 200 and 300 mg/g of quercetin-diet for 7 days. Behavioral assessment of negative geotaxis was determined from the climbing activities of flies (Feany *et al.*, 2000). Finally, flies were killed, homogenized and centrifuged for supernatant used for the assay of levels of CAT, SOD, GSH, MDA and total thiol.

2.3. Determination of survival rate of flies

A pilot study (containing an initial corresponding cohort of Quercetin-feeding experiment) in order to monitor the number of dead *D. melanogaster* during the whole life span was conducted. The survival rate was studied using 50, 100, 200, 300 and 400 mg/g of quercetin treatment for 14 days, wherein a daily record of the number of live and dead flies was taken. The data obtained was then analyzed. This pilot (survival) study informed our choice of doses and duration of the subsequent experiment (Abolaji *et al.*, 2014).

2.4. Climbing Assay

Negative geotaxis is a behavior in *D. melanogaster* that reveals the intactness of the locomotor system. The method as described by (Feany *et al.*, 2000) with modification in the duration by Abolaji *et al.* (2014), was employed. Flies of both sexes were collected from both the Quercetin-treated and control groups. Using the labeled vertical glass columns of diameter and length 1.5 cm and 15 cm respectively, 50 flies were quickly placed inside a glass column and gently knocked to the base. The flies were allowed to get accustomed to the vial and after 20 min recovery period, the number of flies that moved up to the glass column's 6 cm mark within a duration of 6 s along with those that remained below this level were counted.

2.5 Preparation of samples for biochemical assays

Flies were anaesthetized using carbon dioxide.The vials containing the flies were inverted and placed on a pad that has been connected to a source of carbon dioxide. The CO2 gas displaced the oxygen in the vial causing the flies to become exposed to carbon dioxide till they become sedated (Shen *et al.*, 2020). The weight of each fly was recorded. Afterwards, they were homogenized in 1.0ml of 0.1 M of PBS for 1 min and was centrifuged for 10 min at 4000x g and $4^{o}C$ in an Eppendorf cooled centrifuge (Sigma Aldrich). The supernatant was separated from the pellet into labeled Eppendorf tubes and used for biochemical assay of total thiol content, CAT, MDA, GSH and SOD activities. The biochemical assay was carried out in triplicate.

2.5.1 Determination of Total Thiol determination

Total thiol content was measured according to Ellman's method as modified by Pam *et al*. (2021). The total reaction mixture was 600µL of (510 µL 0.1 M PBS pH 7.4, 30 µL of 10mM DTNB and 25µL of sample). After incubating for 30 minutes, the absorbance score was taken at 412 nm using the 752S UV-Visible spectrophotometer.

2.5.2 Determination of Glutathione (GSH) activity

GSH activity was measured according to the method of Habig and Jacoby as modified by Abolaji *et al.* (2014). The total reaction mixture was 600µL of (510 µL solution A (0.25 M PBS pH 7.0 with 2.5 mM EDTA, and 0.1 M GSH) at 25°C, 60 µL of sample (1:5 dilution) and 30 µL of 25 mM CDNB). Absorbance measurement was taken at 340 nm for 2 min. Results were presented in mmol/min/mg.

2.5.3 Determination of Supeoxide dismutase (SOD) activity

A method originally described by Misra and Fridovich (1972) was employed. This method involves inhibition of epinephrine auto-oxidation, in an alkaline medium at 480 nm in an ultraviolet spectrum. The enzyme SOD inhibits the auto-oxidation of adrenaline by catalyzing the breakdown of superoxide anions. The degree of inhibition is thus a reflection of the activity SOD and is determined at one unit of the enzyme activity.

2.5.4 Determination of Catalase (CAT) activity

CAT activity was assayed according to the method of Aebi (1984) as modified by Abolaji *et al.* (2014). 590 µL Solution A (194 mL of 300 mM H_2O_2 in 100 mL of potassium phosphate buffer (pH 7.0) was reacted with 10 µL of the sample. The fall in H_2O_2 was measured by a UV-visible spectrophotometer for 2 min at 240 nm and presented as mmol of H_2O_2 used/min/mg protein.

2.5.5 Determination of Malondialdehyde activity

Lipid peroxidation was determined by measuring the levels of Malondialdehyde produced during lipid peroxidation according to the method described by Varshney and Kale (1990). An aliquot of 400µl of the sample was mixed with 1.6ml of tris-KCl buffer to which 500 µl of 30% TCA was added. Then 500 µl of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^0C. This was then cooled on ice and centrifuged at 3000 g for 5 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. Lipid peroxidation expressed as MDA formed/mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ Cm^{-1}$

2.6. Statistical analysis

The data obtained were analysed and presented as the Mean ± SEM (Standard Error of Mean). Using Graph Pad Prism 5.0 software, one-way analysis of variance (ANOVA) was used to determine significant differences across groups, and the individual comparisons were obtained by Tukey's post hoc test. Statistical significance was set at $p \leq 0.05$. However, data from survival rate of flies was analyzed using the Kaplan-Meier analysis method and the log rank test was used to make comparisons among groups.

3. Results

3.1 Effects of graded-dose of quercetin on the survival rate of D. melanogaster

As shown in figure 1, various concentrations of quercetin were administered to determine the survival rate of flies. We observed a dose-dependent decrease in survival rate. Survival rate was significantly ($p<0.001$) reduced from day 3 in the 400 mg quercetin treated flies compared to control. There was no significant difference in survival rate of flies among 50-300 mg quercetin-treated groups. However, it was significantly increased in 50 mg ($p<0.001$), 100 mg ($p<0.001$), 200 mg ($p<0.001$) and 300 mg ($p<0.01$) compared with 400 mg quercetin-treated group. The lowest survival or highest death rate was observed at the highest concentration of 400 mg/g quercetin treatment (16.9%) while the control flies had the highest survival rate (92.9%). Doses of 50-300 mg/g compared favorably with control flies which eventually determined the use of 100-300 mg/g quercetin for further studies.

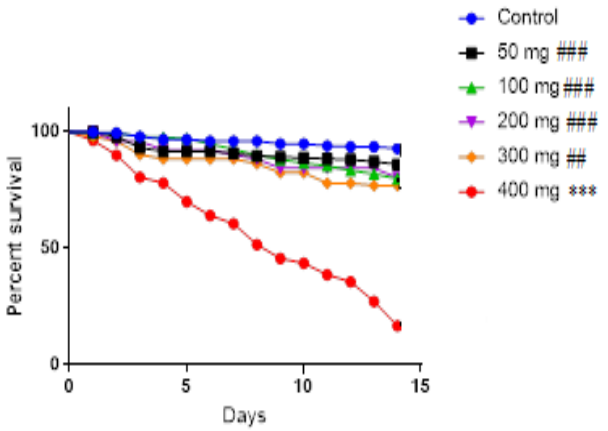

Figure 1. Survival rate of *D. melanogaster* following 14 days of quercetin treatment. (n=50) ***P<0.001 compared to control, $^{##}$P<0.01 compared to 400 mg quercetin, $^{###}$P<0.001 compared to 400 mg quercetin.

3.2. Effects of graded-dose of quercetin on negative geotaxis (Climbing activity)

In figure 2, quercetin treatment at high doses of 200 (p<0.05) and 300 p<0.05) mg/g significantly attenuated the negative geotaxis of flies compared to control. At the lowest dose of 100 mg/g, the climbing activity was not significantly different from control (p>0.05). Likewise, there was no significant difference in the negative geotaxis of flies across the quercetin-treated groups. Similarly, we observed that the higher the concentration of quercetin, the lower the negative geotaxis of flies.

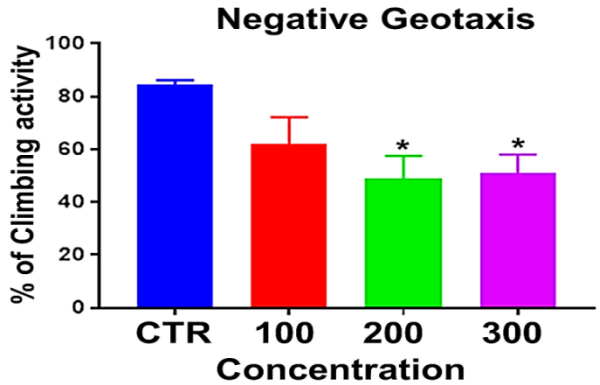

Figure 2. Effects of quercetin on the climbing activity of *D. melanogaster*. Values are expressed as Mean ± SEM. (n=3). CTR- Control. 100-300 mg/g quercetin. *P < 0.05 compared to control.

3.3. Effect of graded-dose of quercetin on total thiol, glutathione (GSH), Catalase (CAT), Superoxide dismutase (SOD) and Malondialdehyde (MDA) level in D. Melanogaster

As shown in fig.3, the level of total thiol (fig.3A) and SOD (fig.3D) were not significant statistically (p>0.05) in all the quercetin-treated groups compared with control. In fig.3B, there was no significant difference (p>0.05) in the level of GSH across all the groups.

It was observed that the 200mg/g quercetin-treated flies compare favorably with control. Control flies had the highest level of GSH and 300mg/g showed the lowest level of GSH. The level of catalase was significantly elevated in 100 (p<0.05) and 200 mg/g (p<0.05) quercetin treated flies compared with control.

The quercetin treatment groups showed elevated CAT level which was highest in the 100mg/ treatment group. Across the group, control had the lowest level of CAT, see fig.3C. Furthermore, administration of 100-200 mg/g quercetin showed no significant difference in the level of MDA compared with control.

Similarly, the level of MDA was not significantly different across the various quercetin treatment groups. However, 300 mg/g of quercetin significantly (p<0.05) decreased MDA level compared with control, see fig.3E.

4. Discussion

Based on the present findings, the survival rate of flies decreases with increase in quercetin concentration, wherein the least concentration of 50 mg showed a survival rate similar to control flies and highest concentration of quercetin was accompanied by highest death rate. Similarly, impairment to the locomotor system observed at high concentration of quercetin corroborates the low survival rate.

Taken together, our results suggest that high dose of quercetin might negatively impact or affect the motor activity and longevity of flies as characterized by a decline in the upward movement behaviour and survival rate respectively. Thus, confirming the toxicity of this flavonoid at high concentrations. The presence of free radicals in *D. melanogaster* has been reported to affect their upward movement (Abolaji *et al.*, 2014). Several flavonoids-rich plant-based extracts have been reported to show toxic tendencies at high concentrations.

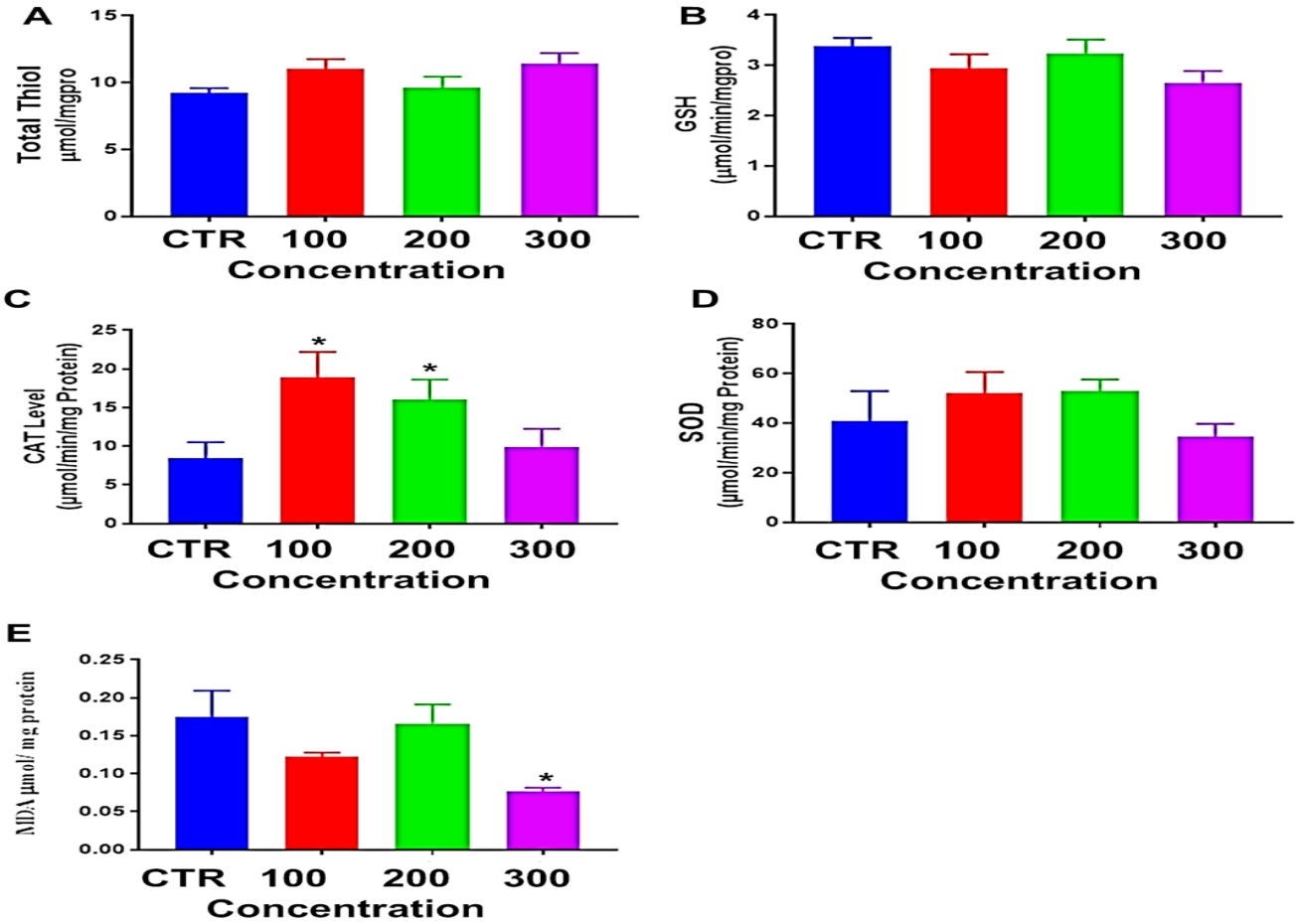

Figure 3. Bar chart presentation of: (A) Total thiol, (B) Glutathione, (C) Catalase, (D) Superoxide dismutase and (E) Malondialdehyde level in *D. melanogaster* exposed to varying concentrations of quercetin. Values are expressed as Mean ± SEM (n=3). CTR- Control. 100-300 mg/g quercetin. *P < 0.05 compared with control.

For example, tests on the toxicity and analgesic activity of the flavonoids of *Achillea wilhelmsii* and *Teucrium stocksianum* in mice, showed the safety of the action of the flavonoids in doses of up to 100 mg/kg, with prominent toxicity in doses above 100 mg/kg (Ali *et al.*, 2016).

Biochemically, catalase (CAT) and superoxide dismutase (SOD) are one of the most efficient enzymatic antioxidants while glutathione (GSH) is a non-enzymatic antioxidant. We reported that, 100 and 200 mg/kg of quercetin supplemented diet significantly increased CAT, it slightly increased and decreased SOD and GSH respectively. 300 mg/kg of quercetin supplemented diet did not cause any significant change to the levels of GSH, CAT and SOD. This could be interpreted to mean

that the antioxidant efficacy of quercetin appears to be potent at lower concentration. This finding corroborates the report of Halina *et al.* (2014), wherein the anti-oxidative and radioprotective activities of quercetin in whole red blood cells were reported. Similarly, Dong *et al.* (2020) showed that quercetin increased the serum GSH which resulted in decrease in reactive oxygen species and apoptosis ratio. The major differences in our finding and these previous reports are that quercetin was checked for at various doses using fruit fly while Dong *et al.* study checked for a single dose using rat model.

GSH has diverse important functions such as storage and transport of cysteine, maintaining the reduced state of proteins and thiols, and protecting cells from toxic compounds such as reactive oxygen species, drugs, or heavy metal ions and to protect against or delay apoptosis triggered by different stimuli (Maduagwuna *et al.,* 2020). An important role in maintaining the highly reduced environment inside the cell is played by the intracellular thiols such as glutathione and thioredoxin (Jones *et al.,* 2000). Total thiol groups are very susceptible to oxidation and considered as one of the most important plasma sacrificial antioxidants. When the cells are exposed to oxidative stress, thiol groups are the first antioxidants that are consumed (Etuh *et al.,* 2019). The elevated total thiol content of the quercetin treated fruit flies further supports its antioxidant impact. In the scavenging processes of ROS, superoxide dismutase plays an interesting role as the first and most important enzyme of the antioxidant system that catalyzes the dismutation of superoxide anions to form hydrogen peroxide (H_2O_2) and water as the end products. H_2O_2, as an end product of superoxide dismutation reaction is toxic to the system. In the second step of this reaction, CAT further breaks down or brings about the decomposition of H_2O_2 to non-toxic end products; water and oxygen (Chelikani *et al.,* 2004). In contrast, the level of MDA, an indirect index of oxidative stress was significantly reduced at high concentration of 300mg quercetin. Malondialdehyde is a mutagenic product of enzymatic and free radical-induced lipid peroxidation (Niedernhofer *et al.,* 2003). Quercetin has been shown to have very potent antioxidant and cytoprotective effects in preventing endothelial apoptosis caused by oxidants (Choi *et al.,* 2003). Additionally, quercetin prevents oxidant injury and cell death by several mechanisms, such as scavenging oxygen radicals (Inal *et al.,* 2002) and protecting against lipid peroxidation (Laughton *et al.,* 1991). The antioxidant activity of quercetin may act through the suppression of superoxide radicals to prevent tissue assault. Taken together, our finding is similar to the report of Unsal *et al.* (2013) wherein treatment using a single dose of quercetin showed protective effect by decreasing significantly the elevated MDA levels. It is however worthy of mention that most of these reports showed the beneficial impacts of quercetin viz-a viz neuroprotection, anti-oxidant, anti-inflammatory and anti-apoptotic effects in disease or compromised conditions. In contrast, our study shows the anti-oxidant impacts of quercetin in a non-disease model which suggests that quercetin supplementation might enhance the levels of anti–oxidant and reduce pro-oxidant in healthy states. However, supplementation at high dosage may affect its beneficial effects.

Conclusion

Quercetin showed better antioxidant activity at lower doses and adversely affected the climbing behavior and survival rate at higher doses. Therefore, quercetin should be used with caution as a supplement. Similarly, future clinical trials of quercetin and its analogs as antioxidant should modify its toxicity for better clinical efficacy.

Conflict of interest: The authors declare no conflicting interest

Acknowledgment: No financial support was received to conduct this research.

Contribution: JAO, LA, OO and PO designed the conceptual frame work of the study. AAB, JIE, MAE, TOA and OOS were responsible for designing the experimental work plan while AP, WA, OS, EO, DO and UJK were involved in executing the experiment and running of protocols. All authors were involved in drafting the manuscript.

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