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Effects of sodium fluoride and *Ocimum sanctum* extract on the lifespan and climbing ability of *Drosophila melanogaster*

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Abstract: Background: Fluoride may induce oxidative stress and apoptosis. It may also lead to neurobehavioural defects including neuromuscular damage. The present study aimed to explore the effects of sub lethal concentrations of sodium fluoride (NaF) on the lifespan and climbing ability of *Drosophila melanogaster*. In total, 0.6 mg/L and 0.8 mg/L of NaF were selected as sublethal concentrations of NaF for the study. Lifespan was measured and climbing activity assay was performed.

Results: The study showed significant decrease in lifespan of flies treated with fluoride. With increasing age, significant reduction in climbing activity was observed in flies treated with sodium fluoride as compared to normal (control) flies. Flies treated with tulsi (*Ocimum sanctum*) and NaF showed increase in lifespan and climbing activity as compared to those treated with NaF only. Lipid peroxidation assay showed significant increase in malondialdehyde (MDA) values in the flies treated with NaF as compared to control. The MDA values decreased significantly in flies treated with tulsi mixed with NaF.

Conclusions: The results indicate that exposure to sub lethal concentration of NaF may cause oxidative stress and affect the lifespan and climbing activity of *D. melanogaster*. Tulsi extract may help in reducing the impact of oxidative stress and toxicity caused by NaF.

Keywords: Sodium fluoride (NaF), *Ocimum sanctum*, Lifespan, Climbing activity assay, Oxidative stress

Background

Fluoride may enter the human body through (i) drinking water, (ii) food and food products (e.g. contaminated with pesticides) and (iii) industrial emission of fluoride dust and fumes (Susheela, 2013). Excessive ingestion of fluoride results in development of dental, skeletal and non-skeletal fluorosis in humans. Acute pesticide poisoning during childhood may lead to neurobehavioural deficits (Berger, Friedman, Jaffar, Kofman, & Massarwa, 2006; Beseler, Bouchard, & London, 2012). Toxic effects of fluoride have been documented in other animals also. Chronic fluoride exposure can alter kidney structure, renal function and induce apoptosis in pigs (Zhan, Wang, Xu, & Li, 2006). It can cause severe health problems in rat, mice and fish due to oxidative stress, DNA damage and apoptosis (He & Chen, 2006; Mukhopadhyay & Chattopadhyay, 2014).

Fluoride is able to induce oxidative stress and apoptosis in both intrinsic and extrinsic pathways in the mammalian cells (Agalakova, Petrova, & Gusev, 2019; Lu et al., 2017; Ribeiro et al., 2017). NaF-induced oxidative damage inhibits enzymes involved in energy production, membrane bound ion transport and neurotransmission (Vani & Reddy, 2000). Pesticides containing fluoride have negative impacts on the biology of non-target organisms (Agalakova & Gusev, 2012). *D. melanogaster* (commonly called fruit fly) is not considered as a pest, as it grows and multiplies on rotten fruits. Instead, it is considered as a non-target organism and is used as a model for toxicity evaluation of chemicals (Rand, Montgomery, Prince, & Vorojeikina, 2014). *Drosophila melanogaster* is an acceptable

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model for understanding the human condition under stress of toxicants because of the abundance of highly conserved genes and pathways controlling development, stress response and xenobiotic metabolism across these two divergent species (Mackay & Anholt, 2006; Misra, Horner, Lam, & Thummel, 2011; Sykiotis & Bohmann, 2010). A number of studies have been conducted on *Drosophila* model using priority environmental contaminants and toxicants including mercury (Rand, Dao, & Clason, 2009), lead (Hirsch et al., 2003), arsenic (Ortiz, Opoka, Kane, & Cartwright, 2009), fluoride (Dutta, Rajak, Khatun, & Roy, 2017; Khatun, Mandi, Rajak, & Roy, 2018), manganese (Bonilla et al., 2012), ethanol (Guarnieri & Heberlein, 2003), nanoparticles (Posgai et al., 2011), pesticides (Gupta et al., 2007; Das, Podder, & Roy, 2010; Chmiel, Daisley, Burton, & Reid, 2019), nanopesticides (Demir, 2020) and solvents (Wasserkort & Koller, 1997).

NaF can cause genotoxic alterations and induce melanotic tumours in adult *D. melanogaster* (Herskowitz & Norton, 1963). Similarly, sublethal exposure of fluoride has been found to cause physiological and reproductive changes in *D. melanogaster* (Dutta et al., 2017; Singh, Chowdhary, Shah, & Yasmin, 2020).

The basic biological, physiological, and neurological properties are conserved between mammals and *D. melanogaster*, and nearly 75% of human disease-causing genes are believed to have a functional homologue in the fly (Lloyd & Taylor, 2010; Reiter, Potocki, Chien, Gribskov, & Bier, 2001). Therefore, *D. melanogaster* is used as a model system for investigating the roots of human diseases such as neurological diseases, including neuromuscular disease.

O. sanctum (Linn) commonly called 'tulsi' belongs to family *Labiatae*. Several medicinal properties have been attributed to the plant not only in Ayurveda and Siddha but also in Greek, Roman and Unani system of medicines. *O. sanctum* has been reported to possess antimicrobial, anti-stress, antidiabetic, hepatoprotective, anti-inflammatory, anti-carcinogenic, immunomodulatory, radioprotective, neuroprotective and cardioprotective properties. The leaves of *O. sanctum* contain alkaloids, flavonoids, glycosides, saponins, tannins, ascorbic acid and carotene (Mondal, Mirdha, & Mahapatra, 2009).

This paper reports about the effects of fluoride toxicity on the lifespan and climbing ability of *D. melanogaster.* Impaired climbing ability may be linked to premature ageing caused by fluoride due to oxidative stress. This is because fluoride inhibits bioenergetic reactions, in particular oxidative phosphorylation, reducing physical activity of muscles (Machoy-Mokrzyńska, 2004). An attempt was made to study the effect of O. sanctum on the lifespan and climbing activity of *D. melanogaster* exposed to NaF.

Methods

Native *D. melanogaster* was cultured in the laboratory at 25 °C in standard cornmeal medium. The standard cornmeal medium consisted of maize powder, sucrose, dextrose, yeast extract and agar. Single line culture (stock) of *D. melanogaster* was maintained to obtain flies of the same age and strain. For determining the sublethal concentration of NaF for *D. melanogaster*, 0.6 mg/L, 0.8 mg/L and 1 mg/L of NaF were tested following Singh et al. (2020) and Mishra, Kumari, Ranjan and Yasmin (2020). Four sets of cornmeal media were prepared. Each set consisted of three bottles. The media were changed after every 4 days.

The four sets of bottles were as follows:

1) *Control set*—fruit flies were cultured in standard cornmeal media.
2) *NaF-treated sets* (*three sets*)—three sets of standard cornmeal media were prepared and 1.0 mg/L, 0.8 mg/L and 0.6 mg/L of NaF were added respectively into these three sets.

Five flies from the stock were transferred into each set and monitored.

Preparation of *O. sanctum* extract

O. sanctum (tulsi) leaf extract was prepared following Mitra et al. (2014). The tulsi leaves were dried in a hot air oven and powdered using mortar and pestle. The dried *O. sanctum* leaf dusts were soaked overnight in distilled water (15 g leaf dust per 100 ml distilled water) and filtered through a fine muslin cloth. The filtrate was centrifuged at 5000 rpm for 10 min. The supernatant thus obtained, was filtered again using a fine muslin cloth and the filtrate was collected in sterile polypropylene tubes and frozen at 20 °C.

Published report on clinical trials conducted on humans till date suggests that tulsi is a safe herbal intervention. Tulsi dosage and frequency in such studies varied from 300 mg to 3000 mg given as 1–3 times per day as tulsi leaf aqueous extract to human subjects (Jamshidi & Cohen, 2017). In the present study, 10% v/v tulsi leaf extract (TLE) was taken which was roughly 50% of the above mentioned dosage. However, higher dose of TLE may also be tried.

For performing the experiments, five sets of cornmeal media were prepared. Each set had three bottles of cornmeal media.

1) Control set with standard cornmeal media
2) NaF (0.6 mg/L) treated cornmeal media
3) NaF (0.8 mg/L) treated cornmeal media
4) *O. sanctum* cornmeal media (with 10% v/v tulsi leaf extract in standard cornmeal media)

Table 1 Life table depicting survival of *D. melanogaster* in control medium

Age interval (in days)	Number surviving at the beginning of age interval (nx)	Number dying during the age interval x to x+1 (dx)	Age-specific mortality rate (qx)	Average number of days lived by flies x to x+1 (lx)
0-7	60	0	0	60
7-14	60	3	5	58.5
14-21	57	16	28	49
21-28	41	15	36.5	33.5
28-35	26	3	11.5	24.5
35-42	23	7	30.4	19.5
42-49	16	4	25	14
49-56	12	7	58.3	2.5
56-64	5	5	100	2.0
64-72	0	0	0	0

5) *O. sanctum* + NaF (0.8 mg/L) containing media (with 10% v/v *tulsi* leaf extract and 0.8 mg/L of NaF in standard cornmeal media)

For studying the lifespan of native *D. melanogaster* in the different media mentioned above, newly ecloded flies were collected from the stock and raised in the respective media at 25 °C. Twenty flies were placed into each bottle and were transferred to bottles with fresh media after every 4 days. The numbers of dead flies were counted every day.

Climbing activity assay was performed following Manjila and Hasan (2018) in a glass cylinder of 50 ml. Batches of 20 flies from each experimental set were used to perform climbing activity assay. Timer of 10 s was set and the number of flies crossing the 50 ml border was counted. Each assay was repeated thrice and average climbing ability of each batch of flies was calculated. Climbing activity of each batch of flies was monitored at interval of 3 days.

Lipid peroxidation assay was performed following Ohkawa, Ohishi, and Yagi (1979) on third generation flies (flies exposed to NaF for three generations). In total, 0.3 g of the flies was taken and homogenised by adding 1 ml of 0.1% trichloroacetic acid (TCA) in a glass homogeniser. The homogenate was centrifuged at 5000 rpm for 15 min at room temperature. One millilitre of the supernatant was transferred into a clean and dry test tube and 2 ml of freshly prepared 0.5% thiobarbituric acid (TBA) in 20% TCA was added into it. This sample was incubated at 90 °C for 30 min and subsequently cooled at room temperature. Absorbance was measured by dual beam spectrophotometer at 532 and 600 nm. All the readings were taken in triplicates.

MDA level was calculated by following formula:

$$\mathrm{MDA} = (\mathrm{OD}\ 532 - \mathrm{OD}\ 600 \times 100/1.56) \times \mathrm{TV}/[\mathrm{dw} \times 1000]$$

Where,

OD = optical density

TV = total volume of the sample

dw = dry weight of sample

Statistical analysis was performed using ANOVA and $p<0.05$ was considered as significant. One-way ANOVA was used to analyse climbing assay data and two-way ANOVA was used to analyse lifespan data.

Results

Flies cultured in media with 1.0 mg/L NaF survived for 1 week only. Some eggs but no larvae were found in these media after 1 week. Flies cultured in media with 0.6 mg/L and 0.8 mg/L NaF continued to survive and produce eggs and larvae. So, 0.6 mg/L and 0.8 mg/L of

Table 2 Life table depicting survival of *D. melanogaster* in media with 0.6 mg/L NaF

Age interval (in days)	Number surviving at the beginning of age interval (nx)	Number dying during the age interval x to x+1 (dx)	Age-specific mortality rate (qx)	Average number of days lived by flies x to x+1 (lx)
0-7	60	10	16.6	55
7-14	50	23	46	38.5
14-21	27	18	66.6	18
21-28	9	9	100	4.5
28-35	0	0	0	0

Table 3 Life table depicting survival of *D. melanogaster* in media with 0.8 mg/L NaF

Age interval (in days)	Number surviving at the beginning of age interval (nx)	Number dying during the age interval x to x+1 (dx)	Age-specific mortality rate (qx)	Average number of days lived by flies x to x+1 (lx)
0-7	60	9	15	83.5
7-14	51	22	43.1	40
14-21	29	28	96.5	15
21-28	3	3	100	1.5
28-35	0	0	0	0

NaF were considered as sub lethal concentrations for the present study. Singh et al. (2020) and Mishra et al. (2020) also reported 0.8 mg/L as the sublethal concentration of NaF for *D. melanogaster* and *Zaprionus indianus* respectively.

Survival of *D. melanogaster* was better in control media (Table 1) as compared to that in the media containing 0.6 mg/L NaF (Table 2), where only a single fly survived for 25 days. In the media containing 0.8 mg/L NaF (Table 3), a single fly survived for 22 days. Survival of flies in the media containing *O. sanctum* extract (Table 4) was similar to those in the control media whereas survival of flies in media containing NaF + *O. sanctum* extract was found to be better than those in media with NaF (Table 5). Fifty percent of the flies was dead by the end of 4th week in control media (Table 1), by the 2nd week in media with NaF (Tables 2 and 3), by the 5th week in media with tulsi extract (Table 4) and by the end of 3rd week in media with tulsi extract+0.8 mg/L NaF (Table 5). Lifespan of *D. melanogaster* was found to be ~75 days in control set, ~76 days in media with tulsi extract, ~40 days in media with 0.6 mg/L NaF, ~37 days in media with 0.8 mg/L NaF and ~57 days in media with tulsi extract+0.8 mg/L NaF. A comparative picture of survival of flies in different media is shown in Fig. 1a and b.

Climbing activity of *D. melanogaster* was maximum after 3 days of eclosion in control media (Fig. 2). On the other hand, the climbing activity of flies reared in media with 0.6 mg/L and 0.8 mg/L NaF was suppressed markedly after 3 days of eclosion. A progressive decline in climbing activity was seen in the flies exposed to NaF after 6, 9 and 12 days of eclosion (Figs. 3, 4 and 5). These flies could not show any climbing activity after 15 days of eclosion (Fig. 6). *D. melanogaster* cultured in media with tulsi extract showed improved climbing activity after 15 days of eclosion (Figs. 2, 3, 4, 5 and 6).

Lipid peroxidation (LPO) assay revealed altered malonyl dialdehyde (MDA) production in the tissues of flies after exposure to sub lethal concentration (0.8 mg/L) of NaF as compared to control and to those treated with tulsi extract+ 0.8 mg/L NaF ($F_{(2,6)}$ =11.95, P<0.05) (Fig. 7).

Discussion

The significant reduction in lifespan of fluoride treated flies was similar to the findings of Khatun et al. (2018), where NaF exposure to *D. melanogaster* in the parental generation led to an increase in adult mortality. Significant reduction in the climbing activity was also seen in the flies treated with NaF. Similarly, Khatun et al. (2018) and Sarkar, Roy, and Roy (2018) also observed alteration in climbing behaviour in flies exposed to sub lethal concentrations of fluoride. Fluoride is thought to inhibit the activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase. Depletion of glutathione results in excessive production of reactive

Table 4 Life table depicting survival of *D. melanogaster* in medium with tulsi extract

Age interval (in days)	Number surviving at the beginning of age interval (nx)	Number dying during the age interval x to x+1 (dx)	Age-specific mortality rate (qx)	Average number of days lived by flies x to x+1 (lx)
0-07 days	60	1	1.6	59.5
07-14 days	59	12	20.3	53
14-21 days	47	1	2.12	46.5
21-28 days	46	19	41.3	36.5
28-35 days	27	7	25.9	23.5
35-42 days	20	4	20	18
42-49 days	16	6	37.5	13
49-56 days	10	6	60	7
56-63 days	4	4	100	2
63-70 days	0	0	0	0

Table 5 Life table depicting survival of *D. melanogaster* in medium with tulsi extract+0.8 mg/L NaF

Age interval (in days)	Number surviving at the beginning of age interval (nx)	Number dying during the age interval x to x+1 (dx)	Age-specific mortality rate (qx)	Average number of days lived by flies x to x+1 (lx)
0-7	60	12	20	54
7-14	48	11	22.9	42.5
14-21	37	15	40.5	29.5
21-28	22	13	59.09	15.5
28-35	9	3	33.3	7.5
35-42	6	3	50	4.5
42-49	3	3	100	1.5
49-56	0	0	0	0

oxygen species at the mitochondrial level, leading to the damage of cellular components. Abolaji et al. (2019) treated *D. melanogaster* with NaF and found altered levels of oxidative stress markers (Glutathione-S-transferase (GST), catalase and acetylcholinesterase (AchE) activities, total thiol (T-SH), nitrites/nitrates and hydrogen peroxide (H_2O_2) levels). These parameters could be balanced by resveratrol (a natural polyphenol with antioxidant and anti-inflammatory properties).

In the present study, when tulsi extract was mixed in the media with NaF, the flies survived and maintained their climbing activity in comparison to the flies cultured in media with NaF only. Similar results were found by Siddique, Faisal, Naz, Jyoti, and Rahul (2014), where the flies exposed to various doses of *O. sanctum* extract showed a dose-dependent significant delay in the loss of climbing ability.

Higher MDA value in the flies treated with NaF indicated high oxidative stress which is similar to the results obtained by Patel and Chinoy (1998); Wang et al. (2004) and Dutta et al. (2017). Treatment with tulsi extract possibly reduced the oxidative stress. Siddique et al. (2014)

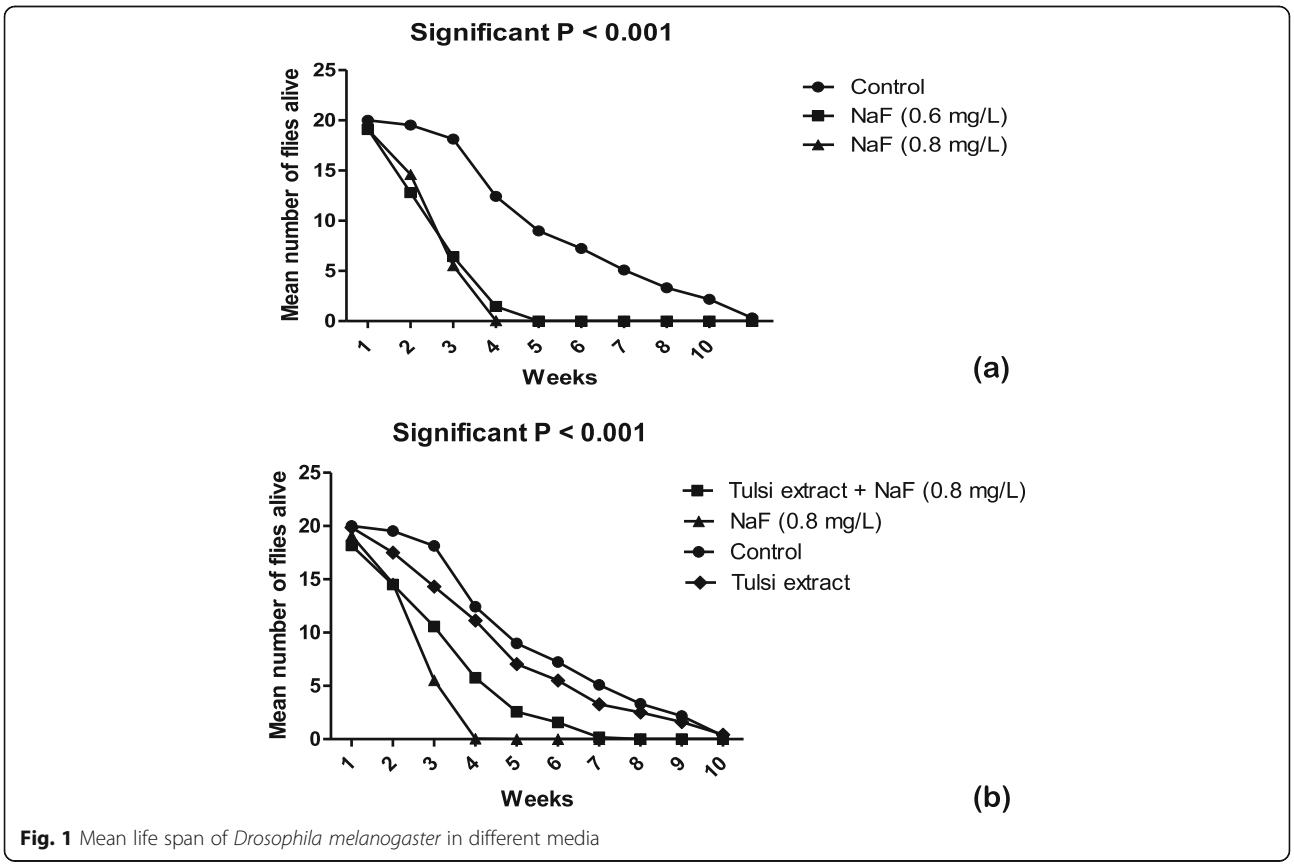

Fig. 1 Mean life span of *Drosophila melanogaster* in different media

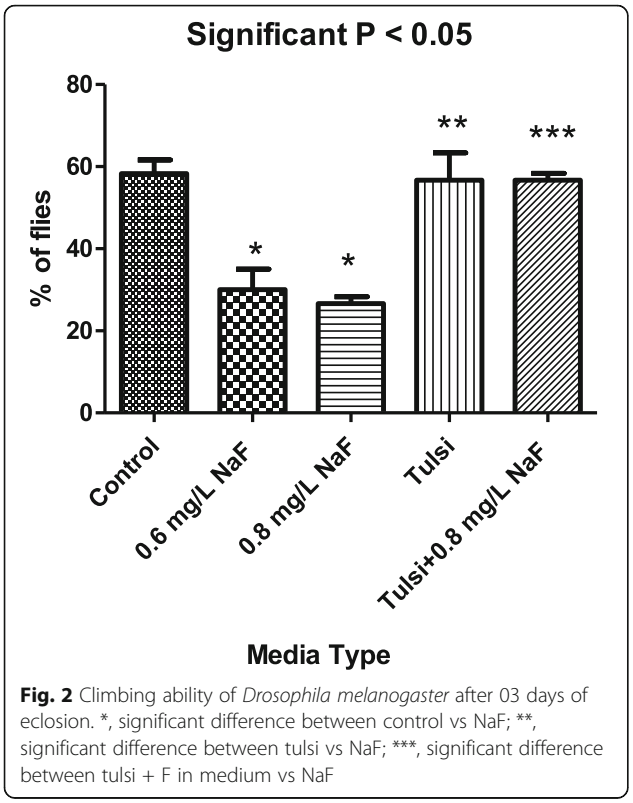

Fig. 2 Climbing ability of *Drosophila melanogaster* after 03 days of eclosion. *, significant difference between control vs NaF; **, significant difference between tulsi vs NaF; ***, significant difference between tulsi + F in medium vs NaF

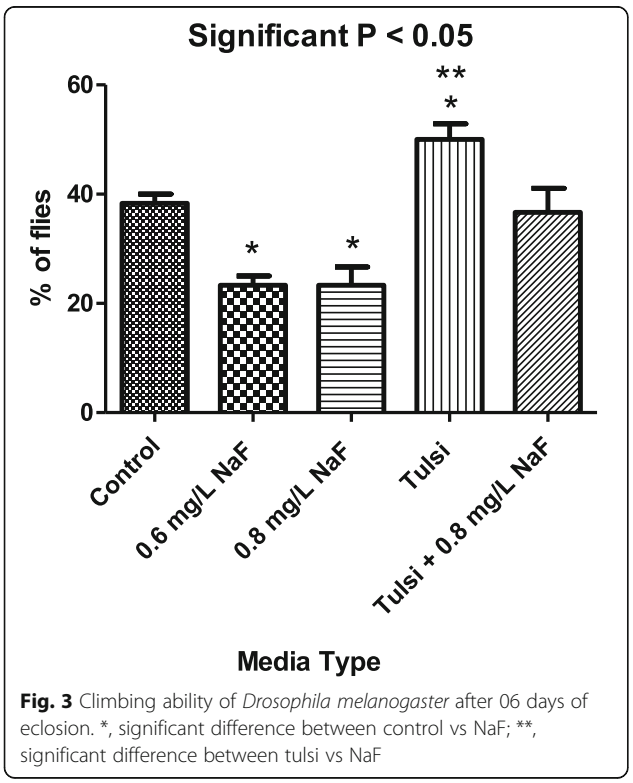

Fig. 3 Climbing ability of *Drosophila melanogaster* after 06 days of eclosion. *, significant difference between control vs NaF; **, significant difference between tulsi vs NaF

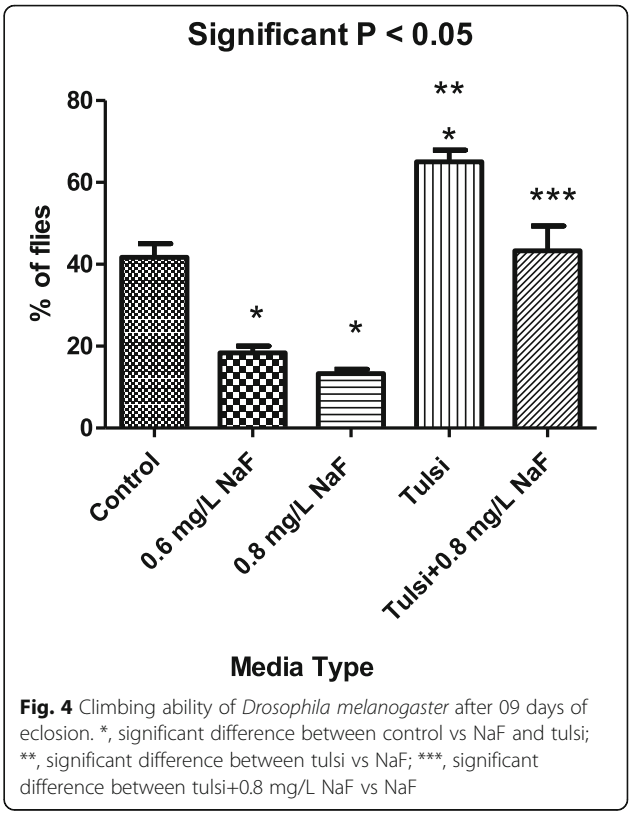

Fig. 4 Climbing ability of *Drosophila melanogaster* after 09 days of eclosion. *, significant difference between control vs NaF and tulsi; **, significant difference between tulsi vs NaF; ***, significant difference between tulsi+0.8 mg/L NaF vs NaF

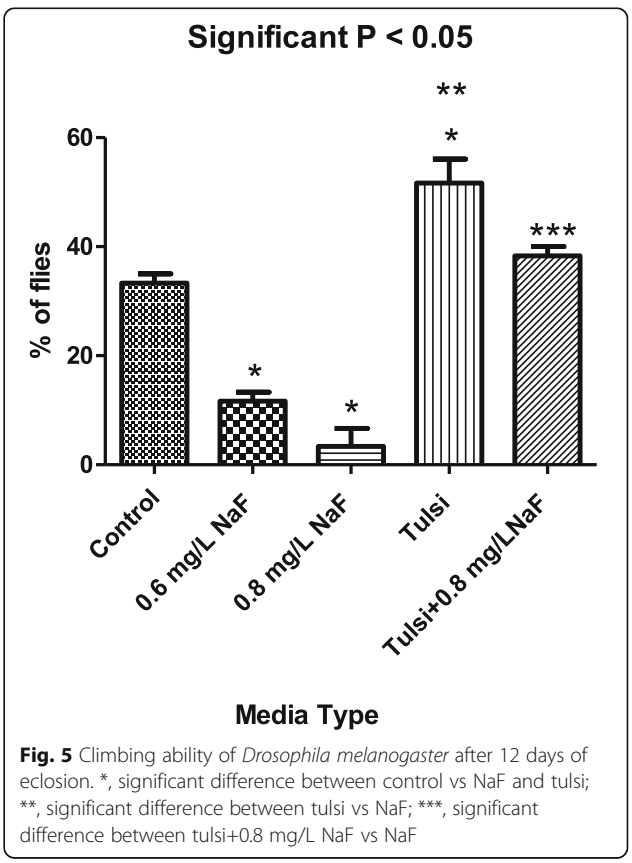

Fig. 5 Climbing ability of *Drosophila melanogaster* after 12 days of eclosion. *, significant difference between control vs NaF and tulsi; **, significant difference between tulsi vs NaF; ***, significant difference between tulsi+0.8 mg/L NaF vs NaF

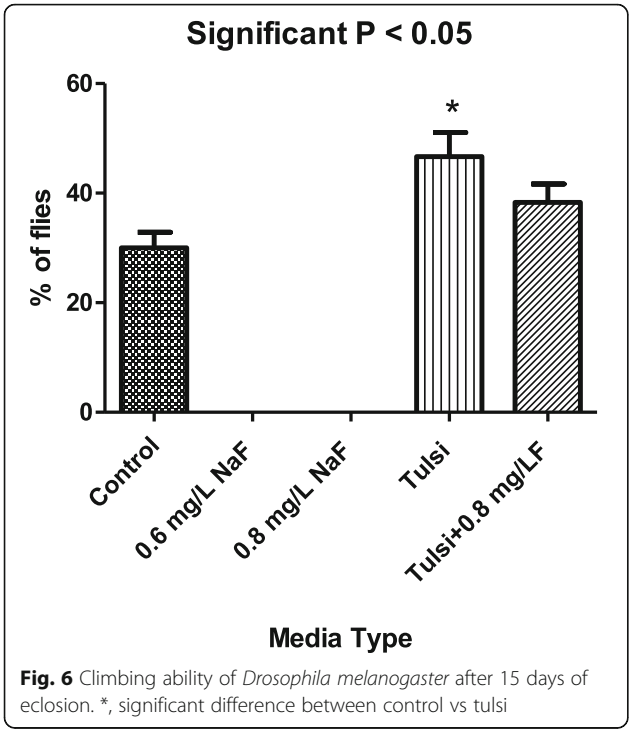

Fig. 6 Climbing ability of *Drosophila melanogaster* after 15 days of eclosion. *, significant difference between control vs tulsi

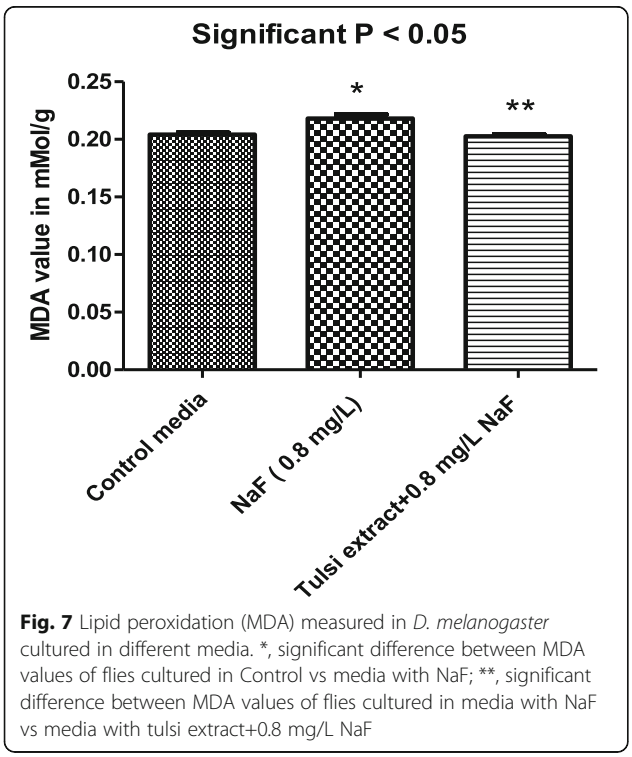

Fig. 7 Lipid peroxidation (MDA) measured in *D. melanogaster* cultured in different media. *, significant difference between MDA values of flies cultured in Control vs media with NaF; **, significant difference between MDA values of flies cultured in media with NaF vs media with tulsi extract+0.8 mg/L NaF

also found that treatment with tulsi extract caused reduction in oxidative stress in the brain of Parkinson's diseased model flies.

Oxidative stress plays a major role in ageing, and is associated with several neurodegenerative diseases. *O. sanctum* (tulsi) leaf extract possesses antioxidative properties (Mitra et al., 2014). *O. sanctum* leaves are rich in polyphenolic flavonoids which act as antioxidants (Hakkim, Gowri Shankar, & Girija, 2007) and are helpful in preventing lipid peroxidation (Geetha & Vasudevan, 2004). Aqueous extract of the *O. sanctum* leaves may function simply by quenching the free radicals generated during oxidative stress or may improve the antioxidant enzyme status of the tissue in the face of the oxidative stress (Mitra et al., 2014). Tulsi has been found to be a chief source of many biologically active compounds like ursolic acid, eugenol, rosmarinic acid, linalool, carvacrol and β caryophyllene and these compounds play a significant role in the treatment and prevention of many diseases (Almatroodi, Alsahli, Almatroudi, & Rahmani, 2020). The antioxidant nature of *O. sanctum* leaf extract might have reduced the oxidative stress caused by NaF in *D. melanogaster* used in this study. Shivananjappa and Joshi (2012) also found that aqueous extract of tulsi had putative potency to enhance the endogenous antioxidant defences in human hepatocyte cell line (HepG2) which can potentially effect faster dissipation of ROS. Free radical scavenging activity is a chief mechanism through which *Ocimum sanctum* products protect against cellular damage. Its role in free radicals scavenging property has confirmed its strong antioxidant activity and free radicals scavenging property (Ganasoundari et al., 1998; Keshari, Srivastava, Verma, & Srivastava, 2016).

In the present study, tulsi extract (alone) was not found to significantly increase the lifespan of flies as compared to control, but lifespan of flies treated with fluoride + tulsi significantly increased as compared to the flies treated with fluoride. The results of this study can be considered preliminary and further investigations are required to prove the worth of tulsi in increasing the lifespan. Research has shown that tulsi reduces stress, enhances stamina, relieves inflammation, lowers cholesterol, eliminates toxins, protects against radiation, prevents gastric ulcers, lowers fevers, improves digestion and provides a rich supply of antioxidants and other nutrients. The nutritional analysis of *Ocimum sanctum* has shown high level of ascorbic acid, N, P, K, total phenol, carbohydrates and proteins in their leaves, which may be very good for health. These properties may help to enhance the lifespan (Patel, 2020). Further, tulsi has been found to mediate a significant reduction in tumour cell size and an increase in lifespan of mice having sarcoma-180 solid tumours (Nakamura et al., 2004).

Fluoridated insecticides may be helpful in targeting the pests (Metcalf, 2015), but the toxic effects of fluoride on non-target animals should not be neglected (Dhar & Bhatnagar, 2009; Sauerheber, 2013). The results of the present study indicate that aqueous tulsi leaf extract acts as antioxidant by possibly scavenging the oxygen free radical and other reactive oxygen intermediates. Thus, *O. sanctum* has the potential to reduce fluoride toxicity in *D. melanogaster*. The study suggests that *O. sanctum* (tulsi) may be of future therapeutic relevance particularly in the area where humans are chronically exposed to fluoride either occupationally or through food chain.

Conclusion

The present study concluded that exposure of *D. melanogaster* to sub lethal concentrations of NaF caused oxidative stress induced damage in its body leading to reduced lifespan and climbing activity. It was also concluded that *O. sanctum* extract may reduce oxidative stress and fluoride toxicity. Therefore, *O. sanctum* can be of therapeutic relevance.

Abbreviations

NaF: Sodium fluoride; TLE: Tulsi leaf extract; MDA: Malondialdehyde

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Authors' contributions

The manuscript was drafted by SP; life tables and graphs were prepared by SK and data were compiled and analysed partly by HR. SY designed the experiment, performed final data analyses and revision of manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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