

Determining the effects of traditional herbs on aggression levels in *Drosophila melanogaster*

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Abstract

Aggression is a common social behaviour found in most living organisms. However, its causes and potential treatment are still not well understood due to its complex nature. Current medication to treat aggression has adverse side effects and limited efficacy. Therefore, we propose the use of traditional chinese herbs, suan zao ren (*Ziziphi spinosae semen*) and bupleurum root (*Bupleuri radix*) as an alternative. In this study, we investigated the effects of the traditional herbs on aggression levels, using *Drosophila melanogaster* as the model organism. Aggression in *Drosophila* was quantified through an aggression assay where two male flies were fought against each other and aggressive behaviour was recorded. The safety of the herbs were evaluated via a toxicity assay where flies were housed in vials containing the herbs and the number of surviving flies were counted after one week. Herbs were found to have limited toxicity even at high concentrations of 5%. The optimal concentration for both herbs where the highest percentage of flies survived was 2.5%. Both herbs also significantly decrease aggression levels in *Drosophila*. Thus, the herbs show promise to be used as a safe and effective method in treating aggression.

1. Introduction

Aggression is common in individuals suffering from neurodegenerative diseases such as dementia. It has been found that dementia affects 80% of home care residents worldwide, and 90% of those patients exhibit elevated levels of aggression, agitation, and psychosis (Rainey, 2015). Despite their efficacious ability to reduce aggression, atypical antipsychotics such as risperidone leave many concerned about their detrimental side effects such as type II diabetes, cardiac rhythm abnormalities, and compromised metabolic functioning (Pappadopulos et al., 2006). This supports the conclusion that herbal, traditional medications such as suan zao ren (*Ziziphi spinosae semen*) and bupleurum root (*Bupleuri radix*) may serve as an advantageous alternative to current medication by eliminating the threat of lethal side effects.

Ziziphi spinosae semen has a long history as an effective traditional Chinese medicine (He et al., 2020). Numerous studies have found that suan zao ren has a wide range of pharmacological activities including ameliorating effect of learning and memory, anti-inflammatory, antioxidant, antihypertensive, hypolipidemic, antiaging, and anticancer properties (He et al., 2020).

More importantly, it is also effective in improving sleep quality and sleep efficiency (Chan, Chen, Yang, Lo & Lin, 2015). Improved quality of sleep leads to a decrease in violence or other aggressive behaviours (Kamphuis, Meerlo, Koolhas & Lancel, 2012). Therefore, we believe that *suan zao ren* (SZR) can potentially reduce aggression in flies.

Bupleuri radix (Chaihu) has been used as a traditional medicine for more than 2000 years in China, Japan, Korea, and other Asian countries (Yang et al., 2017). One of its properties is to relieve the mental disorders such as depression, anxiety, fear (Liu et al., 2015). Depression, anxiety, aggression and insomnia are frequently found together in a single patient and they share some neurological basis (Liu et al., 2015). Therefore, we believe that bupleurum root (BR) can also potentially reduce aggression in flies since it is effective in treating other related disorders.

Drosophila have DNA content 50 times greater than *E. coli* and only 30 times smaller than mammals (Echalier, Perrimon & Mohr, 2018). In addition, approximately 50% of human genes have *drosophila* ortholog (Isabel & Preat, 2008). Thus, they provide a balance between complexity and simplicity (Echalier, Perrimon & Mohr, 2018). Studies have also developed a quantitative framework for studying aggression *Drosophila melanogaster* (Chen, Lee, Bowens, Huber, & Kravitz, 2002). This makes *Drosophila* an ideal model organism for studying the effect of herbs on aggressive behaviour.

1.1 Objectives

This study aimed to investigate the optimal concentration of herbs that would be given to the *Drosophila* without killing them and if individual herbs could decrease aggression in *Drosophila*.

1.2 Hypotheses

This study hypothesized that both herbs are not toxic to the flies and both herbs would significantly decrease aggressive behaviours in the flies.

2. Materials and Methods

2.1 Materials

Suan zao ren and bupleurum root herbs were bought from Fuhua, a local TCM shop. Flies and fly food were procured from Carolina Biological Supply.

2.2 Toxicity Assay

Suan zao ren and bupleurum root herbs were ground into powder, mixed with deionised water, stirred and heated for 1 hour. The herb extracts were centrifuged and concentrated to 2.5% and 5% by mass of water by evaporation to dryness. The various concentrations of herb extract were then mixed into 5 different fly food preparations and was fed to 5 different groups of 15 flies for 1 week. The concentration of herbs where most flies survived was recorded as the optimal concentration and used in the aggression assay.

2.3 Aggression Assay

2 male flies were isolated in a vial for 3 to 7 days while receiving food containing water (the control), suan zao ren or bupleurum root herbs. After that, the flies were fought in a 5cm by 5cm arena containing a 1cm by 1cm piece of agar mixed with 10% sucrose and apple juice for 10 minutes. Videos were taken for the fight.

2.4 Fight Analysis

Videos were viewed frame by frame using a software Framegrabber to determine the number of occurrences of these aggressive actions, namely approach, low level fencing, wing threat, high-level fencing, chasing, lunging, holding, boxing and tussling (table 2.4). Aggressiveness increases down the table. Chen, Lee, Bowens, Huber and Kravitz (2002) first proposed this table. The number of occurrences and timestamp were recorded in another table.

Table 2.4 Classification of Aggressive behaviours

Low level aggressive behaviours	High level aggressive behaviours
Approach	High level fencing
Low level fencing	Chasing
Wing threat	Lunging
	Holding
	Boxing
	Tussling

3. Results and Discussion

3.1. Herbs displayed non-lethal effect on flies

Toxicity assay was done to determine the optimal concentration of suan zao ren and bupleurum root. There was no significant difference in survival rate of flies fed water, suan zao ren and bupleurum root, as seen in Fig. 3.1, with the p-value of the one tailed Mann-Whitney U-test between suan zao ren and water and between bupleurum root and water being 0.8469 and 0.4942 respectively. This shows that both herbs are not toxic to flies, even at high concentrations of 5%. Since suan zao ren and bupleurum root at 2.5% concentration had the highest percentage of flies surviving, it was used in the subsequent aggression assay.

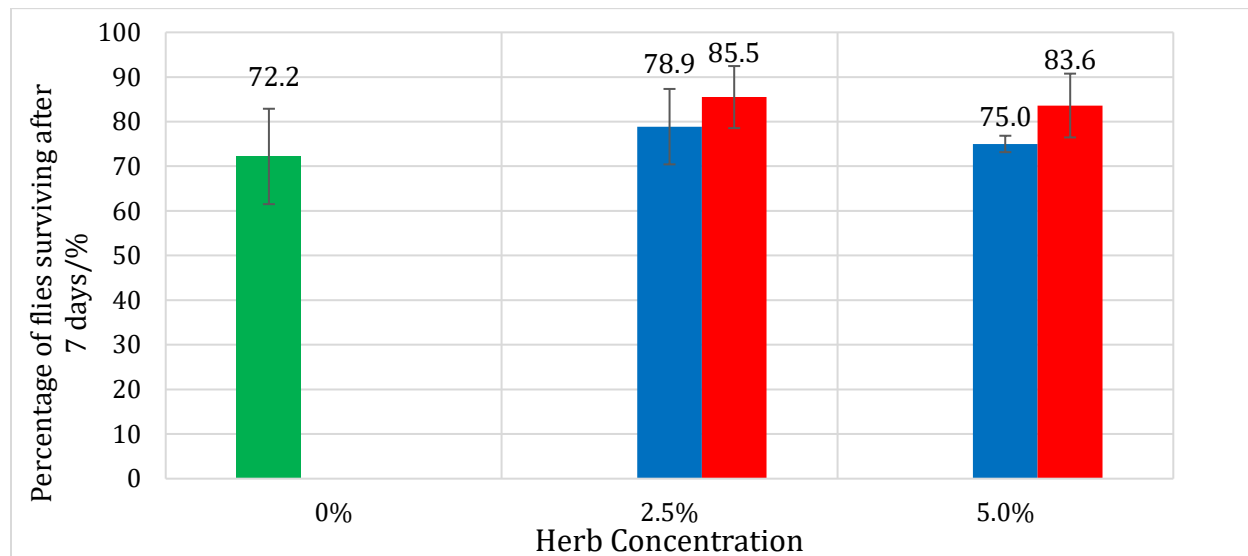


Fig. 3.1 Effect of different concentrations of suan zao ren and bupleurum root on percentage of flies surviving after 7 days

The green bar represents flies fed water, the control, for 7 days. Sample size = 54 flies

The blue bar represents flies fed suan zao ren for 7 days. Sample size = 71 flies (2.5%) and 56 flies (5%). Percentage of flies surviving is not significantly less than control (p-value = 0.8469).

The red bar represents flies fed bupleurum root. Sample size = 62 flies (2.5%) and 61 flies (5%). Percentage of flies surviving is not significantly less than the control (p-value = 0.4942).

Our results concur with other studies, which shows that suan zao ren is only toxic at high doses and the chronic toxicity test in rats showed that suan zao ren's toxicity is very low (He et al., 2020). Lin, Zhang and Su (2016) found that bupleurum root could lead to hepatotoxicity only in high doses and with long-term use.

3.2. Aggression Assay

Aggression assay was done to determine if both herbs can significantly decrease aggressive behaviours in flies.

3.2.1 Approach

The least aggressive behaviour is approach, where one fly lowers its body and advances in the direction of the other fly, as seen in Fig. 3.2.1A and Fig. 3.2.1B. From Fig. 3.2.1C, though not statistically significant compared to the control, flies fed with suan zao ren (p-value = 0.3500) and bupleurum root (p-value = 0.1500) displayed fewer number of approaches.

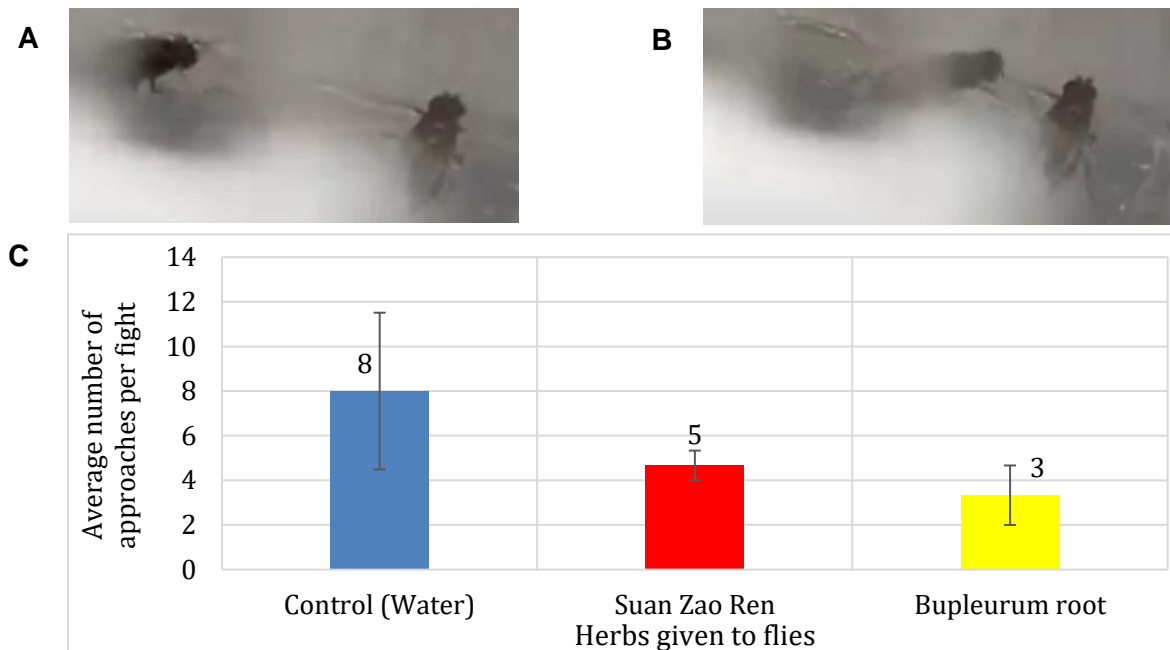


Fig. 3.2.1. Results of aggression assay (Aggressive behaviour: Approach)

A: Approach of left fly (1s)

B: Approach of left fly (2s)

Video link: <https://drive.google.com/drive/folders/1RLGCNJJa63muwCFRJsdI2xIpaouVffYVn>

C: Suan zao ren and bupleurum root on average number of approaches per fight, compared to the control (water). There were 3 fights for each data set, with each fight lasting 10 minutes. There was no significant difference between suan zao ren and the control (p-value = 0.3500) and between bupleurum root and the control (p-value = 0.1500).

3.2.2 Low Level Fencing

The second least aggressive behaviour is low level fencing, where both flies extend one leg and tap the opponent's leg, as seen in Fig. 3.2.2A. Flies fed herbs displayed fewer instances of low level fencing than the control, as seen in Fig. 3.2.2B. This was significantly lower for bupleurum root (p-value = 0.0318) but not significantly lower for suan zao ren (p-value = 0.0500).

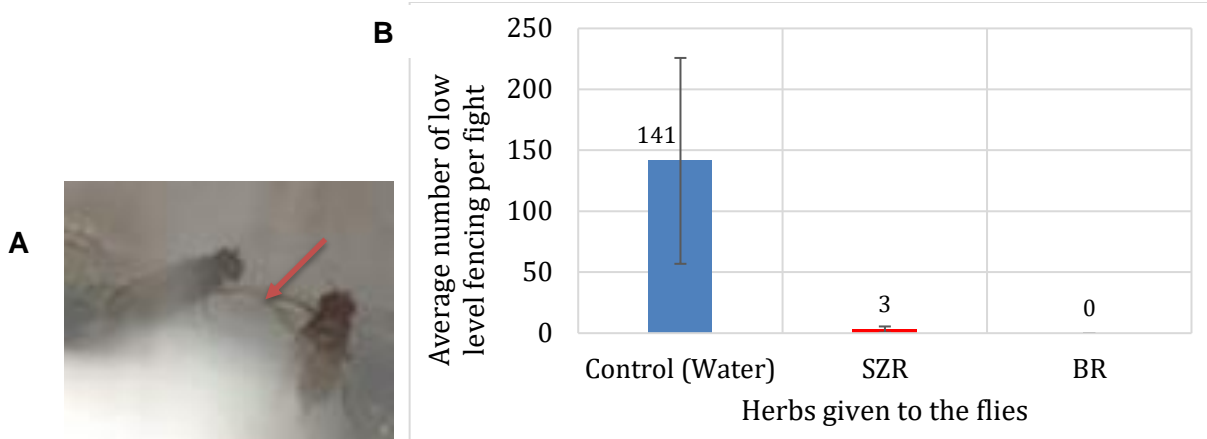


Fig 3.2.2 Results of aggression assay (Aggressive behaviour: Low level fencing)

A: Low level fencing of flies

B: Effect of suan zao ren and bupleurum root on average number of low level fencing per fight, compared to the control (water). There were 3 fights for each data set, with each fight lasting 10 minutes. There was no significant difference between suan zao ren and the control (p -value = 0.0500) but a significant difference between bupleurum root and the control (p -value = 0.0318).

3.2.3 Wing Threat

The third least aggressive behaviour is wing threat, where one fly quickly raises both wings at a 45 degree angle towards the other fly, as seen in Fig. 3.2.3A. There was no statistical significance between both herbs and the control. Flies fed suan zao ren displayed fewer instances of wing threats (p -value = 0.3214), as seen in Fig. 3.2.3B.

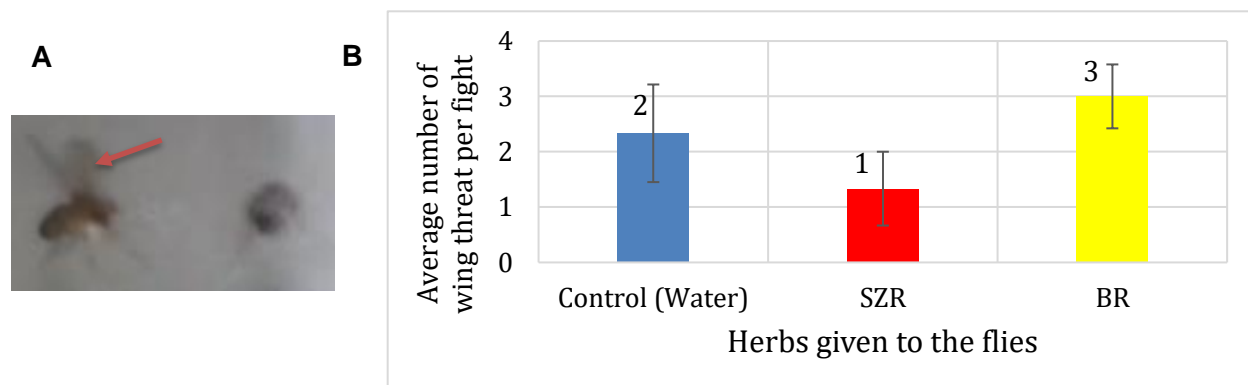


Fig 3.2.3 Results of aggression assay (Aggressive behaviour: Wing Threat)

A: Wing threat by fly on the left

B: Effect of suan zao ren and bupleurum root on average number of wing threats per fight, compared to the control (water). There were 3 fights for each data set, with each fight lasting 10 minutes. There was no significant difference between suan zao ren and the control (p -value = 0.3214) and between bupleurum root and the control (p -value = 0.3265).

Interestingly, flies fed bupleurum root displayed more instances of wing threats than the control (p -value = 0.3265), though not statistically significant, as seen in Fig. 3.2.3B. Upon further examination, we found that for flies treated with bupleurum root, the opponent fly walks away after the wing threat, as seen in Fig. 3.2.3C and Fig. 3.2.3D. However, for the control, the flies start fighting after the wing threat, as seen in Fig. 3.2.3E and Fig. 3.2.3F. Therefore, despite higher instances of wing threat for flies fed bupleurum root, there is less aggression overall.

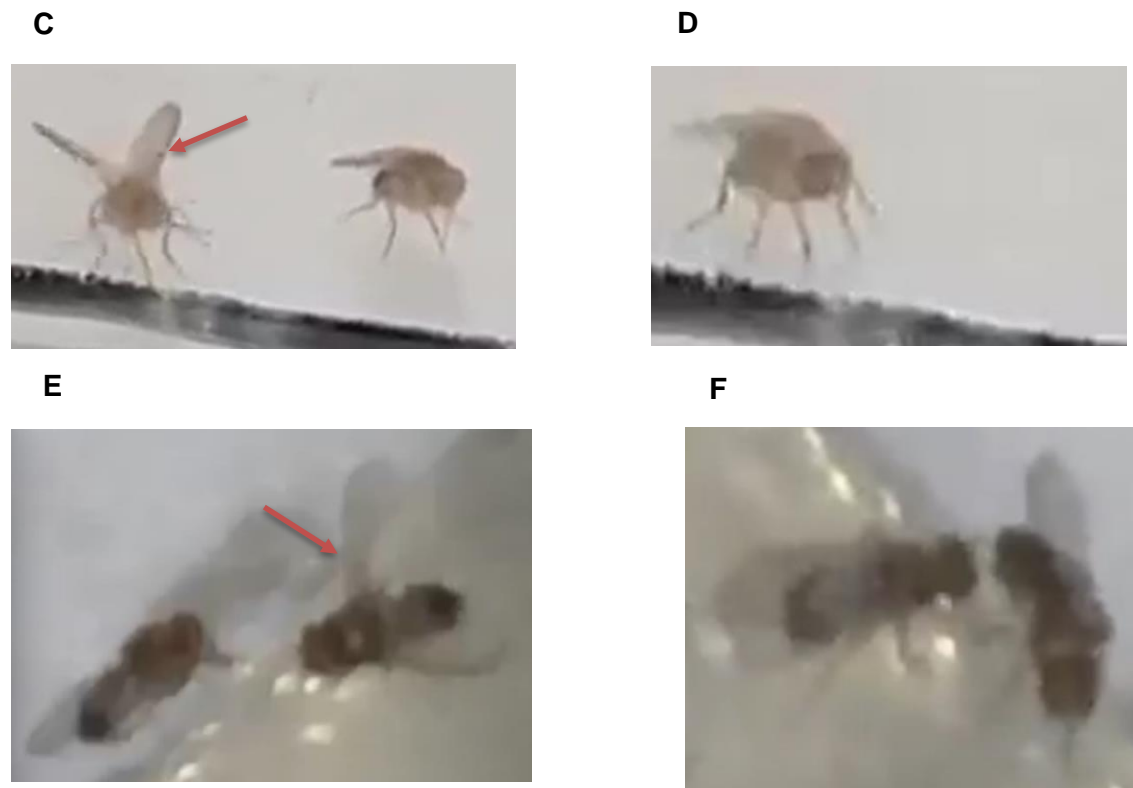


Fig 3.2.3 Differences between fly behaviour after wing threat

C: Flies given bupleurum root, wing threat observed for left fly (1s)

D: Flies given bupleurum root, right fly flew away (2s)

E: Flies given water, wing threat observed for right fly (2s)

F: Flies given water, fighting observed between both flies (4s)

Video link: <https://drive.google.com/drive/folders/1RLGCNJJa63muwCFRJsdl2xlpauVffYVn>

3.2.4 Overall Low Level Aggressive Behaviour

Approach, low level fencing and wing threat are classified under low level aggressive behaviour. The control had significantly greater low level aggressive behaviour than flies given suan zao ren (p -value = 0.0320) and bupleurum root (p -value = 0.0092), as seen in Fig. 3.2.4. This shows that suan zao ren and bupleurum root significantly decreased the degree of aggression in flies.

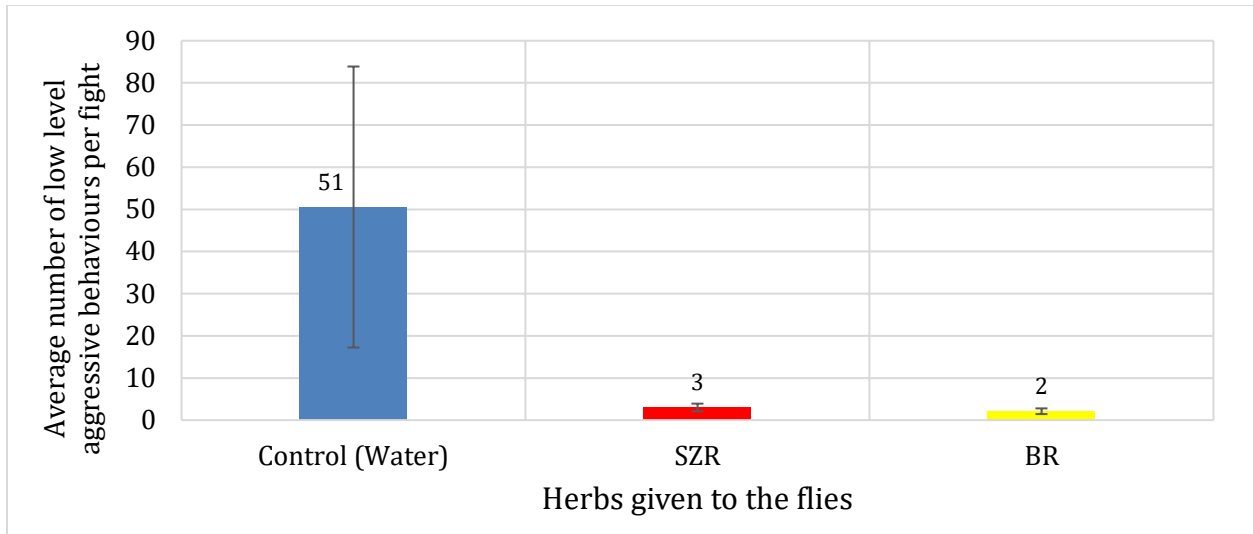


Fig 3.2.4: Suan zao ren and bupleurum root on average number of low level aggressive behaviour per fight

There were 3 fights for each data set, with each fight lasting 10 minutes. There was a significant difference between suan zao ren and the control (p-value = 0.0320) and between bupleurum root and the control (p-value = 0.0092).

3.2.5 All High Level Aggressive Behaviour

High level aggressive behaviours include high level fencing, where both flies face each other, extend leg forward and push opponent, chasing, where one fly runs after the other, as seen in Fig. 3.2.5A and Fig. 3.2.5B, lunging, where one fly rears up on hind legs and snaps down on the other, holding, where one fly grasps the opponent with forelegs and tries to immobilize it, boxing, where one fly rears up on hind legs and strike the opponent with forelegs and tussling, where both flies tumble over each other.

Although not statistically significant, suan zao ren (p-value = 0.1002) and bupleurum root (p-value = 0.0728) had fewer instances of high level aggressive behaviour than the control, as seen in 3.2.5C, which shows that both herbs decreased the degree of aggression in flies.

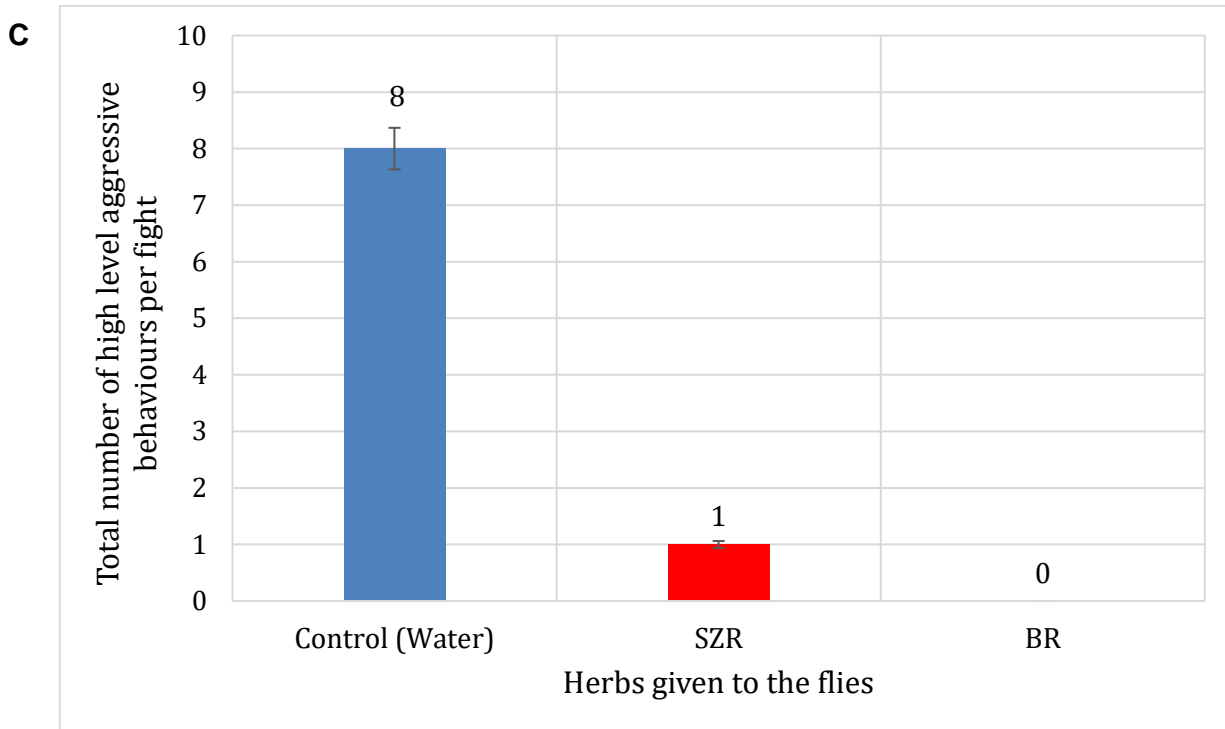
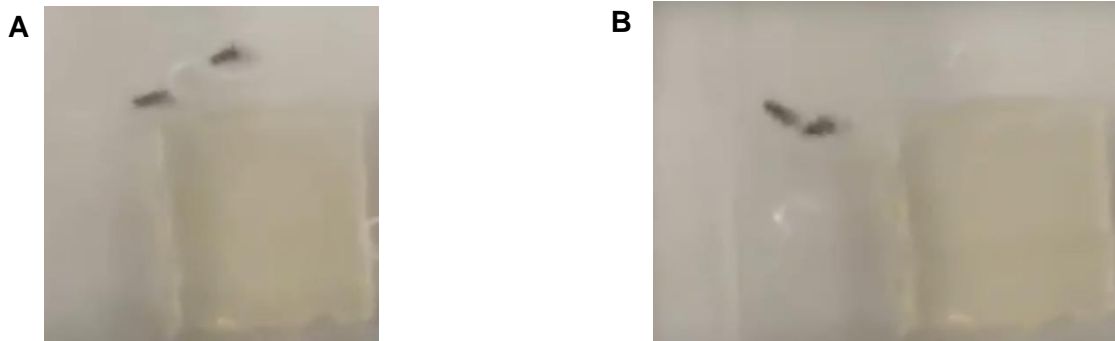


Fig 3.2.5 Results of aggression assay (High level aggressive behaviour)

A: Right fly chases left fly (2s)

B: Right fly chases left fly (3s)

Video link: <https://drive.google.com/drive/folders/1RLGCNJa63muwCFRJsdl2xIpaouVffYVn>

C: Suan zao ren and bupleurum root on total number of high level aggressive behaviours per fight, compared to the control (water). There were 3 fights for each data set, with each fight lasting 10 minutes. There was no significant difference between suan zao ren and the control (p -value = 0.1002) and between bupleurum root and the control (p -value = 0.0728).

Suan zao ren decreases aggression via partial agonist activity of spinosin, the main ingredient in suan zao ren, on 5-HT_{1A} receptors (Kitagawa et al., 2015). Spinosin would activate 5-HT_{1A} receptors, which suppresses serotonergic neuronal activity, leading to decreased

serotonin release (Heisler et al., 1998). With lower levels of serotonin in the brain, less aggression is detected (Edwards & Kravitz, 1997).

For bupleurum root, aqueous extracts of this herb have affinity for 5-HT_{1A} receptors (Seo et al., 2012). Bupleurum root also significantly decreases Corticotropin-releasing factor (CRF) immunoreactivity in the hypothalamus (Seo et al., 2012). Since CRF controls fight or flight reactions and the activation of the sympathetic nervous system, by decreasing CRF immunoreactivity, bupleurum root decreases aggression.

4. Conclusion and Future Work

Suan zao ren and bupleurum root herbs are not toxic to the flies, even at high concentrations of 5% by mass of water. The optimal concentration of herbs where the highest percentage of flies survived is 2.5% for both suan zao ren and bupleurum root. Both herbs can also significantly decrease aggression in flies.

Therefore, these herbs could be used alongside Western medicine as a safe and effective method to reduce aggression in patients with dementia and other neurodegenerative diseases.

The scope of this study could be expanded by investigating other effects of suan zao ren and bupleurum root on flies, such as locomotive effects, as well as investigating the combinatorial effects of both herbs on aggression in flies. The ability of other herbs such as valerian root and gou teng to reduce aggression could be investigated too. To improve the accuracy of the results, more fights could be conducted for each data group (water, suan zao ren and bupleurum root). In addition, the combinatorial effects of both suan zao ren and bupleurum root herbs on aggression can be investigated. Last but not least, the optimal dosage of herbs (volume of herbs that should be fed to the organism per unit mass of organism) could be further studied.

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