GENOTOXICITY AND BEHAVIOURAL EFFECTS OF SODIUM BENZOATE AND SOME FUNGAL STRAINS ON *Drosophila melanogaster*

MANAR ELDESOKY[1], AZZA ABDELRAHMAN[2], A. ABDEL KHALKHAL[3] AND AMIRA ELKEREDY[1]

1 Genetics Department, faculty of Agriculture, Tanta University, Egypt.

2 Environmental and Biological Sciences Department, Faculty of Home Economics, El-Azhar University, Tanta, Egypt.

3 Plant protection and Biomolecular Diagnostic Dep., The Scientific Research City, Borg El-Arab, Alexandria, Egypt.

Sodium benzoate (E211) is a preservative in food industry. Benzoic acid is found in some plants. It is also used as an anti-fungal (Hong *et al.,* 2009). Sodium benzoate (SB) as the European nomenclature E211 is a salt of benzoic acid and is easy soluble in water, tasteless, odourless, as well as it has antifungal and antibacterial properties. It inhibits the growth of bacteria, yeast, and mold (Davidson *et al.*, 2021). Using *Drosophila* as ideal model for geneticists, toxicology and behavioural studies (Rand *et al.,* 2015). Genotoxicity assays include mortality and chromosomal aberrations, DNA damage, disorder behaviour, and mutations (El-Keredy 2014 and 2017; Nohmi *et al*. 2012).

Drosophila melanogaster is a standered genetic model (lifespan, SMART, behaviour, ect.) in diseases of human, mammalian especially fly proteins (Tasset *et al*.,2010; Bourg. 2011; Aysal *et al.*, 2012 and Aysal *et al.*,2013).

The immune system of *Drosophila* distinguishes between different types of infections and activates signal transduction pathways to combat invading microorganisms (Gottar *et al.,* 2006). *Drosophila* social attraction larvae to fungal-infected sites leading to suppression of mould growth may reflect an adaptive behavioural response that increases insect larval fitness and can thus be discussed as an anti-competitor behaviour. The relationship between spatial oviposition patterns, allee effects and the suppression of mould, spatial aggregation in *Drosophila* can be interpreted as an adaptive behaviour against competing fungi on larval feeding sites in order to enhance offspring survival, (Marko 2005). Characterization of the genetic variation underlying gene expression can easily be compromised by lack of environmental control (Hodgins-Davis. and Townsend, 2009). More DNA damage in comet assay resulting treated by benzoic acid, boric acid and sodium sulphite concentrations indicating muta-

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Web Site (*www.esg.net.eg*)

genicity and genotoxic materials (El-Hefny *et al*., 2020).

MATERIALS AND METHODS

The experiments of this study were carried out at the faculty of Agriculture, Tanta University and Technological Application (SRTA) City (Department of plant protection and biomolecular diagnostic) 2017-2021. To examined the effect of food additives sodium benzoate (SB) on larva and adult of *Drosophila melanogaste*. Also measuring behaviour of larvae under the influence of different concentrations of sodium benzoate (SB), the effect of some fungal species on *D. melanogaster* was studied.

Drosophila Medium

The best media was corn flour media for breeding *Drosophila* in the local environment (El-keredy, 2017). After cooling one drop of yeast suspension was spread on the surface of the media.

SB effective line point determination of Petri dishes

Whereas the other half added equal number were used to it 0.007 or 0.075g SB. For half of the control and SB treates cases. These larvae in each plat were growing for a time points (1, 2,4, 8min) as mentioned in the results, data was recorded as the number of larvae located at the control and SB treated using the following equation

$$\text{PREF}_{\text{Gustatory}} = \frac{\#SB - \#PURE}{\#TOTAL}$$

Thus, PREF values were confined between 1 and -1, positive values indicating preference for SB and negative values indicating hatred of the SB according to (König *et al.*, 2014).

The used fungal species

Three *Aspergillus* species *A.pergillus flaves, Aspergillus niger* and *Aspergillus terreus* were isolated from different soil samples in addition to three *Trichoderma* species (*Trichoderma cremeum, Trichoderma viride* and *Trichoderma citririnoviride*) as well as *Penicillium spp* were used to infect the different *Drosophila* flies strains.

Infected flies with fungi

Females and equal number of males were infected with each fungus in the flask. The flies inside the flask loaded with spores for 3-4 minutes and then were transferred back to the culture bottles. The rate of death, flight rate, egg laid and activity were recorded daily. The infected flies were kept in liquid nitrogen and then saves it in -80ºC until the required analyses for immunity gene expression.

RNA extraction

RNA was extracted according to (Mangalathu *et al.*, 2001) from *Drosophila* flies.

cDNA extracted and PCR reaction

cDNA was synthesized using Moloney Murine Leukemia Virus Reverse

Transcriptase Enzyme (Fermentas, USA). Reverse transcription reactions were performed using primer oligo dT primer (Table 1). Each 25 µl reaction master mix containing 2 µl of 5X buffer with 6 µl of H2O, 2 µl of mM dNTPs mix, 5 µg of primer, 1 µg RNA and 2 µl Reverse Transcriptase Enzyme. RT-PCR amplification was performed in a thermal cycler (Eppendorf, Germany) programmed at 37ºC for 20 min and 95ºC for 10 min. Amplification products were visualized using gel documentation system (Syngene, USA) in 1.5% agarose gel that was electrophoresed in 0.5X TBE buffer. cDNA was then stored at -20ºC until used. Protocol for cDNA synthesis (Mangalathu *et al.*, 2001).

Statistical analysis

Analysed by using one-way ANOVA followed by LSD test through SPSS 16 (version 4). The trait means were compared using least significant difference (LSD) tested at significant levels of 5% as described by **(**Gomez and Gomez, 1984). Real-time Q-PCR data analysis: The relative expression ratio was accurately quantified and calculated according to Livak and Schmittgen, (2001).

RESULTS AND DISCUSSION

This experiment was conducted to know how sodium benzoate with different concentrations and fungi strains had affected on different strains of *Drosophila melanogaster* in different generations from flies or larvae.

In the ninth generation (F9) the mortality was 97.14%. While the sexual ratio was zero, as the flies died and did not complete the tenth generation in Tanta flies (Fig. 1).

Figure (2) showed that the effect of sodium benzoate with different concentrations on *Drosophila* which collected from *Kafr el-Sheikh* strain. It is also clear that *Kafr El-Sheikh* flies dynasty was more affected, while the mortality in the highest concentration was 67.69% compared to the lowest concentration (0.007 g) while reached 41%,53% and (sexual ratio was 0.91). The effect of SB was significant in strain *Kafr El-Sheikh*, reached 84.37%, as well as in the case of the sexual ratio, which reached up to 0.87.

The same results were obtained in Fig. (3) to Fig. (6) where the sodium benzoate affected both flies' mortality and sexual ratio was zero in most strains. In the highest concentration of (SB), also, the 0.157mM, 0.35mM, 0.5mM, and 0.7mM concentrations from (SB) resulted in increase DNA tail and decrease DNA head with Comet assay (Sahin *et al.*, 2015) which was led to the genetic mutation, and genotoxic, cytotoxic and proapoptotic effects (Tasset *et al.*, 2010).

Determination of the itemize point (choice behavior)

Larvae third- instar feeding – stages *Drosophila melanogaster* were used. Choice-behaviour differs in their dose-effect characteristics. Those results rev-

elled that different sets of gustatory receptors *Gr*-gene family (El-Keredy., 2017).

The results of behaviour experiments in the Figures (5 to (7A-12A)) which explained the relationship between sodium benzoate concentrations and its preference in the different Egyptian *Drosophila* strains in the fifth generation after 8 minutes treatment for each strain. The sodium benzoate was affected on larval behaviour similarly in different strains (*Tanta, Kafr El-Sheikh, Canton-S*) in the highest SB concentration 0.075g although *Canton-S* (wild type strain) was highly diverged compared to the Egyptian local strains in Africa (Khatab *et al.,* 2015), which indicates the extent to behaviour effects of genotoxicity and mutation with SB treatment. In some studies, like (Walczak-Nowicka and Mariola, 2022) were discussed sodium benzoate and their relationship to neurodegenerative diseases (autism spectrum disorder ASD, Schizophrenia, major depressive disorder MDD, and pain relief. Electrical system in *Drosophila* nervous system was played essential roles in neuronal function (Ammer, *et al.,* 2022).

The effect of treatment with sodium benzoate concentrations on gene expression

The mRNA expression of *Im1* and *Im2* genes in the different *Drosophila* strains which used (*Tanta, Kafr El-Sheikh, Mansoura, Alex, Canton-S,* and *OR*) with 0.007 and 0.075g concentrations in middle and last generation of each *Drosophila* strain. In Tables (2 and 3) which recorded gene expression for each gene in *Drosophila* strain for 0.007g and 0.075g SB concentrations at middle and last generation of each strain, where we find a significant difference between the decrease within one generation (*Tanta, Kafr El-Sheikh, Mansoura, Alex,* and *OR* flies) as well as between generations. The results were recorder will be Aledwany *et al.,* (2018) where be reported sodium benzoate was affected on lymphocytes, inhibited DNA synthesis also increased micronuclei and anaphase bridges formation. More differences were recorded between *Drosophila* strains in two generations for *Im2* gene.

With more than 60% of human disease were similarity to morphology of eucaryotic organism, so, *Drosophila* was used as a model organism of genetic experiments (Sahin. *et al.,* 2015), also in the modern studies like (Ganglberger, *et al.,* 2022) included the *Drosophila* larvae, human, and mouse for brain network visualization.

Gene expression studies in these experiments from Figs. (11 to 16) recorded the differences of gene expression between *Im1* and *Im2* in different *Drosophila* strains in the lowest (0.007g) and highest (0.075g) SB concentrations compared to control at two generation for each strain. Sodium benzoate was affected on P^{21}, homocysteine levels, tryptophan metabolism, inhibited of microglia activation and inhibited of neopterin production (Łucja and Mariola 2022 and Klapoetke *et al.,* 2022).

Influence of gene expression in *Drosophila* strains by infection with different species of fungi

In *Drosophila melanogaster*, fungal infections depends on invariant microbial patterns and the virulence on the host, because *Drosophila* immune system detected kinds of infections and activated signal pathways (like *Toll* pathway) to combat microorganisms which were invading (Gottar *et al.,* 2006). Data in Table (4) monitored RT-PCR for *Im1* gene in different *Drosophila* flies (*Tanta, Kafr El-Sheikh, Mansoura, Canton-S* and *OR*) infected with different fungal *ssp*. (*Penicillium, Trichoderma, Aspergillus*).

The gene expression of *Im1* gene was significantly affected in *Tanta* and *Kafr El-Sheikh* flies when infected with *T. citrinoviride*, while *Penicillium ssp, and T. viride* affected in gene expression on *Mansoura strain*, but gene expression on *Alx*. and *OR* strains effected of infection with *A. terreus.*

Table (5) recorded significant *Im2* gene expression effect to *T. viride* for both *Kafr El-Sheikh and Mansoura* flies. *Alex*. strain was affected with *T. viride*, *A. flavus*, and *A. terreus*. *Canton -S* flies was more affected with *Penicillium ssp* and *A. flavus* infection. The *OR Drosophila* strain was the most affected in the lower gene expression of *Im2* gene for infection with different species of fungi except *A. niger*. Antifungal response in *Drosophila* was studied using human pathogenic yeast, entomopathogenic fungi, and resulted that gene expression levels of *Toll*-dependent *Drosophila* gene (Gottar *et al.,* 2006). In *Drosophila* Toll receptors activation in larval fat body by infection, which caused reduction of insulin-like growth factor1 (IGF1).

Toll pathway activation led to growth reduced and there was a relationship between innate immune signalling and endocrine regulation of growth (Suzawa *et al.,* 2019). Also, antifungal immunomodulator downstream of Toll improving our knowledge of *Drosophila* antimicrobial response (Hanson *et al*., 2021).

Infection of *Drosophila* strains with different species of fungi led to the death of a large proportion of flies. This affected the gene expression of both *Im1* and *Im2* genes, it turned out to be clear from Table (2) to Table (5).

PCR products were electrophoretic ally analysed confirm these results for *Im1* gene 187bp which determined in *Drosophila* flies by Leader (L) for *Drosophila* stains (*OR, Canton-S*, and *Alexandria*) which infected with Fungal *ssp*: *Penicillium* (P), *Trichoderma* (T), *Aspergillus* (A) in Fig. (17).

About PCR product gel electrophoresis for Im2 gene 90bp which located in Drosophila (Tanta, Kafr El-Sheikh, and Mansoura) strain with different sodium benzoate concentrations in Fig. (18). Based on experiments which Drosophila were infected with fungal ssp. and the Im1, Im2 genes were expressed to combat attacking fungi. Marko Rohlfs (2005) re-

ported that fungi competed Drosophila flies on resources and led to suppression of mould growth may adaptive behavioural response. In Drosophila investigating the relationship between the inter-kingdom competition and the behaviour in insects.

Pathological condition of Alzheimer diseases AD controlling bacteria in the oral cavity and the body (Matsushita *et al.*, 2020).

SUMMARY

Sodium benzoate (E211) used as a food additive was researched on *Drosophila melanogaster* (*Tanta, Kafr El-Sheikh, Mansoura, Alexandria, Canton-S,* and *OR* strains) and fungi strains from (*Aspergillus* species, *Trichoderma* species, *Penicillium spp*). Adult and larvae in third larvae stages were treated with medium of *Drosophila* which was mixed with different concentrations of sodium benzoate (SB) 0.007, 0.012, 0.037. 0.050. 0.075g. Mortality and sex ratio were affected with this treated so in fifth generation F5 in the number of flies and the sexual ratio that reached zero (0%) in the highest concentration of benzoate (0.075 g) in *Kafr El-Sheikh* flies and the mortality reached its highest rate in the highest concentration of sodium benzoate which was 98.03%. In the ninth and final generation (F9) of the *Tanta* flies. Behaviour experiments choice were curry out on third larvae after treated with different fungal species concentrations. Sodium benzoate (SB) concentrations from 0.007 g to 0.075 g recorded avoidance in different generations about more than -7 in *Tanta, Mansoura,* and *Alexandria* strains at 8 minutes. Rail time PCR (Rt. PCR) was used to determine gene expression of *Im1* and *Im2* genes, gene expression was zero for highest BS concentration for *Im1* gene in *Alexandria* flies in the sixth generation, while was 4.52 compared the control (1.0) for *Im2* gene. *Im1* and *Im2* genes (PCR product) were run in gel electrophoresis. Results led to genetics, behaviour and toxicity effects to SB on *Drosophila melanogaster* and the over load to fungi strains on the *Drosophila* behaviour through the effect of their genes. Thus, it affected flies mortality, sex ratio and behaviour, as well as the gene expression of its immune response *Im1*and *Im2* genes.

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Table (1): The primer was used to determined genes.

Gene name	Primer sequence 5 - ⟶3 -	Reference
Im1	F-TGTGGCCAATGGTGAGTAAA R –TTTTTCGAATCCTTGGGTTG	Pal, (2006)
Im2	F-TGGCCAACGCTGTTCCC R –CCTACTTTCCACCGTGCACAT	Suzawa *et al.*, *(*2019)

Table (2): RT-PCR for IM1 gene affected with sodium benzoate of middle and last generation on *Drosophila* strains.

D. strains	Generation	*Tanta*	*Kafr EL-Sheikh*	*Mansoura*	*Alex*	*canton s*	*OR*
0.007 g	middle	2.323576628	0.017398302	1.89181317	3.006417392	2.094824112	0.155374189
0.075 g		0.956336728	0.524673919	4.19896760	15.69313031	2.243684691	1.281744628
0.007 g	Last	0.649376282	0.250121189	1.2198549	0.065537208	9.782524247	2.715778697
0.075 g		2.609789863	0.341669569	2.46767067	0.055615733	1.202189604	0.351897834

Table (3): RT-PCR for IM2 gene affected with sodium benzoate of middle and last generation on *Drosophila* strains.

D. strains	Generation	Tanta	Kafr El-Sheikh	Manoura	Alex	Canton- S	OR
0.007 g	middle	1.4421854	1.014803	2.2808611	1.026922	3.2843106	0.035498
0.075 g		2.0157366	0.308685	1.765406	0.773355	7.0141152	0.1738421
0.007 g	Last	1.549387	0.488369	1.5107136	3.194129	2.0228763	0.7581166
0.075 g		1.7003876	0.130516	1.6908913	4.520974	6.7602381	0.3725139

Table (4): RT-PCR for *Im1* gene affected with fungal species on *Drosophila* strains.

Fungi Strains / ***D*. strains**	***Penicilli-um spp***	***Trichoder-ma creme-um***	***T.viride***	***T.citrinovir ide***	***Aspergil-lus flavus***	***A.niger***	***A.terreus***
Tanta	2.5224760	2.6551941	2.0641988	0.4469937	1.8381355	0.9428623	2.4923231
Kafr EL-Sheikh	0.1321307	0.1218611	0.2495725	0.1196591	0.5289880	179.61769	0.2900998
Mansoura	0.7868657	2.9785481	0.8017627	2.7251718	2.6126504	1.0604094	3.0948537
Alex.	1.8435548	0.0825481	1.5550947	0.5679740	1.2301358	754.81543	0.0018094
Canton S	2.412298	2.0668264	0.7465376	1.2481404	0.5298889	300.7808	3.5126718
OR.	0.358164	0.2266982	0.2513814	0.2154741	0.7634411	146.97095	0.1804719

Table (5): RT-PCR for *Im2* gene affected with fungal species on *Drosophila* strains.

Fungi Strains / ***D*. strains**	***Penicillium spp***	***Trichoderma cremeum***	***T.viride***	***T.citrinoviride***	***Aspergillus flavus***	***A.niger***	***A.terreus***
Tanta	1.5555444	1.6349001	2.021730	0.444933	1.9651255	1.047759	2.598037
Kafr EL-Sheikh	0.266161	0.151730	1.111547	0.354497	0.528988	179.6176	0.290100
Mansoura	0.290100	1.5823242	1.113428	1.9181273	1.8072784	1.060409	3.094853
Alex.	22.769389	1.829020	0.773093	1.053535	0.722500	694.3785	0.818635
Canton S	0.0248605	7.0128458	4.963395	0.7115672	0.2404112	151.1706	0.624165
OR.	0.2226971	0.399363	0.223904	0.1582196	0.1220537	76.81911	0.559855

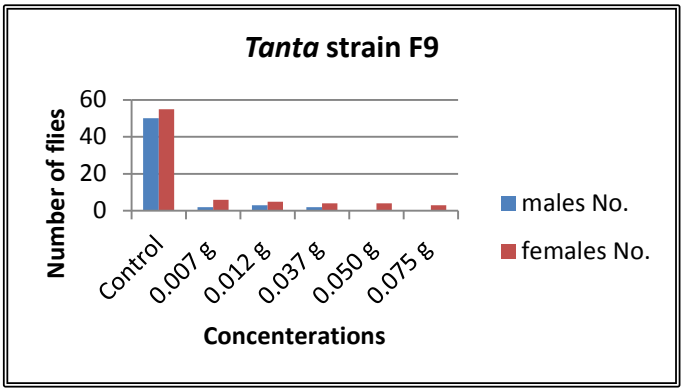

Fig. (1) The progeny number of *Drosophila melanogaster* in the last generation (F9) with sodium benzoate concentrations males, females were counted in *Tanta* strain.

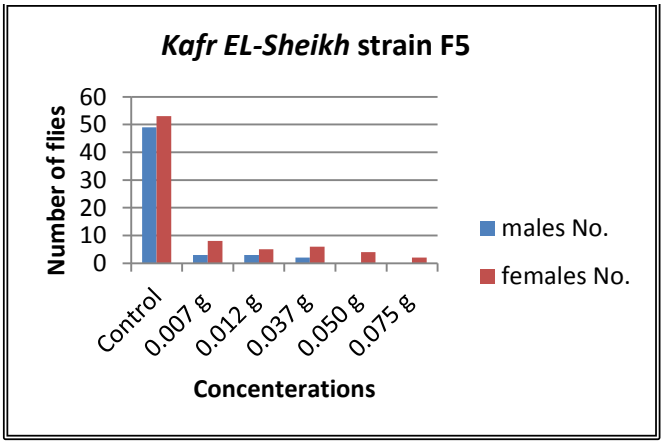

Fig. (2) The progeny number of *Drosophila melanogaster* in the last generation (F5) with sodium benzoate concentrations males, females were counted in Kafr El-Sheikh strain.

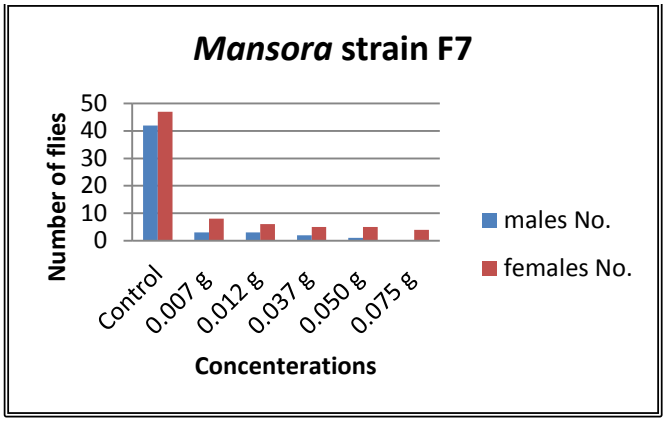

Fig. (3) The progeny number of *Drosophila melanogaster* in the last generation (F7) with sodium benzoate concentrations males, females were counted in Mansoura strain

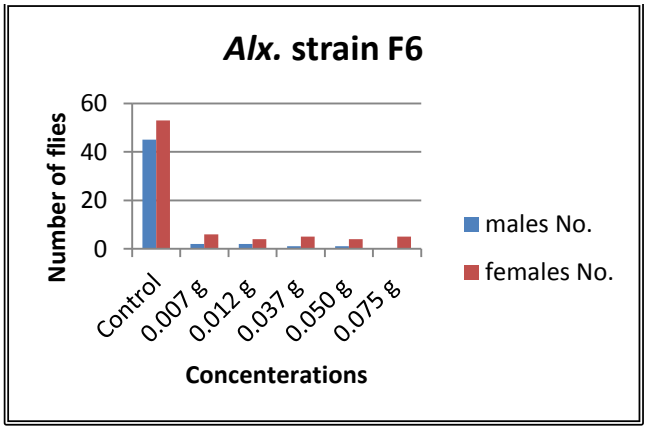

Fig. (4) The progeny number of *Drosophila melanogaster* in the last generation (F6) with sodium benzoate concentrations males, females were counted in Alexandria strain.

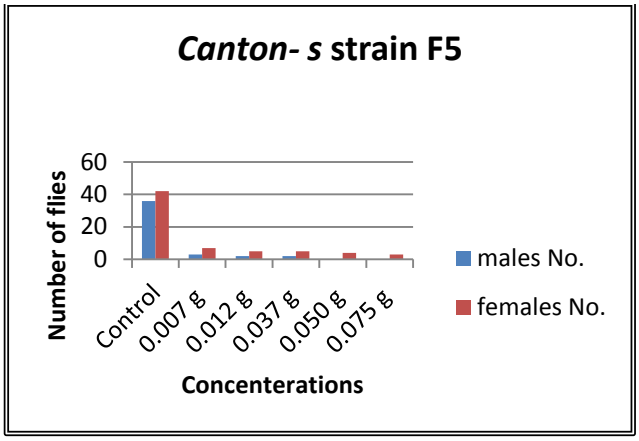

Fig. (5) The progeny number of *Drosophila melanogaster* in the last generation (F5) with sodium benzoate concentrations males, females were counted in Canton-S strain

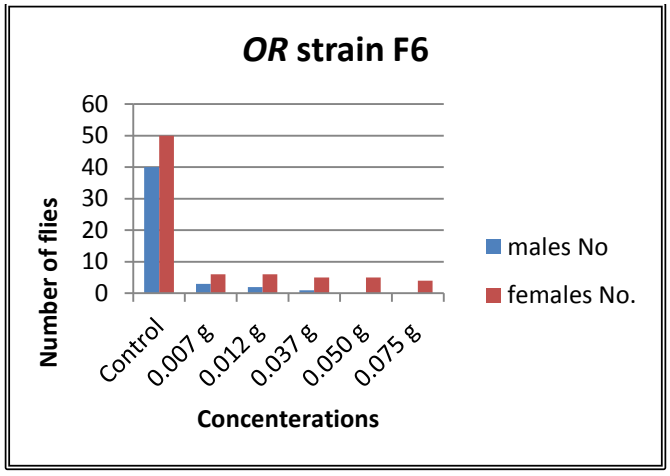

Fig. (6) The progeny number of *Drosophila melanogaster* in the last generation (F6) with sodium benzoate concentrations males, females were counted in OR strain

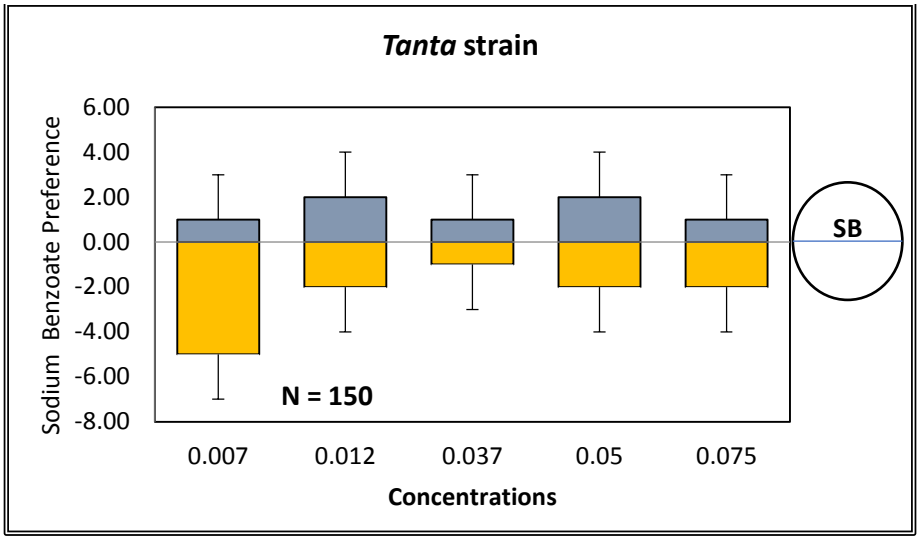

Fig. (7A) Histogram of the average of preference for sodium benzoate concentration on *Tanta* strain larvae in the F5 after 8 minutes.

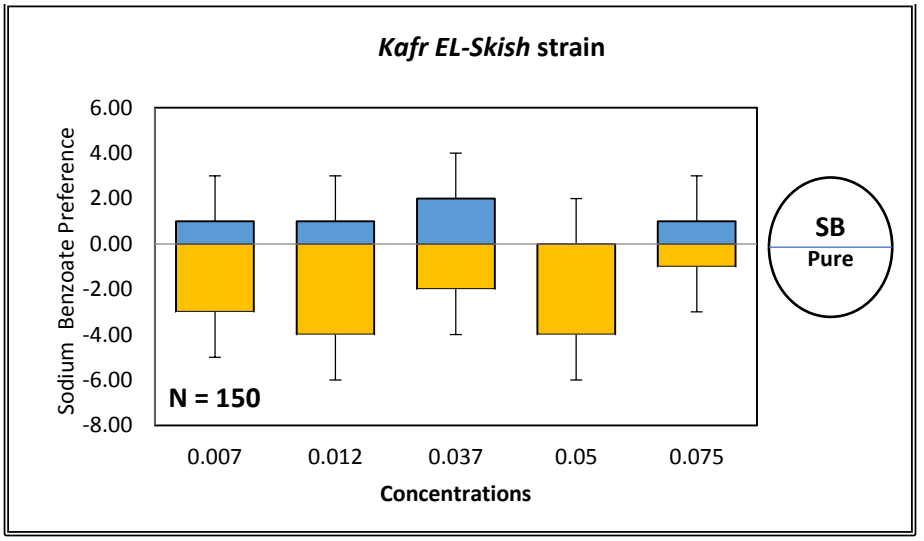

Fig. (8A) Histogram of the average of preference for sodium benzoate concentration on *Kafr El-Sheisk* strain larvae in the F5 after 8 minutes.

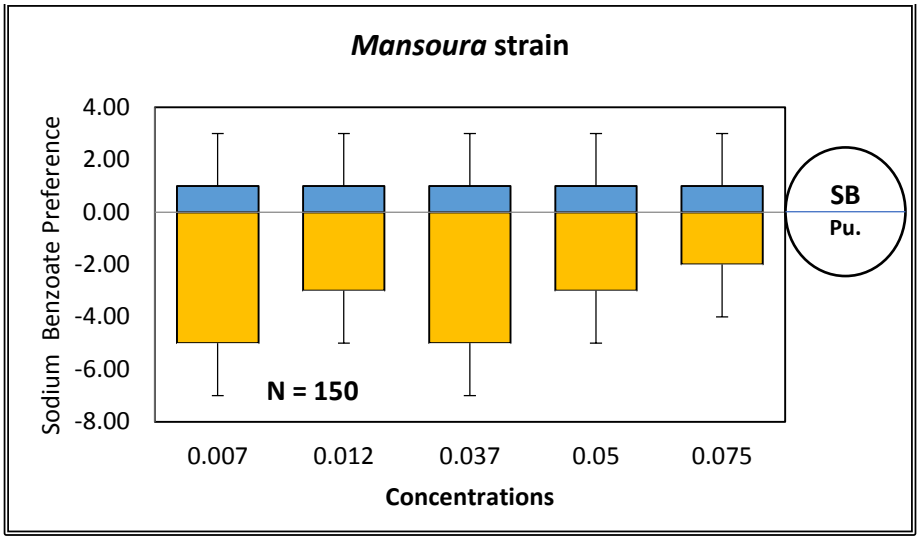

Fig. (9A) Histogram of the average of preference for sodium benzoate concentration on *Mansoura* strain larvae in the F5 after 8 minutes.

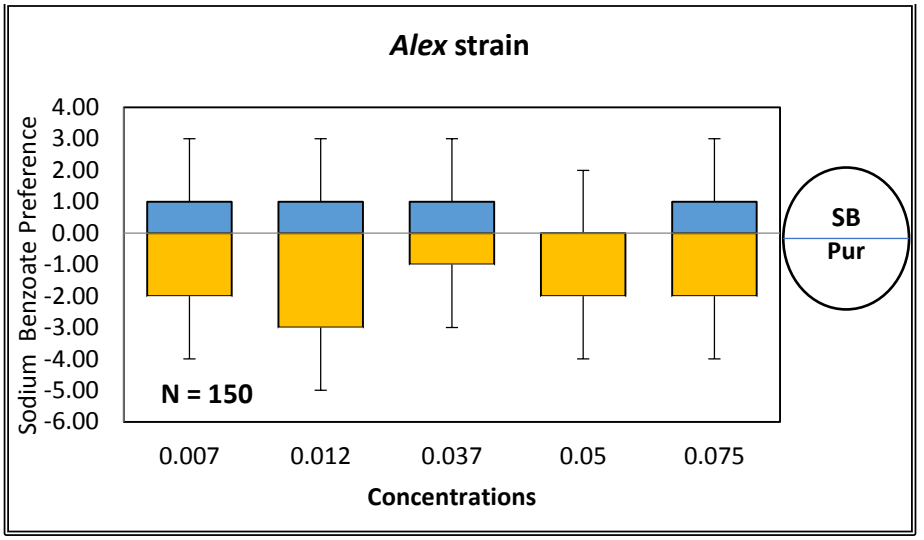

Fig. (10A) Histogram of the average of preference for sodium benzoate concentration on *Alexandria* strain larvae in the F5 after 8 minutes.

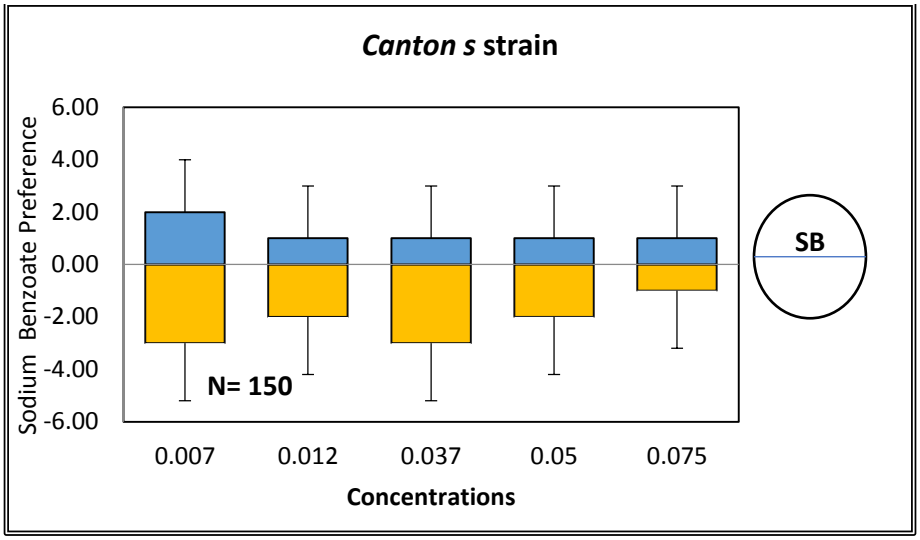

Fig. (11A) Histogram of the average of preference for sodium benzoate concentration on *Canton-S* strain larvae in the F5 after 8 minutes.

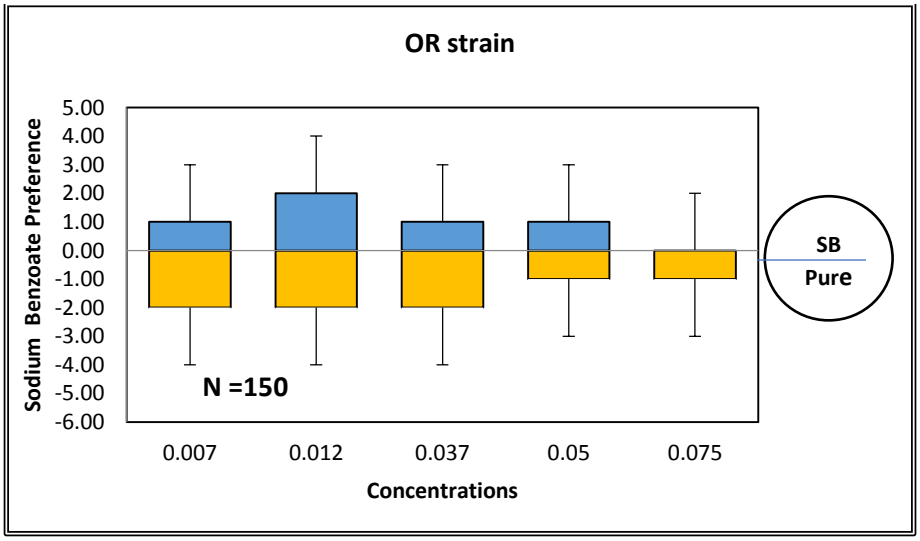

Fig. (12A) Histogram of the average of preference for sodium benzoate concentration on *OR* strain larvae in the F5 after 8 minutes.

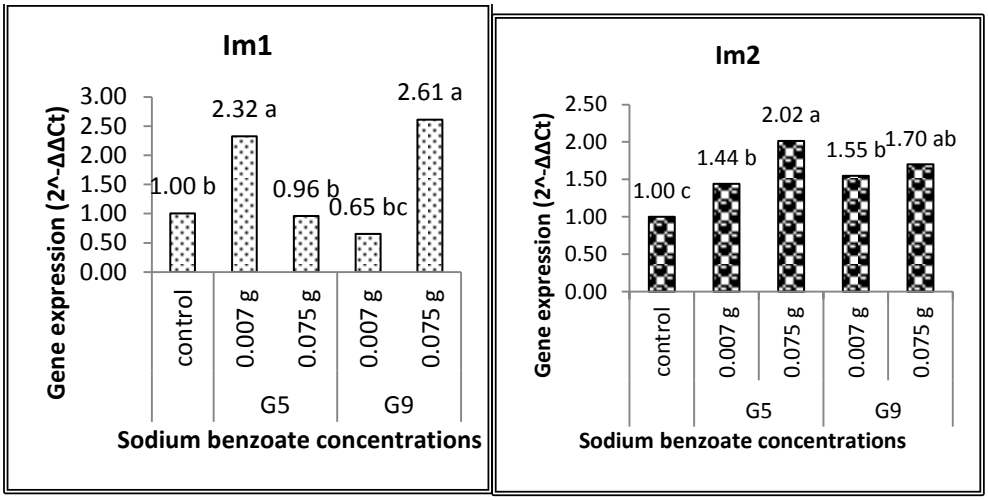

Fig. (11) Gene expression for both gene I*m1* and *Im2* result of the effect of sodium benzoate concentrations in *Tanta* strain.

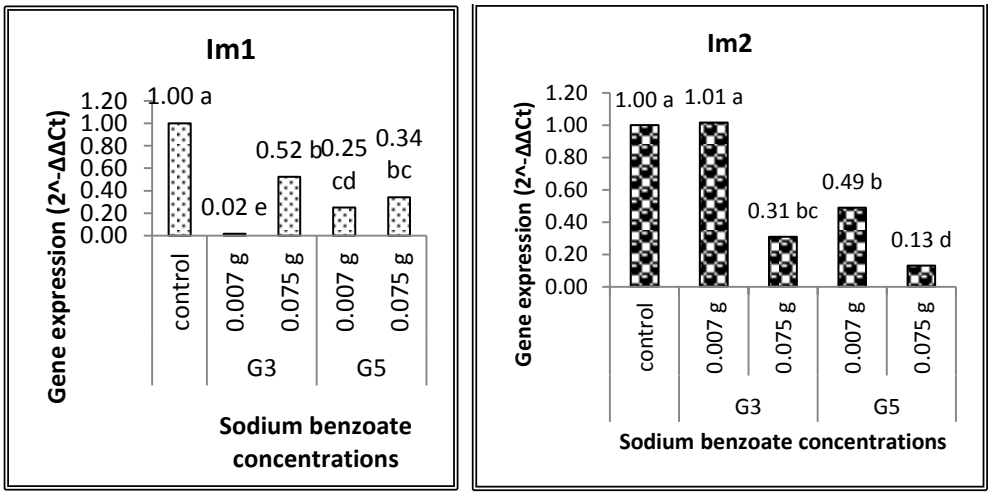

Fig. (12) Gene expression for both gene *Im1* and *Im2* result of the effect of sodium benzoate concentrations in *Kafr El-Sheikh* strain.

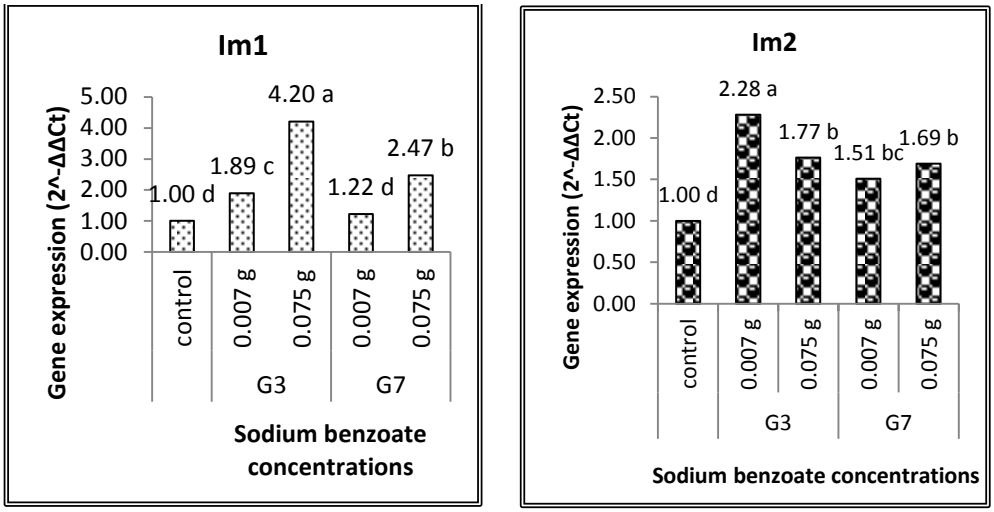

Fig. (13) Gene expression for both gene *Im1* and *Im2* result of the effect of sodium benzoate concentrations in *Mansoura* strain.

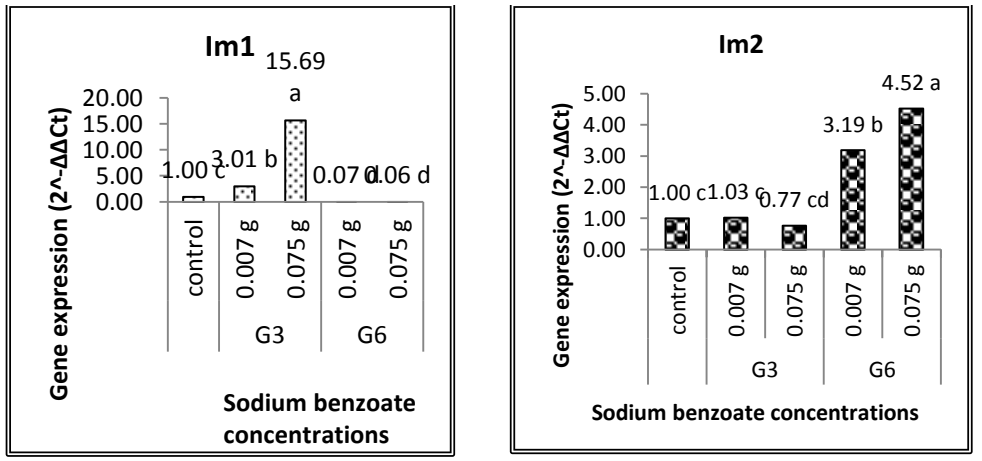

Fig. (14) Gene expression for both gene *Im1* and *Im2* result of the effect of sodium benzoate concentrations in *Alexandria* strain.

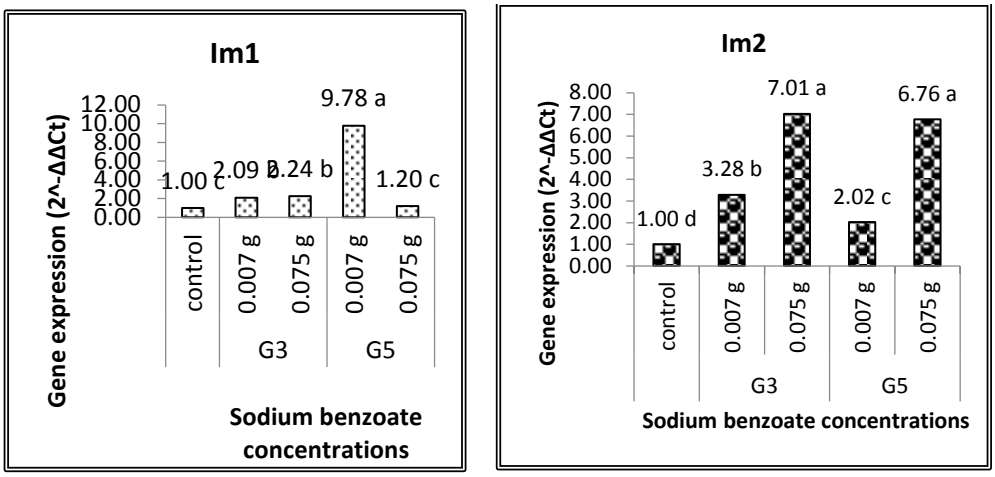

Fig. (15) Gene expression for both gene *Im1* and *Im2* result of the effect of sodium benzoate concentrations in *Canton-s* strain

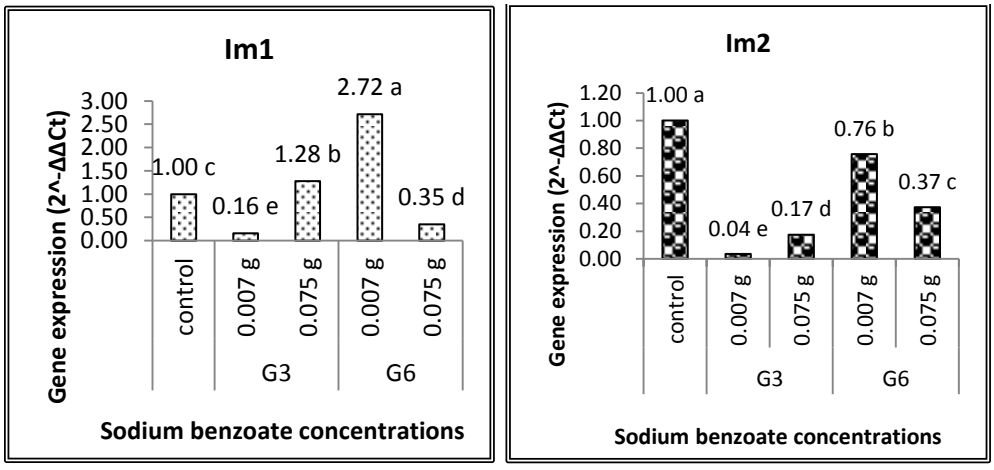

Fig. (16) Gene expression for both gene *Im1* and *Im2* result of the effect of sodium benzoate concentrations in *OR* strain.

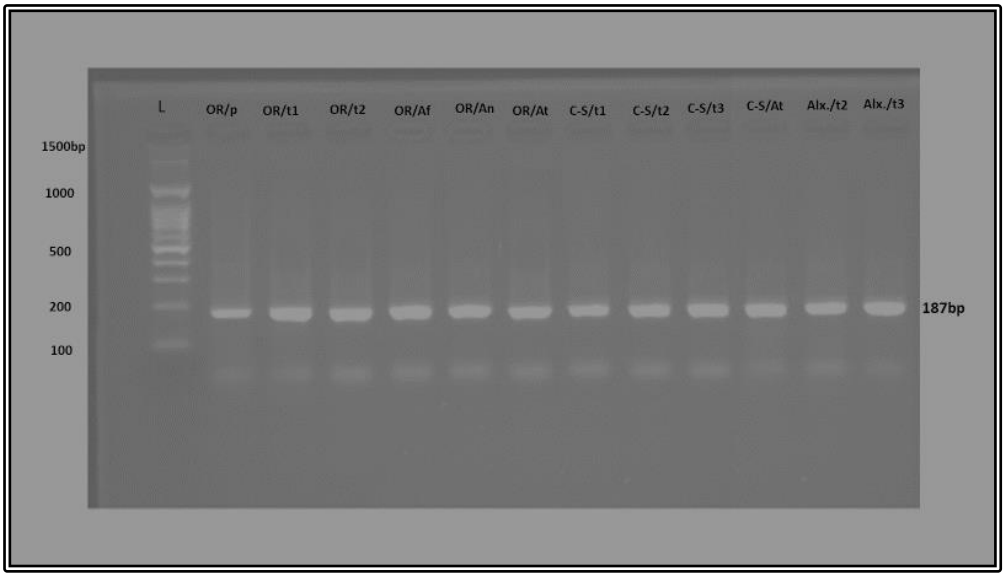

Fig. (17) PCR product gel electrophoresis for *Im1* gene at the last generation of each strain for the effect of sodium benzoate and fungal strains on different *Drosophila* strains

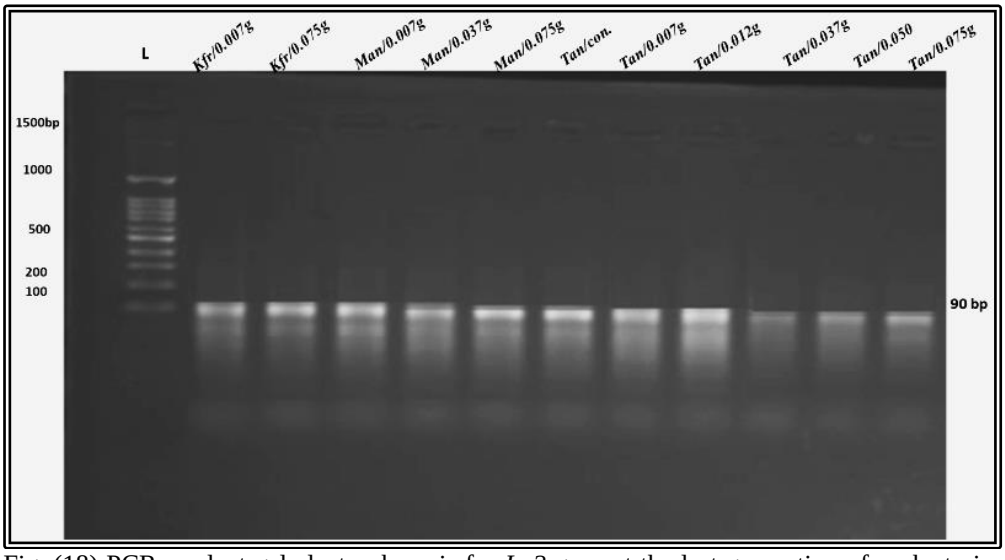

Fig. (18) PCR product gel electrophoresis for *Im2* gene at the last generation of each strain for the effect of sodium benzoate concentrations on different *Drosophila* strains