# Protective Effect of Apigenin against Ethinylestradiol induced Toxic Effects in the Third Instar Larvae of Transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$

Yasir Hasan Siddique[1], Gulshan Ara[2], Smita Jyoti[1], Falaq Naz[1], Mohammad Afzal[2]

[1]Drosophila Transgenic Laboratory, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh-202002 (U.P.).
[2]Human Genetics and Toxicology Lab, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh-202002 (U.P.)

## Summary

All living organisms under stressful condition respond by synthesizing heat shock proteins (HSPs). In recent years, *hsp70* has been considered to be one of the candidate genes for predicting the cytotoxicity against environmental chemicals. The effect of apigenin was studied against the toxic effects induced by ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$ using *hsp70* expression and dye exclusion test as a parameter. The effect of 0.1, 0.50 and 1.0 µl/ml of apigenin was studied against the toxic effects induced by 0.50 and 1.0 µl/ml of ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$ using *hsp70* expression and dye exclusion test as a parameter. The selected doses of apigenin for the estimation of *hsp70* expression in the third instar larvae (i.e. 0.1, 0.5 and 1.0 µl/ml) were not toxic though reduced significantly, the expression of *hsp70* and tissue damage induced by 0.5 and 1.0 µl/ml of ethinylestradiol. The results demonstrates the protective role of apigenin against the toxicity of ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$ and also promotes the use of alternative to higher laboratory animals such as mice/rats for the initial screening of the chemical agents for their possible toxic or protective effects.

**Keywords:** Apigenin; *hsp70; Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$

## Introduction

Heat shock proteins are the important part of the cell for protein folding. It also protects cells from the stress [1]. The genes coding the heat shock proteins are highly conserved and many of them are assigned to families on the basis of sequence homology and molecular weight [2]. Now-a-days, *hsp70* is used as a candidate gene for predicting the cytotoxicity against environmental chemicals [3, 4, 5, 6]. Estrogens are used to cure of many types of sexual disorder as well as in oral contraceptive formulations [7]. There are sufficient evidences of the estrogens carcinogenicity and genotoxicity in various experimental models [8].

Apigenin is one of the several active ingredients found naturally in many fruits and vegetables. It is found in several popular spices, including basil, oregano, and parsley [9]. Apigenin is recognized in traditional or alternative medicine for its pharmacological activity [10]. In the recent years, the use of animals in toxicological/ pharmacological research and testing has become an important issue for both science and ethics. For this the emphasis has been given to the use of alternative to mammals in testing, research and education [4].

The European Centre for the Validation of Alternative Methods (EVCAM) has recommended the use of *Drosophila* as an alternative model for scientific studies [11, 12]. In the present study an attempt has been made to validate this model for the evaluation of the chemotherapeutic/natural plant product for their protective action. The protective effect of apigenin was studied against the toxicity induced by ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$.

## Materials and methods

### Fly strain

A transgenic *Drosophila melanogaster* line that expresses bacterial β-galactosidase as a response to stress was used in the present study. The flies and larvae were allowed to grow on the diet containing agar, cornmeal, sugar, and yeast at 24$^{o}$C [13].

### Experimental Design

Ethinylestradiol at 0.5 and 1.0 µl/ml of food concentration alone and along with 0.1, 0.5 and 1.0 µl/ml of apigenin (dissolved in DMSO) were established. The third instar larvae were allowed to feed on them for different time intervals i.e. 12, 24 and 48 hrs.

### Soluble O-nitrophenyl-β-D-galactopyranoside (ONPG) assay

The method followed was as described by Nazir et al. [13]. The larvae were washed in phosphate buffer and then were taken in a micro centrifuge tube (20 larvae/tube; 5 replicates / group). For permeabilization the larvae were kept for 10 min in acetone. After removing the acetone the larvae were incubated overnight at 37$^{o}$C in 600 µl of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 µl $Na_2CO_3$. The extent of reaction was quantified by measuring the absorbance at 420 nm.

### Trypan blue exclusion test

The tissue damage in the larvae exposed to different concentrations of ethinylestradiol separately and in combination with apigenin was assayed by the dye exclusion test [14]. The internal tissues of larvae were explanted in a drop of phosphate buffer (PB), rotated in trypan blue stain for 30 min, washed thoroughly in PB and scored immediately for dark blue staining. Total 50 larvae per treatment (10 larvae per dose; 5 replicates per group) were scored for the trypan blue staining on an average composite index per larvae; no color, O; any blue 1; darkly stained nuclei, 2; large patches of darkly stained cell, 3; or complete staining of most cells in the tissue, 4 [14].

### Statistical analysis

Statistical analysis was carried out by one way analysis of variance (ANOVA) using commercial Software Statistica Soft Inc (2007).

## Results

The exposure of third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$ to 0.50 µl/ml of ethinylestradiol for 12, 24 and 48 hrs showed a exposure dependent increase

in the mean absorbance values and were 0.2821±0.0133, 0.2985±0.0144 and 0.3049±0.146, respectively (Table 1). Similarly, the exposure of larvae to 1.0 µl/ml of ethinylestradiol for 12, 24 and 48 hrs showed a exposure dependent increase in the mean absorbance values and were 0.3132±0.0148, 0.3264±0.0153 and 0.3289±0.0159, respectively (Table 1). The exposure of larvae to 0.50 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin for 12 hrs showed a dose dependent reduction in the mean absorbance values i.e. 0.2461±0.0068, 0.2318±0.0053 and 0.2302±0.0050, respectively (Table 1). Similarly, the exposure of larvae to 1.0 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin showed a dose dependent reduction in the mean absorbance values i.e. 0.2743±0.0121, 0.2614±0.0113 and 0.2516±0.0093, respectively (Table 1). The exposure of larvae to 0.50 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin for 24 hrs showed a dose dependent reduction in the mean absorbance values i.e. 0.2618±0.0134, 0.2576±0.0098 and 0.2413±0.0042, respectively (Table 1). Similarly, the exposure of larvae to 1.0 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin for 24 hrs was associated with the dose dependent reduction in the mean absorbance values i.e. 0.2810±0.0132, 0.2762±0.0125 and 0.2631±0.0117, respectively (Table 1). The exposure of larvae to 0.5 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin for 48 hrs was associated with the dose dependent decrease in the mean absorbance values i.e. 0.2772±0.0128, 0.2613±0.0130 and 0.2582±0.0183, respectively (Table 1). Similarly, 1.0 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin for 48 hrs showed a dose dependent reduction in the mean absorbance values i.e. 0.2913±0.0142, 0.2844±0.133 and 0.2757±0.0122, respectively (Table 1).

The regression analysis was also performed to study the dose effect of apigenin on the third instar larvae exposed to 0.5 and 1.0 µl/ml of ethinylestradiol. The exposure of larvae for 12 hrs to 0.5 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin was associated with the β-coefficient of -0.88 (F=3.412). For 24 and 48 hrs of exposures the β-coefficient values were -0.97 (F=13.645) and -0.91 (F=4.630), respectively (Table 2). The exposure of larvae to 1.0 µl/ml of ethinylestradiol along with 0.1, 0.5 and 1.0 µl/ml of apigenin for 12 hrs was associated with β-coefficient of -0.99 (F=48.411). The exposure of larvae for 24 and 48 hrs was associated with the β-coefficient of -0.98 (F=24.969) and -1.0 (F=165.675), respectively (Table 2).

The tissue damage induced by 0.5 and 1.0 µl/ml of ethinylestradiol alone and in combination with 0.1, 0.5 and 1.0 µl/ml of apigenin for the exposure of larvae to 48 hrs was evaluated by trypan blue staining. About 95% of the untreated larvae were negative to trypan blue staining. About 90% of the larvae exposed to 0.5 and 1.0 µl/ml of ethinylestradiol showed damage in brain ganglia, midgut, salivary glands, malpighian tubules and the hind gut. The exposure of larvae to ethinylestradiol, along with various doses of apigenin results in the reduction of the tissue damage. About 40% of larvae show light staining in the midgut and brain ganglia and no damage was observed in the midgut, malpighian tubule, and midgut.

Table1: β-galactosidase activity measured in transgenic *Drosophila melanogaster* (*hsp70-lacZ)Bg*$^{9}$ third instar larvae exposed to different concentrations of ethinylestradiol and apigenin for various durations

| **Treatments** | **O.D. (Mean±SE) after 12 hrs** | **O.D. (Mean±SE) after 24 hrs** | **O.D. (Mean±SE) after 48 hrs** |
|---|---|---|---|
| EE (µl/ml) | | | |
| 0.50 | 0.2821 ± 0.0133[a] | 0.2985 ± 0.0144[a] | 0.3049 ± 0.0146[a] |
| 1.0 | 0.3132 ± 0.0148[a] | 0.3264 ± 0.0153[a] | 0.3289 ± 0.0159[a] |
| EE (µl/ml) + Apigenin (µl/ml) | | | |
| 0.50 + 0.1 | 0.2461 ± 0.0068[ab] | 0.2618 ± 0.0134[ab] | 0.2772 ± 0.0128[ab] |
| 0.50 + 0.5 | 0.2318 ± 0.0053[ab] | 0.2576 ± 0.0098[ab] | 0.2613 ± 0.0130[ab] |
| 0.50 + 1.0 | 0.2302 ± 0.0050[ab] | 0.2413 ± 0.0042[ab] | 0.2582 ± 0.0103[ab] |
| 1 + 0.1 | 0.2743 ± 0.0121[ab] | 0.2810 ± 0.0132[ab] | 0.2913 ± 0.0142[ab] |
| 1 + 0.5 | 0.2614 ± 0.0113[ab] | 0.2762 ± 0.0125[ab] | 0.2844 ± 0.0137[ab] |
| 1 + 1 | 0.2516 ± 0.0093[ab] | 0.2631 ± 0.0117[ab] | 0.2757 ± 0.0122[ab] |
| Apigenin (µl/ml) | | | |
| 0.1 | 0.2136 ± 0.0015 | 0.2148 ± 0.0015 | 0.2176 ± 0.0028 |
| 0.5 | 0.2147 ± 0.0016 | 0.2109 ± 0.0009 | 0.2219 ± 0.0034 |
| 1.0 | 0.2166 ± 0.0019 | 0.2214 ± 0.0030 | 0.2233 ± 0.0039 |
| Untreated | 0.2013 ± 0.0011 | 0.2057 ± 0.0017 | 0.2136 ± 0.0021 |
| DMSO (1µl/ml) | 0.2034 ± 0.0018 | 0.2083 ± 0.0019 | 0.2156 ± 0.0024 |

EE: Ethinylestradiol; DMSO: Dimethylsulfoxide; OD: Optical density; SE: Standard error
[a]P<0.05 as compared to untreated.
[b]P<0.05 as compared to EE treatment

Table-2: Regression analysis for the dose effect of apigenin along with 0.5 and 1.0 µl/ml of ethinylestradiol for various duration of exposure.

| S. No. | Treatments EE (µl/ml) | Duration (hr) | Regression Equation | β-coefficient | SE | P-value | F-value |
|---|---|---|---|---|---|---|---|
| 1. | 0.50 | 12 | Y = 0.24514 – 0.0171 X | - 0.88 | 0.0059 | < 0.0156 | 3.412 |
| 2. | 0.50 | 24 | Y = 0.26593 – 0.0232 X | - 0.97 | 0.0040 | < 0.0097 | 13.645 |
| 3. | 0.50 | 48 | Y = 0.27650 – 0.0205 X | - 0.91 | 0.0060 | < 0.0142 | 4.630 |
| 4. | 1.0 | 12 | Y = 0.27576 – 0.0250 X | - 0.99 | 0.0023 | < 0.0054 | 48.411 |
| 5. | 1.0 | 24 | Y = 0.28418 – 0.0201 X | - 0.98 | 0.0026 | < 0.0059 | 24.969 |
| 6. | 1.0 | 48 | Y = 0.29305 – 0.0173 X | - 1.0 | 0.0000 | < 0.0001 | 165.675 |

## Discussion

The results of the present study reveals that the apigenin reduced the toxic effects of ethinylestradiol in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*$^{9}$. In our earlier study the effect of ethinylestradiol was studied at 0.25, 0.50, 1.0 and 2.0 µl/ml and was found to express β-galactosidase significantly at 0.50, 1.0 and 2.0 µl/ml [5]. Our earlier study with ethinylestradiol in cultured human lymphocytes showed that the metabolic activation and possible conversion of ethinylestradiol to reactive species is responsible for the genotoxicity [15]. Flavonoids, a complex group of aromatic compounds have been found to be protective against the toxicity of various compounds [16]. The transgenic *D. melanogaster* has been used as an alternative animal model for toxicological research [17]. Medicinal plants and their products have been used for centuries to cure various ailments [18]. *Drosophila* as a model in pharmaceutical research is easy to handle, culture, time efficient and cost effective in comparison to rodents [19]. The high microsomal oxidase activity has been reported in the midgut tissues of insects [20]. In our present study the damage has been first observed in the mid gut tissues, probably the metabolic activation of ethinylestradiol takes place in the mid gut. The supplementation of apigenin reduces the expression of *hsp70* as well as the tissue damage. It may be due to the scavenging of reactive oxygen species by the apigenin that causes stress in the tissue as a result the *hsp70* expression increases. A dose dependent decrease in the β-galactosidase expression clearly demonstrates the protective effect of apigenin. The studies related to the protective role of natural plant products using *Drosophila melanogaster* are limited. Although the modulatory effects of some plant extract have been studied using as a model [21, 22]. The results of the present study support the use of *D. melanogaster* as it is also capable of activating promutagens and procarcinogens [23,24].

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