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Original Article

Larvicidal activity and *in vitro* effects of green tea (*Camellia sinensis L.*) water infusion

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***Abstract*:**
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In this study green tea water infusion was tested on *Drosophila melanogaster* wild-type larvae *in vivo*, also an *in vitro* antihemolytic and hemolytic tests were performed. Three different concentrations were used 7.5 mg/ml, 37.5 mg/ml and 75 mg/ml, the lowest dose representing the recommended dose followed by five times and ten times higher doses. Effect of these three concentrations was monitored and tested *in vivo* on *Drosophila melanogaster* (Meigen, 1830) wt (wild type) larval development and surviving. All three concentrations showed toxic effect for larvae, with toxicity being increased in dose – depended manner. The time needed for larvae to fully develop was delayed. This decrease of developmental time was in dose – dependent manner, too. Amount of hemolysis caused by the lowest concentration was very small when compared with the percent of spontaneous hemolysis. Other two higher concentrations, 37.5 mg/ml and 75 mg/ml, showed higher hemolytic effect. During the four hour incubation period percent of hemolysis grew in time – dependent manner. The highest hemolytic effect was recorded for the concentration of 37.5 mg/ml. Antihemolytic test showed that the lowest concentration had the highest inhibitory effect to H_2O_2 induced hemolysis. The 37.5 mg/ml and 75 mg/ml concentrations had lower inhibitory effect when compared with the dose of 7.5 mg/ml. According to our study it can be concluded that the high concentrations of green tea water infusion exhibit larvicidal activity against *D. melanogaster* larvae, don't have protective effect to RBC membrane and cause greater hemolysis.

Key words: green tea; *Drosophila melanogaster* wt; larvicidal activity; RBC; hemolytic test; antihemolytic test.

Introduction

One of the most consumed beverages in the world is green tea. Caffeine is one of the active compounds present in tea, and its content is around 3 to 4 % (Willson et al., 1992). Caffeine may play an important role against some pathogens (Van Breda et al., 2012). Green tea is also a source of polyphenol antioxidants such as epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate (Zhang et al., 1997, Hodgson, 2008). Catechins have similar backbone but differ in number and location of hydroxyl groups. Catechins are water – soluble, colorless compounds that make up to 30% of the dry leaf weight (Graham, 1992). These natural antioxidants have been reported to have the ability of preventing cancer (Li et al., 2008), are effective scavengers of free radicals (Nanjo et al., 1996), inhibit HBV *in vitro* (Xu et al., 2008). It has been

reported that one cup of tea can provide 150 – 200 mg of flavonoids. Also, lower risk of heart disease and stroke are related to the flavonoids intake (Hodgson, 2008). Isolated flavonoids at high physiological concentrations can reduce platelet aggregation and markers of platelet activation (Rein et al., 2000). Green tea extract can delay and inhibit oxidation of low-density lipoprotein LDL catalyzed by copper *in vitro* (Yokozawa et al., 1997). Some authors report that drinking green tea may result in lower level of total triglyceride and cholesterol in serum together with deceased atherogenic index (Imai et al., 1995).

In view of variety of reports considering green tea, we have conducted a study to evaluate the effects of high concentrations of green tea water infusion. *Drosophila melanogaster* is frequently used in larvacidal and insecticidal studies (Miyazawa et al., 1998; Miyazawa et al., 2000). Our aim was to see whether these high doses have beneficial effects on RBC membrane and on *Drosophila melanogaster* wt larvae development. To evaluate green tea infusion effect RBC membrane the hemolytic test with Drabkin's reagent was used. Also, to see whether high concentrations of green tea water infusion could inhibit lipid peroxidation caused by H_2O_2, the antihemolytic method was employed.

Materials and methods

Test organisms

In this experiment, the *Drosophila melanogaster* reference type 5 wt, originated from „Bloomington Drosophila Stock Centre", Indiana University, USA was used. Fruit flies were cultivated on a standard Drosophila medium (9% sugar, 10% corn meal, 2% agar, 2% yeast, with addition of fungicide Nipagin®), in a standard cultivating 200 ml flasks on 25 °C, 60% humidity, with 12h/12h day/night cycle. Adults were allowed to oviposit eggs during the eight hour period, after that they were removed from the flasks. Then, after three days larvae which were 72±4h old, have been removed from the cultivating medium, washed with distilled water, and placed on the new medium containing green tea infusion. Concentrations of green tea in medium were 7.5 mg/ml, 37.5 mg/ml and 75 mg/ml. Experiment was done in triplicate. There were 15 larvae from the same hatch in each replicant flask, and there were 12 replicant flasks including the water control (negative control). Replicants were incubated in standard way, already described. Alive specimens were collected 10-16 days after the egg laying.

RBC preparation

A modified method from Noudeh et al. (2009) was used. Briefly, blood was collected in heparinized tubes from male Wistar rats via cardiopuncture. Whole blood was centrifuged at 2000 rpm for 10 minutes at 4 ºC. Plasma and the buffy coat were removed and an equal volume of PBS (pH 7.4) was added. This was repeated three times, to obtain washed erythrocytes. At the end packed erythrocytes were diluted with PBS to obtain 4% suspension. RBC prepared in this way were used both for hemolytic and antihemolytic test.

Solutions of green tea infusion and PBS (0.9%, pH=7.4) were made to give the final concentrations of 7.5mg/ml, 37.5mg/ml and 75mg/ml.

Hemolytic test

The following method was employed: 200 µl of green tea water infusions (concentrations 7.5 mg/ml, 37.5 mg/ml, 75 mg/ml) were incubated with 200 µl of RBC suspension at room temperature for one, two, three and four hours. After each hour of incubation samples were centrifuged at 1500 rpm for 10 minutes at 4 ºC. Then, 200 µl of resulting supernatant was added to 3 ml of Drabkin's reagent and the absorbance was measured at 540 nm on spectrophotometer (Shimadzu UV-VIS 1650, Tokyo, Japan). Positive control consisted of 200 µl of distilled water and 200 µl RBC suspension and negative control were 200 µl of PBS and 200 µl RBC suspension. Everything was done triplicate. Percentage of hemolysis was calculated by the equation:

% Hemolysis = $[(Ab_{(sample)} - Ab_{(negative\ control)}) / Ab_{(positive\ control)}] * 100\%$ (Gould et al., 2000).

Antihemolytic test

Antihemolytic test was done by modified method previously described (Ebrahimzadeh et al, 2010.) 2 ml of RBC suspension was pre-incubated with 0.5 ml of green tea water infusion. The final volume was made to be 5 ml with PBS, and the final concentrations of green tea infusions were 7.5 mg/ml, 37.5 mg/ml and 75 mg/ml. This mixture was incubated for 15 minutes at 37 ºC. Then 0.5 ml of H_2O_2 dissolved in PBS was added to the mixture. Concentration of H_2O_2 was made to give 90% of hemolysis after 4 hours. At the end of the incubation period of 4 hours, different concentration samples were spun at 1500 rpm for 10 min at 4 ºC. Then 200 µl of supernatant was added to 3 ml of Drabkin's reagent and the absorbance was measured at 540 nm. Positive control consisted only of PBS and

H_2O_2 without green tea infusion. Experiments were done in triplicate. Percentage of inhibition was calculated by equation: %Inhibition = [(Ab $_{(H2O2)}$ - Ab$_{(sample)}$) / Ab $_{(H2O2)}$] *100% .

Results and Discussion

The absolute mortality was monitored - number of hatched adult specimens that were scored and compared to total transferred larval specimens. It was observed that the mortality increases with the increase in green tea concentration in the medium (Fig. 1).

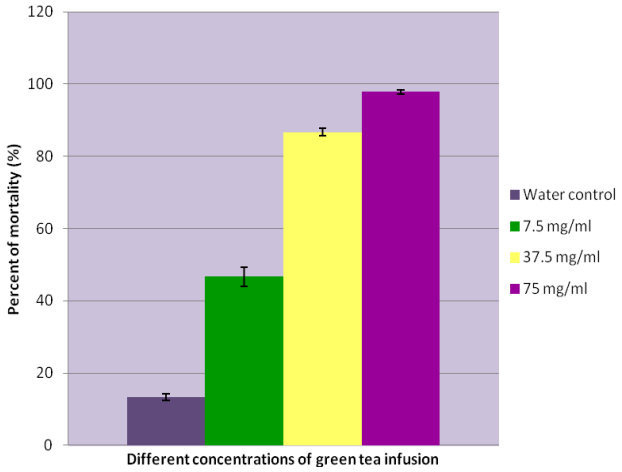

Fig. 1. Absolute mortality of *D. melanogaster* larvae in presence of green tea infusion

Development was also observed and number of pupae that were scored from ninth to sixteenth day of larval life (data not shown). In comparison with water control, developmental time from larvae to adults prolongs if the concentration of green tea infusion is higher. One of the active compounds of green tea is caffeine. According to the literature, content of the caffeine is around 3 to 4% (Willson et al., 1992). It is supposed that caffeine, localized in the vascular bundles of young *Camellia sinensis* L. leaves plays a crucial role in the defense against various pathogens (Van Breda et al., 2012.). Itoyama and Bicudo (1992 and 1997) showed harmful effects of caffeine on *Drosophila prosaltans* D., related to fecundity, egg lying capacity and longevity which is consistent with our results. On the other hand, some studies show that *Camellia sinensis* L., inhibits the accumulation of iron and thus extends the life span of *Drosophila melanogaster* (Massie et al., 1993). Also, these results are consistent with previous studies of other authors who showed that polyphenols as antioxidants affect larval development in Drosophila causing its time delay (Rice-Evans et al., 1996; Aruoma, 2003; Kim et al., 2004).

Results for both antihemolytic and hemolytic test are accordant. In both experiments the lowest doses of green tea infusion exibited slightly beneficial effect on RBC membrane (Fig. 2 and 3).

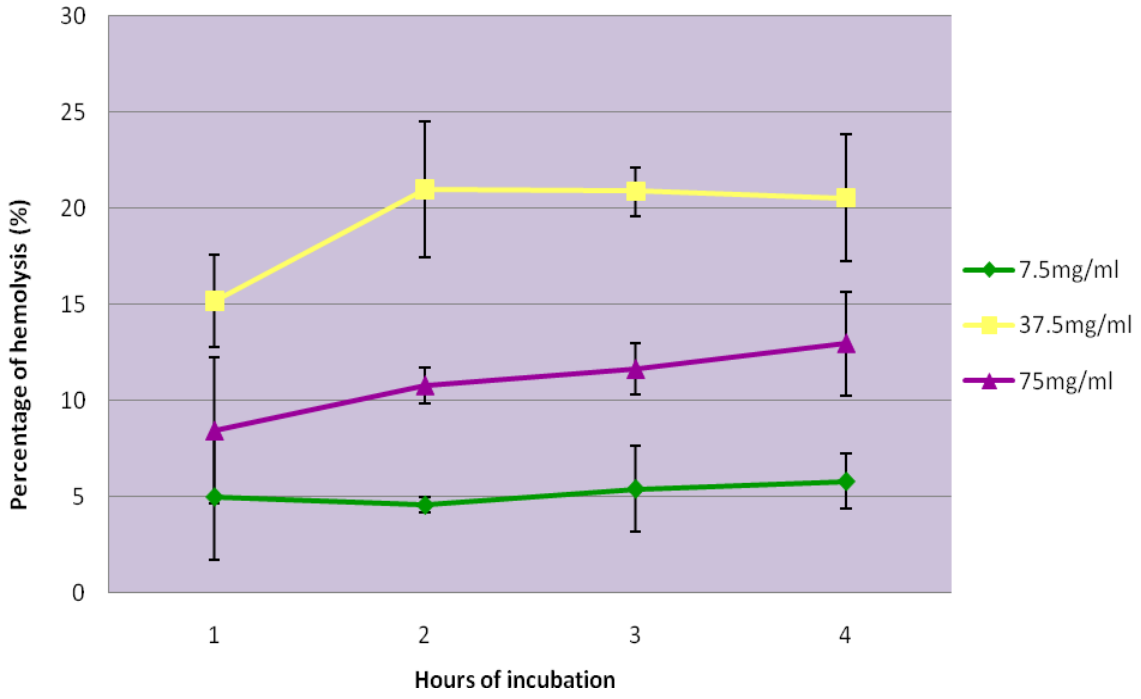

Fig. 2. Percentage of RBC hemolysis in presence of green tea water infusion

Percentage of hemolysis was the lowest for the concentration of 7.5 mg/ml, and it grew as the time of incubation prolonged (after first hour it was 4.99% ± 3.28, second 4.56% ± 0.4, third 5.41% ± 2.22, fourth 5.8 % ± 1.43). Other two higher doses showed an increase in RBC hemolysis also in time dependant manner. Hemolysis was highest for the 37.5 mg/ml concentration. Percent of hemolysis for the 37.5 mg/ml concentration was 15.16% ± 2.4 after first hour, and for the second, third and fourth hour of incubation it was 20.96 % ± 3.55, 20.86 % ± 1.27 and 20.52 % ± 3.3 respectively. Percent of hemolysis for the concentration of 75 mg/ml was 8.45 % ± 3.79, 10.79 % ± 0.95, 11.63 % ± 1.33, 12.96 % ± 2.69 after first, second, third and fourth hour respectively.

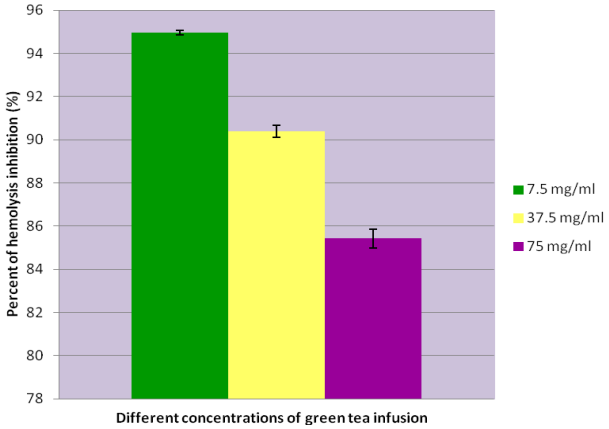

Fig. 3. Antihemolytic effect of green tea infusion

According to the Fick's law, diffusion flux from a membrane is proportional to concentration difference of both sides. So, an increase in concentration of green tea infusion in outer membrane leads to diffusion to internal membrane until it gets to specific concentration which can promote membrane disruption and induce hemolysis (Kleszczynska et al., 2005.).

Lipid peroxidation of rat RBC induced by H_2O_2 creates membrane damage and hemolysis. The highest percentage of inhibition was recorded for the 7.5 mg/ml concentration (94.96 % ± 0.1). Other two concentrations had lower inhibitory effect, and for 37.5 mg/ml dose it was 90.39 % ± 0.29, while for the 75 mg/ml it was 85.42 % ± 0.45. Certain polyphenols may interact with membrane, leading to the decrease in its fluidity and the diffusion of free radical into the RBC (Costa et al., 2009.). In higher doses effects differ (Fig. 3.), percentage of hemolysis inhibition is lower. High concentrations of green tea infusion don't have ameliorative effect against RBC free radical hemolysis.

Conclusion

High concentrations of green tea water infusion exibit toxic effect to Drosophila larvae. The time of development was prolonged. This is probably due to high concentration of active compounds present in green tea water infusion such as caffeine and catechins. Also these results indicate that high doses of green tea infusion can cause a change in tonicity of the erythrocyte membrane. Change in tonicity is probably due to the interactions of polyphenols with the outer lipid monolayer of the erythrocyte membrane. Therefore, it is necessary to continue the research further.

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