Evaluation of cytotoxicity of sodium benzoate and fresh and boiled green chili pepper (*Capsicum annuum* L.) on human red blood cells

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ABSTRACT

Peppers (*Capsicum* spp.) are a rich source of different bioactive compounds with potential health-promoting properties. Pepper fruits have numerous uses in culinary preparations that make this one of the most important vegetables and spices. This study investigated the evaluate the antioxidant activity of green chili pepper extracts on human red blood cells by comparison of different extraction method and treatments. Two solvents (water and methanol) was used to extract from green pepper compounds (fresh and after boiling process). The cytotoxic effect of sodium benzoate and fresh and boiled green chili pepper on human red blood cells was investigated. The obtained results show the protective effect of the extract from hot chili peppers on the negative effect of sodium benzoate. This effect was significantly dependent (p<0.05) on the type of source (fresh or boiled), not on the concentration of extract. Used type of solvent had statistical significant matter in C3 concentration of fresh vegetable. The highest protection gave fresh chili pepper extract.

**Keywords:** green chili pepper, *Capsicum annuum*, red blood cells, antioxidant, cytotoxicity, toxicity test, extracts
1. INTRODUCTION

Pepper (*Capsicum annuum* L.) belongs to the *Solanaceae* family and it is widely cultivated in Asia, Africa, and Mediterranean countries. *C. annuum* fruits have numerous uses in culinary preparations that make this one of the most important vegetables and spice [18]. There are many varieties of pepper which are characterized by different sizes and shapes. Their maturation process is distinct and for human consumption their fruits are collected in different stages of maturation. The green fruits which are immature and the red ones (full mature) are the most used in culinary preparations. Depending on the flavor intensity and texture, the culinary application changes from as a spice (hot chili pepper) as well as vegetable (sweet pepper or bell pepper). More than 30 different pigments have been identified in pepper fruits [14]. The color of sweet bell peppers is the major factor associated with consumer purchasing decisions, green and red fruits being the most consumed in the worldwide [21].

![Pepper](image)

**Figure 1.** Pepper (*Capsicum annuum* L.).

Green chili pepper (*Capsicum* spp.) is a rich source of diverse bioactive compounds such as phenols, flavonoids, vitamin C and capsaicinoids with potential health-promoting properties [2,27]. Phenolic compounds, especially phenolic acids and flavonoids, are considerably present in vegetables and fruits; thus, they are an integral part of the human diet.

These bioactive compounds have a really strong antioxidant effect and play an important role in prevention of numerous diseases, such a cancer, ischaemic heart disease. Furthermore, antioxidant properties were deeply investigated namely in inhibition of lipid peroxidation [15]. Capsaicinoids and flavonoids have been shown to act as anti-cancer, antioxidant, anti-inflammatory, anti-viral and anti-bacterial compounds [8,9,11,12].

Furthermore, processing of food (boiling, freezing) has a negative effect on content of bioactive compounds in pepper [10].
Sodium benzoate is food additives and preservatives commonly used in a different product, including beverages, fruit juices, jams, sauces, and other preserves. The addition of food preservatives such as sodium benzoate prevent the growth of bacteria and fungi [13,16]. However, there have been several studies related to synthetic antimicrobial agent like sodium benzoate which can cause liver damage, mutagenicity and neurotoxicity [7]. Sodium benzoate in combination with ascorbic acid may form benzene well known as strong carcinogen [17].

Therefore, the aim of the present study was to evaluate the protection property of green chili pepper extracts on human red blood cells by comparison of different extraction method and treatments.
2. MATERIALS AND METHODS

2.1. Pepper samples and extraction procedures

Fresh green chili pepper (*Capsicum annuum* L var. *annuum*) was bought from a local fruit market (Bogor, Indonesia).

![Figure 2C. Green chili pepper (*Capsicum* spp.).](image)

![Figure 3. Extraction and filtration of pepper samples.](image)
Thirty grams of fully mature peppers were analyzed fresh and after boiling process. Boiling procedure was made by 50 g putting whole fresh peppers in 400 ml of boiling water for 10 min, blotted dried and used. Methanol and water extract of peppers were used for the analysis. To extract the compounds soluble in methanol and water, 50 g fresh plant var annuum and boiled samples were chopped and soaked and mixed in methanol or water at room temperature. The samples were extracted three times, with fresh methanol (or water) each time, over period of 3 days. All the extracts were filtered. The combined filtrates were concentrated on a rotary evaporator (Heidolph, Germany) under vacuum at 40 °C.

2. 2. Preparation of red blood cells (RBC)

This study has received ethical approval from the Research Ethics Committee of Bogor Agricultural University, Indonesia. RBC were isolated from blood of a healthy volunteer who signed the informed consent approval forms. The blood was centrifuged at 6500g for 10 min to separate the erythrocytes. The cell pellets were collected and diluted 20 times with phosphate-buffered saline (PBS). The cell viability should be 95% living. Cell calculation and viability was done using hemacytometer and tryphan blue.

![Figure 4. Cultures of red blood cells](image)

2. 3. Cytotoxicity assay

Suspension of RBC cells (1×10⁶ cells/ml), 80 µL were added in microplate 96 wells and 20 µl of each extract (in three dilution C1: 0.385 mg/ml, C2: 0.75 mg/ml, C3: 1.25 mg/ml) were added as S1. In separated wells, 60 µL of RBC suspension was added in wells and 20 µl of each extract and 20 µl sodium benzoate acid (BA) with concentration 10mg/ml were added (S2). For S3, 80 µL of RBC suspension were added in wells and 20 µl of RPMI that function as controls. The cultures were incubated at 37 °C and RH 90% for 2h. After 2 hours the cultures were added with 10 µl MTT (3-[4,5-dimetithiazol-2-y]-2,5-difenil tetrazolium bromide) at concentration 5 mg / ml. Two hours before the last incubation period,
to Absorbance value was read using microplate reader at $\lambda = 595$ nm. The activity of extract protection was expressed as the protection index (PI):

$$\text{PI} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100\%$$  \hspace{1cm} (1)

**Figure 5.** Distribution of components used in the study.

### 3. RESULTS AND DISCUSSION

Exposure to chemicals often results in toxicity to living organisms. This is not equal for all cells. Toxicity of chemical compounds can be different for individual cell components. Many organs, including the kidney, are capable to decomposition toxic chemicals to less toxic compounds. The toxicity effects of chemicals in blood can be diminish by phytochemical (plants substances which plants produced for self-defense). Polyphenols, flavonoids as well as phenolic acids belong to this group. Using more than one method for analyses of bioactive compounds gives more statistical significant results. [7]. In this paper cytotoxicity assay using human red blood cells was used. That method has not been documented previously [13,24]. In this study two kind of solvents (water and methanol) and two kinds of chili pepper treatment (fresh and boiled) were used for testing survivability of
blood cells and calculate for protection index (PI). Results from absorbance measurement are presented in Table 1.

**Table 1.** Absorbance value of control and tested samples after 2 and 4 hours incubation.

<table>
<thead>
<tr>
<th></th>
<th>methanol extract</th>
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<th>water extract</th>
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<tbody>
<tr>
<td></td>
<td>2 hours incubation</td>
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<td>2 hours incubation</td>
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<tr>
<td>Tested: RBC+E</td>
<td>Tested: RBC+E+BA</td>
<td>Tested: RBC+BA</td>
<td>Control: RBC+RPMI</td>
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<td>4 hours incubation</td>
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<td>Tested:</td>
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<td>C1</td>
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<td>fresh</td>
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<td>fresh</td>
<td>2.757^a</td>
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<tr>
<td>boiled</td>
<td>fresh</td>
<td>boiled</td>
<td>control</td>
<td>boiled</td>
<td>2.943^b</td>
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<td>2.418^a</td>
<td>2.077^b</td>
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</tbody>
</table>
Means with different superscript capital letter in the same column were significantly different (p≤0.05). Means in different superscript small letter in the same row were significantly different (p≤0.05).

*** no cell detection.

RBC + E – red blood cells + extract
RBC + E + BA – red blood cells + pepper extract + sodium benzoate
RBC + BA – red blood cells + sodium benzoate
C1 – concentration of pepper extract: 0.375 mg/ml
C2 – concentration of pepper extract: 0.750 mg/ml
RBC + RPMI – red blood cells + RPMI medium

Positive absorbance result means there are still living cells. Basing on results from Table 1 it can be seen that almost whole cells from every sample were dead after 4 hours incubation, but they were still alive after 2 hours. Statistical significant differences between samples from other type of processing after 2 hours and both solvent was obtained (p≤0.05). It is interesting how absorbance value was changed in RBC + BA samples, what can clearly show impact of sodium benzoate for blood cells. The obtain data show positive effect of added extract in protection against sodium benzoate. Although in some case results were changed rapid (RBC + BA after 4 hours in C1). Results after 2 hours were higher than after 4 hours, where most cells were dead, because normally RBC has very short life time in culture [25]. Hu et al. [26] studied lymph node cells isolated from mice treated with different concentrations of sodium benzoate and compared to control cells they founded that sodium benzoate can caused change the structure of lymphocytes and damage the cell membrane. Particularly important data in present study was absorption value after 4 hours of boiled pepper in C3 (B + E + BA, water extract). After this incubation only, the highest concentration was able to protect red blood cells against sodium benzoate. PI was calculated and presented in Table 2. Statistical analysis was obtained statistically significant differences between solvent in the highest concentration of pepper extract (C3 = 1.250 mg/ml). Results show distinct trends for fresh pepper depending on the solvent in every concentration. The obtained PI results changed unregularly in case of methanol, but in case of water and fresh sample PI was stable. Boiled peppers with water extract gave extreme results of activity for C1 (1.019% - maximum value) and for C3 (0.018% - minimum value). Samples tasted after 4 hours of incubation by spectrophotometric method were not alive in case of C3 extract.

The results obtained were dependent on whether aqueous or methanol extract was used for the treatment. One of the most important issues is choosing the proper solvent for a specific group of biologically active compounds. Wide range of solvents like acetone and methanol or hexane were used in other research with chili peppers. The smallest capability for pepper extract was obtained for acetone [2,3]. Presented PI results show that water has the highest capability than methanol for C1 and C3. Another research also investigated an extract from chili pepper extracted with methanol [22]. It has shown strong antioxidant and antidiabetic properties and the inclusion of hot pepper in a full-day diet promotes maintaining high quality of health. Hot peppers contain high quantity of bioactive compounds, which was strongly correlated with antioxidant activity. The most important bioactivity effect has contents of capsaicinoids, carotenoids, flavonoids and phenolics [2]. Most studies show that the most widely investigated are flavanoid [5,6,20].
Flavanoid content in peppers are around 0.66 mg/g which is compound in the highest content after capsaicin, but in other research peppers are considered as a source of plant phenolics [1]. Special property of phenolics is role in modulating oxidative stress, which takes important place in protection against cancer [13,23]. Others reported the most active compounds from chili pepper is only capsaicin. Already after 24 h, the use of capsaicin in relation to tumor cells showed a significant reduction in cell division. [4]. The results obtained indicate that the biologically active compounds contained in chili pepper have a protective effect on cells treated with sodium benzoate. The mechanism of RBC death by sodium benzoate still need further study. Furthermore, excess uptake of benzoic acid is eliminated from body as hypuric acid, but still can cause the negative effects of many organs in people of all ages [25].

**Table 2.** Antioxidant activity of the pepper extract in three concentrations on human blood cells, expressed in PI (Protection index) after 2 and 4 hours incubation.

<table>
<thead>
<tr>
<th>Average PI ±SD</th>
<th>Methanol</th>
<th>Water</th>
</tr>
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<tbody>
<tr>
<td>2 hours incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>Boiled</td>
<td>Fresh</td>
</tr>
<tr>
<td>C1</td>
<td>0.949 ±0.10&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>0.97 ±0.08&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>C2</td>
<td>1.001 ±0.12&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>0.931 ±0.12&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>C3</td>
<td>0.668 ±0.47&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>0.844 ±0.08&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 hours incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>4.509±0.19&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>***bA</td>
</tr>
<tr>
<td>C2</td>
<td>2.104±0.02&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>***bA</td>
</tr>
<tr>
<td>C3</td>
<td>1.559±0.11&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>***bA</td>
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</tbody>
</table>

Means with different superscript capital letter in the same column were significantly different (p≤0.05). Means in different superscript small letter in the same row were significantly different (p≤0.05).

C1 – concentration of pepper extract: 0.375 mg/ml
C2 – concentration of pepper extract: 0.750 mg/ml
C3 – concentration of pepper extract: 1.250 mg/ml

*** - no PI value, because living cells were not detected.
4. CONCLUSIONS

The results of the toxicity test performed on human red blood cells show that the green chili pepper has high protection properties. It can contribute to increasing the quality of health and protecting our body against diseases. Statistically significant is the type of raw material processing used but not the thermal treatment. Rather than the amount of extract acting on blood cells. Fresh material showed the highest protection properties, which were demonstrated even after 4 hours of incubation (RBC+E+BA for both solvent). The obtained data showed that fresh green peppers had the same bioactivity for every concentration (C1, C2, C3) of water extract. In the highest concentration (C3) significant differences of used solvent were detected for both kind of vegetable (fresh and boiled). Human red blood cells (RBC) were total degraded after 4 hours of incubation, except for fresh pepper samples in methanol (RBC+BA). It can be assumed that fresh vegetables have the highest protecting influence for cells, because of bioactive compounds contained. Bioactivity of green chili pepper should be subjected to further research by various methods in order to determine the antioxidant activity on the human blood cells.

References


