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Extraction, identification and anti-inflammatory activity of carotenoids out of *Capsicum Anuum* L.

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ABSTRACT

Introduction: Carotenoids extracted from dried peppers were evaluated for their anti-inflammatory activities.

Methods: Determining the concentration of carotenoids was carried out by spectrophotometry. Anti-inflammatory activity was studied on the model adjuvant-induced inflammation. In addition, the total number of white blood cells was studied by microscopic method in Gorjaev's chamber. The biochemical parameters of blood - cholinesterase activity and total number of seromuroids in blood plasma were determined by the commercial test kits for rapid analysis

Results: Peppers had a substantial carotenoid content: Ukrainian bitter 2076 ± 10 µg/g of sample in dry weight basis. The yellow fraction was 69.3%, the red fraction was 30.7%. The linear decrease of inflammatory edema in the course of therapeutic use of carotenoid extract ranged from 20% to 50%. The application of carotenoid extract reduced levels of activity acetylcholinesterase and concentration of seromuroids in serum of rats with adjuvant-induced inflammation. The use of carotenoid extract in rats with adjuvant-induced inflammation resulted in reduction of serum cholinesterase activity by 1.3 times and double decrease in the serum seromuroid concentration.

Conclusion: Ukrainian bitter pepper carotenoid extract exhibited good anti-inflammatory activity, with inhibited adjuvant-induced oedema formation and progression. The results suggest that the carotenoids in dried Ukrainian bitter peppers have significant anti-inflammatory benefits and could be useful for pain and inflammation relief. The results of this study allow to recommend carotenoid extract for further investigation as active part of anti-inflammatory drugs.

Implication for health policy/practice/research/medical education:

The results of this study show that carotenoid can be as active part of anti-inflammatory drugs

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Introduction

The plant raw materials are the most important source of new biologically active substances which potentially have different types of pharmacological activities. The most high-demand compounds in the medical practice are the substances with anti-inflammatory effect. The formulations with Cayenne pepper (*Capsicum anuum* L.) have been used for a long time as anti-inflammatory drugs of plant origin (1). Therapeutic action of *Capsicum anuum* L. is primarily attributed to the presence of capsaicinoids which are the agonists of the transient receptor potential for vanilloid (TRPV-1) (2). Nevertheless the use of pepper extracts in experimental inflammation enabled to achieve

effects which could not be explained solely by the action of capsaicinoids (3). The other groups of substances contained in pepper and having anti-inflammatory effects include antioxidants-carotenoids (4,5), vitamin E and vitamin C (6).

At the alteration inflammation stage the reactive oxygen intermediates (super-oxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen) - with high oxidation potential are accumulated and consequently may induce high damaging effect on biological membranes. Carotenoids (Figure 1) have a system of conjugated double bonds providing them with a powerful antioxidant effect, reducing the oxidative stress that accompanies the inflam-

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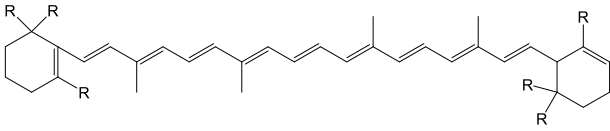


Figure 1. General formula of carotenoids, where R – various substitute.

mation processes (6).

Thus, when using the multicomponent extracts of *Capsicum anuum* L. as anti-inflammatory agents the synergistic pharmacological effect shall occur due to the effect of capsaicin and antioxidant substances on different links of the inflammation process (7).

The aim of this study was to investigate the anti-inflammatory activity of the carotenoids extract of *Capsicum anuum* L. In addition, the total content of carotenoids in generated extracts – one of the main parameters which determine the suitability of the applied plant material as a basis for the production of the pharmaceutical preparations was defined.

Materials and Methods

The plant raw materials were fresh ripe fruits of Ukrainian Cayenne pepper. For extraction of carotenoids, 25 g of finely divided plant raw material (air dried pepper pods without seeds and green part) were filled with 500 mL of acetone at 5°C for several minutes. The obtained extract was filtered under vacuum pump through paper filters No. 4. The filtrate was transferred to a separator funnel containing petroleum ether (mixture of heptane and hexane 1:1) and water. Mixing was performed without shaking, whereupon the aqueous phase was separated. The remaining mixture was washed with water several times, followed by removal of remaining water by means of anhydrous sodium sulfate. Further, the solution was stored in a tightly sealed container in a dark place at a temperature of 5°C. In order to determine the concentrations of carotenoids of various fractions, the petroleum ether was distilled off in rotary evaporator and the resulting oily residue was dissolved in acetone. Then, the absorbance was measured at the transmitted light wavelength of 472 and 508 nm. The concentration of carotenoids of yellow and red fractions was calculated using the following formulas:

$$C_{\text{red fraction}}, \text{ mcg/mL} = \frac{21444 \times A_{508} - 403,3 \times A_{472}}{270,9}$$

$$C_{\text{yellow fraction}}, \text{ mcg/mL} = \frac{1724,3 \times A_{472} - 2450 \times A_{508}}{270,9}$$

Where: A_{508} and A_{472} - absorbance of the solutions for the corresponding wavelengths.

The base for the formulation was produced by mixing 10 parts of paraffinic oil and 1 part of petrolatum. The base was supplemented with concentrated extract of the carotenoids of Cayenne pepper. The final concentration of carotenoids in the formulation was two milligrams per 1

gram of ointment.

Experiments were carried out in young male Wistar rats weighing 180-220 g, managed in standard vivarium conditions with free access to food and water. All studies conformed to the rules of the “European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes” (8) and the principles of the Ukrainian National Congress on Bioethics (9).

Inflammatory arthritis model was reproduced by administration of Freund's complete adjuvant manufactured by FIA (the United States) underneath the plantar aponeurosis of the right limb in a volume of 0.1 mL (10).

The animals were divided into control and experimental groups by a randomized method, 10 animals per group. Treatment was carried out by applying the soft formulation with carotenoid extract to the area of limb inflammation once per day, in 24 hours after administration of phlogogen until full recovery. The control group of animals received treatment in the form of a soft base for the formulation without any carotenoid extract.

The effectiveness of the treatment was assessed by change in the morphological signs of inflammation - thickness and size of limb in the lesion area. The thickness of the limbs was measured by electronic caliper YT-7201 manufactured by YATO company, Poland, the changes in limb size were measured by means of digital Plethysmometer 37140, UgoBasile (PRC). In addition, the total number of white blood cells was studied by microscopic method in Gorjaev's chamber.

The biochemical parameters of blood - cholinesterase activity and total number of seromucoids in blood plasma were determined by the commercial test kits for rapid analysis manufactured by Research and Production Enterprise “Filicit-Diagnostika” Ltd. (Ukraine).

Results

The total content of carotenoids in the dry pods of Ukrainian Cayenne pepper was $2076 \pm 10 \mu\text{g/L}$ g of plant tissue. At the same time the yellow fraction of carotenoids was 69.3%, and the red fraction 30.7%. Red fraction consisted mainly of capsanthin and yellow one of lutein (both fractions were also characterized by a high content of β -carotene).

Investigation of the effect of soft formulation, comprising carotenoid extract of Cayenne pepper fruits as active ingredient, on morphological indicators of adjuvant inflammation process development is shown in Figure 2.

Application of the studied soft formulation with carotenoid extract caused positive therapeutic dynamics reflected in decreased edema in the area of inflammation. Noticeable effect significantly different from the control was observed in 2-3 days of treatment. Linear and volumetric dimensions of the inflamed area of the limb in the experimental group were 20% to 50% lower than the corresponding indicators of the control group. On the 10th day of treatment, the size of the limb in the inflamed area in the treated group of animals was approaching the size recorded before the introduction of phlogogen. The func-

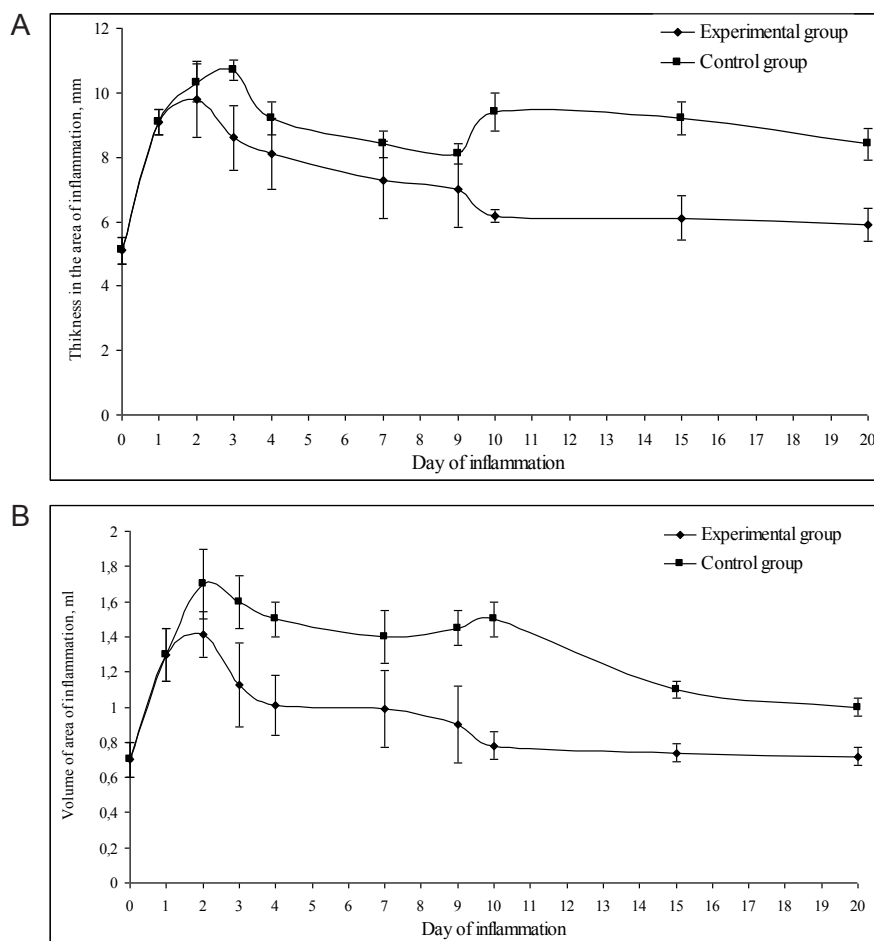


Figure 2. Effect of the formulation with soft carotenoid extract on thickness (A) and volume (B) of limb in adjuvant-induced inflammation area.

tion of the damaged limb was fully recovered. On palpation the areas of administered phlogogen were painless. In contrast, in the control group the lesion area was significantly thickened even on the 20th day after phlogogen administration. On examination, the redness and pain were detected (symptoms that completely absent in the experimental group). Thus, one can conclude that carotenoid extract of Cayenne pepper has not only antiedemic but also complex anti-inflammatory effect.

Dynamics of changes in quantitative indicators of white blood cells after inflammation induction is shown in Figure 3.

After inflammation induction the significant rise in the quantity of white blood cells was observed in the first 2 days of the experiment. The maximum content of white blood cells was observed on the second day after the phlogogen administration and reached 51 and 55 g/L for the experimental and control groups, respectively. Further, there was a decrease of white blood cells to about 30 and 40 g/L, from third to eighth days, and then the regrowth of their quantity was recorded. In the group treated with carotenoid extract ointment the quantity of white blood cells on 20th day was significantly lower than in control group. On 20th day of the inflammatory process, the number of white blood cells both in experimental and

control groups exceeded the corresponding values before phlogogen administration by 2 and 2.6 times, respectively. Biochemical parameters of blood are both important markers of various pathological reactions and indicator of dynamics of the disease development. In adjuvant-induced inflammation the indicators of serum cholinesterase activity and serum seromucoid concentration are of interest (Tables 1 and 2).

Dynamics of changes in the serum seromucoid concentrations were similar to the dynamics of changes in cholinesterase activity. Maximum values of seromucoid concentrations were observed on third day for the experimental group (1.44 absorbance units) and on seventh days for the control group of animals (1.69 absorbance units). It should be noted that on 20th day of treatment the seromucoid serum concentration in the experimental group was close to intact values, while in the control group it exceeded the intact values by more than 2 times.

Discussion

Depending on the color, the quantity of carotenoids contained in the pepper fruits can vary greatly. According to data, the least quantity of carotenoids is found in green fruits, red fruits are intermediate in terms of quantity of carotenoids, and the highest concentration is observed in

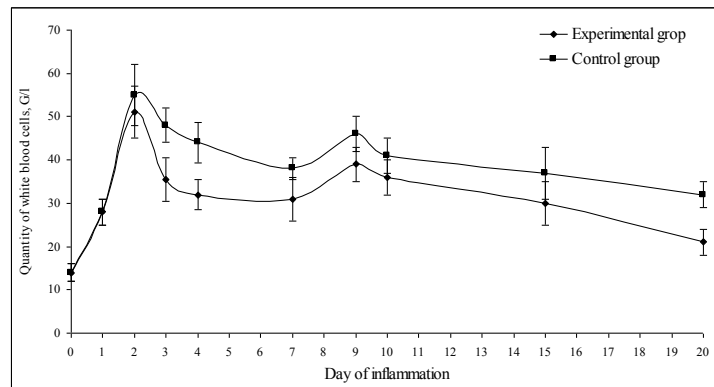


Figure 3. Dynamics of changes in the quantity of white blood cells in treatment of adjuvant-induced inflammation by carotenoid extract of *Capsicum anuum* L.

Table 1. Serum cholinesterase activity in adjuvant-induced inflammation, $\mu\text{mol/s}^{\text{l}}$

Day of inflammation	Control group	Treatment with soft formulation of carotenoid extract
0	28.2 ± 3.6	28.2 ± 3.6
1	83.6 ± 4.3	83.6 ± 4.3
2	129.7 ± 6.7	118.6 ± 7.1
3	138.4 ± 8.1	105.7 ± 11.2*
4	122.9 ± 6.9	92.7 ± 4.1*
7	120.5 ± 5.8	80.0 ± 9.9*
9	127.4 ± 3.1	87.4 ± 9.3*
10	130.6 ± 2.7	85.2 ± 7.6*
15	111.8 ± 7.3	70.4 ± 10.1*
20	54.9 ± 8.6	35.7 ± 8.4*

* Significant differences from control.

Table 2. The serum seromuroid concentration in treating the non-specific inflammation in rats, absorbance units

Day of inflammation	Control group	Treatment with soft formulation of carotenoid extract
0	0.47 ± 0.08	0.47 ± 0.08
1	0.91 ± 0.1	0.91 ± 0.1
2	1.24 ± 0.18	1.17 ± 0.2
3	1.52 ± 0.14	1.44 ± 0.17
4	1.57 ± 0.21	1.22 ± 0.1*
7	1.69 ± 0.16	1.08 ± 0.22*
9	1.62 ± 0.1	0.82 ± 0.18*
10	1.50 ± 0.12	0.75 ± 0.25*
15	1.33 ± 0.15	0.70 ± 0.11*
20	1.05 ± 0.1	0.62 ± 0.07*

* Significant differences from control, $P < 0.05$.

yellow fruits (11). At the same time, the color does not depend only on the total carotenoid content, but on percentage of different carotenoid fractions. In some cases related to the features of growing (low-volume hydroponics), total increase in carotenoid content can be accompanied by a fall in the quantity of yellow-orange component, which affects the final color of the fruits (12).

The quantity of carotenoids in Ukrainian Cayenne pepper is close by values to the same parameters established for chilly varieties (5). In general, we can conclude that by phytochemical composition, the fruits of Ukrainian Cayenne pepper are suitable source of bioactive com-

pounds for the subsequent production of pharmaceutical preparations.

Pronounced anti-inflammatory effect of carotenoids of Cayenne pepper can, in part, be attributed to the effect on reactive oxygen intermediates that occur in the course of the inflammatory process. Relating to non-enzymatic antioxidant system, the carotenoids are always present in the organism providing mainly protection against the damaging effect of singlet oxygen ($^1\text{O}_2$) and peroxide radicals ($\text{ROO}\cdot$). The antioxidant activity of carotenoids is directly dependent on the system of conjugated double bonds connected with different end groups. In the course of antioxidant protection provided by carotenoid the physical and chemical mechanism of utilization of $^1\text{O}_2$ is possible. At the same time the carotenoids refer to the one of the most effective natural utilizers of the singlet oxygen (13,14). Another protection feature of carotenoids is the inhibition of the Maillard reaction - glycosylation of the amino groups of proteins resulting in breach of their functional activity (15). Antinociceptive effect is associated with the α - and β -carotenes and lycopene (16,17).

Increase in the quantity of white blood cells is one of the major systemic reactions to the local inflammatory process. At this stage of primary non-specific inflammation the area of inflammation is infiltrated with neutrophils and macrophages, which is also accompanied by an increase in their quantity in the systemic circulation. In case of rheumatoid lesion due to specific autoimmune processes, there is a growing population of lymphocytes (18). Decrease in the total quantity of white blood cells in the course of the treatment demonstrates its effectiveness. Nevertheless, the absence of external local manifestations of the inflammatory response was not accompanied by the restoration of the system indicators such as the level of white blood cells. The inflammation development at the opposite limb which is sign of autoimmune lesions, typical for rheumatoid arthritis, has not been fixed. Thus, one can assume that the activation of the leukocytic cells in the focus of inflammation caused by phlogogen administration resulted in continued increase in the level of pro-inflammatory and inflammatory cytokines, which led to a stable increase in the quantity of white blood cells.

The existence of several forms of cholinesterase (acetylcholinesterase, butyrylcholinesterase, pseudocholinesterase) determines the predominant location for each type of enzyme. High concentrations of acetylcholinesterase are typical for brain, nerve fibers and erythrocytes, butyrylcholinesterase and pseudocholinesterase are found in blood serum, pancreas and liver (19). Therefore, the observed increase in cholinesterase activity may be a consequence both of direct breach of the cell integrity in the area of inflammation (particularly if skeletal muscle fibers are damaged), and activation of cellular component of the inflammatory cascade. In case of interaction of the acetylcholine with α -7H-acetylcholine receptors located on the surface of blood monocytes, the release of proinflammatory cytokines by latter is decreased, the cholinergic anti-inflammatory pathway occurs (20,21). Increase in the cholinesterase activity leads to an acceleration of acetylcholine utilization and, consequently, increase in inflammatory manifestations. In our study, the increase in cholinesterase activity correlated well with the intensity of the inflammatory response.

Subsequent decreased cholinesterase activity in animals treated with the carotenoid extract ointment in comparison with control group may be both consequence of decrease of alteration process in the inflammation area, and decreased systemic inflammatory response.

α 1-Acid glycoprotein (seromuroid, orosomuroid) refers to a group of acute-phase proteins. The seromuroid level can be increased by several times in the course of inflammatory process development, which makes it an effective biomarker reflecting the development of the acute phase of inflammation (22). Seromuroid has a sufficiently long period of half-life (unlike C-reactive protein, which is 5 days). This enables it to be used as an indicator of the effectiveness of the applied treatment (23). The factors activating the seromuroid synthesis by hepatocytes are interleukin-1 α (IL-1 α) and interleukin-6 (IL-6). The observed decrease in serum seromuroid concentration in the experimental group can be a consequence of decreased level of IL-1 α and IL-6. This assumption seems logical not only because of the direct influence of above-mentioned interleukins on the products of seromuroid, but also due to the proinflammatory nature of IL-1 α and IL-6. We suppose that conducted therapeutic effect has led to a decrease in the level of IL-1 α and IL-6, which in turn reduced the symptoms of the acute inflammation phase and contributed to decrease in the serum seromuroid concentration.

Conclusion

The high content of carotenoids in extract of *Capsicum annuum* L. of Ukrainian Cayenne pepper makes possible its use as the base for the production of pharmaceutical anti-inflammatory products. The formulations of mixed nature containing both carotenoid extract and other antioxidants (vitamin C, E) and capsaicinoid extract are of special interest. Based on the received data on the distribution of different fractions of the carotenoids, we can

recommend the use of yellow fruits due to higher carotenoid content in them.

Using of carotenoid extract as a local anti-inflammatory agent is capable of substantial decrease in all external manifestations of the inflammatory process, thus one can give findings on comprehensive anti-inflammatory effect of these drugs. The most probable mechanism of anti-inflammatory activity of carotenoids is their antioxidant effect resulting in decrease of alteration action of reactive oxygen intermediates in the area of inflammation.

Decreased serum cholinesterase activity and reduced serum seromuroid concentration in the course of inflammation process treatment by carotenoid extract may be testimony both of reduced reactions of the secondary lesion in the area of inflammation, and the general decrease in systemic inflammatory response.

Authors' contributions

BYA prepared introduction, experimental part, analysis and discussion of results, conclusion, preparation for publication. KIA and SAA analyzed the data and prepared discussion as well as conclusion of results. BIA did experimental part. All read and confirmed the article.

Conflict of interests

None of the authors has any conflict of interest to disclose.

Ethical considerations

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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