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# Chemical and biological studies of β-carotene after exposure to *Cannabis sativa* smoke

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ABSTRACT

Considering the increase in consumption of *Cannabis sativa* and the use of the compound β-carotene (BC) as supplement, we investigated potential changes in the chemical and biological proprieties of BC after exposure to *C. sativa* smoke (CSS). Our results showed that the BC exposed to CSS underwent 98.8% degradation and suffered loss of its antiradical activity. The major degradation products identified were 3-hydroxy-2,4,4-trimethylpentyl)2-methylpropanoate and (2-ethyl-3-hydroxyhexyl)2-methylpropanoate compounds. These are found in higher levels in the exhalations of colorectal cancer patients and are similar to the toxic products associated with lipid peroxidation of polyunsaturated fatty acids. In toxicological assays using micro-crustacean *Artemia salina* the BC was non-toxic, while the BC degraded by CSS had a toxicity of $LC_{50}$ = 397.35 μg/mL. In Wistar rats, females treated with BC degraded by CSS (BCCSS) showed whitish liver spots, alterations in liver weight and in bilirubin and alkaline phosphatase levels, and decrease in the number of leukocytes associated with atypical lymphocytosis. In male rats, there was an increase in the number of leukocytes when compared to the control group. In the histopathological analysis, the cortical region of the kidneys showed the presence of discrete amorphous eosinophilic material (cylinders) in the lumen of the proximate and distal convoluted tubules. In general, the BC in contact with CSS undergoes chemical changes and exhibits toxicity to rats and *Artemia salina*.

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## 1. Introduction

Carotenoids are widely studied due to their beneficial biological activities in health. More than 700 carotenoids have been identified [1]. Only about 50, however, are precursors of vitamin A [2]. The β-carotene (BC), besides being one of the most abundant carotenoids in nature, is the only one that is converted into two molecules of vitamin A. The concentration of BC in human serum is about 0.4 μM [3,4].

In the past two decades, the pharmacological potential of BC for the prevention and treatment of many types of cancer has received growing attention. The action mechanisms are still not fully understood, but this carotenoid is believed to induce an apoptotic effect in oncogenic cells, such as in colon cancer cells, leukemia cells and breast cancer cells [4–6]. However, previous studies, as, for example, the "α-tocopherol, β-carotene Cancer Prevention Study" (ATBC) and the "β-carotene and Retinol Efficacy Trial" (CARET) both showed that smokers and nonsmokers alike, with an intake of 40 and 30 mg/day of BC, respectively, increased the risk of cancer in heavy smokers. Since then, various hypotheses have been proposed to explain the negative results associated with tobacco smoking [7–10]. The BC degradation products from cigarette smoke, such as reactive aldehydes and epoxides are thought to help raise the risk of cancer in smokers who have a carotene-rich diet.

Tobacco smoking has declined in recent years in many countries due to the clear evidence of adverse health effects. The use of *Cannabis sativa*, for therapeutic or recreational purposes, however, has been growing in the world. This constitutes a public health problem due to the harmful effects of the various toxic chemical substances released by the burning of the leaves and flowers, known to be the source of a variety of diseases such as lung cancer [11].

*C. sativa* is a bush in the Cannabaceae family. Its parts contain varying levels of more than 480 substances, distributed in 18 chemical classes, of which more than 60 are cannabinoids. This plant has important therapeutic potential as well as psychotropic properties

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[12,13]. Its pharmacological activity is associated with cannabinoid compounds, which are not found in other plant species. Cannabinoid $\Delta$-9-tetrahidrocanabinol (THC) is its main psychoactive component.

The medicinal use of this plant is legal in many American states and in countries such as Holland, Belgium and Canada, to alleviate symptoms of diseases such as cancer, AIDS, multiple sclerosis, glaucoma and Tourette's syndrome. In Brazil, its use is still illegal. The therapeutic application of *C. sativa* is still controversial due to its effects, generally associated with its intoxicating properties and chronic systemic effects on people who are heavy users, besides the possible side effects [14,15].

Another problem of the regular smoking of *C. sativa* is the high THC level in the bloodstream, which can induce anxiety attacks, schizophrenia and precocious psychotic alterations in people who are genetically predisposed [16]. One study showed that the use of *C. sativa* is associated with higher susceptibility to infections and increased rates of head and neck cancer [17]. Those authors also reported that THC has immunomodulatory properties, by reducing the ability of macrophages to produce cytokines, limiting their capacity to recognize antigens, in turn weakening the cytolytic and proliferative response of the T lymphocytes and the production of antibodies by the B lymphocytes.

Since there are no studies of the effect of BC associated with *C. sativa* smoke (CSS), the aim of the present study was to compare the possible alterations in the chemical and biological properties of BC after contact with *C. sativa* smoke.

## 2. Materials and methods

### 2.1. Materials

The $\beta$-carotene (95% purity) was acquired from Sigma-Aldrich. The *C. Sativa* was supplied by the Judicial Office of the city of Limoeiro, state of Pernambuco, Brazil, after authorization by Judge José Claudionor da Silva Filho and by the Forensic Institute of Pernambuco, on the decision of its manager Dr. Luiz Carlos Soares da Silva. The experiment of degradation of BC by CSS was carried out in the Police Science Laboratory of Pernambuco.

### 2.2. Animals

Swiss mice (25–30 g) and Wistar rats (250–260 g) obtained from the laboratory animal facilities of the Federal University of Pernambuco were used. The animals were housed in group cages with free access to food and water. The animals were treated according to the ethical principles of the National Council for Experimental Animal Control (CONCEA). The university's animal study committee approved the experimental protocols with number 23076.012173/2007-77.

### 2.3. Kinetic analysis of degradation of BC by CSS

The method used was that proposed by Lowe with modifications [10]. *C. sativa* smoke was generated artificially using a flexible silicone hose measuring 10 cm in length. A cannabis cigarette was inserted at one end, and the other end was attached to a 50-mL disposable syringe. The cigarette was lit and the smoke was collected in the syringe by suction. The syringe, filled with smoke, was then connected to another hose, whose other end was attached to a glass Pasteur pipette. This injected the smoke into a BC solution in hexane, contained in a round-bottom flask. The flask was then closed and the solution was gently shaken. This process was repeated each 20 min for 2 h. With each smoke injection, an aliquot of 0.2 mL of the solution was removed and diluted in 2.8 mL of a solution containing hexane/dichloromethane/methanol (3:3:2) for analysis with an Agilent 8453 UV–vis spectrophotometer, in intervals from 300 to 600 nm, using 10-mm quartz cuvettes. The samples were analyzed at a concentration of 0.67 mg/mL in hexane/dichloromethane/methanol. Two other curves were plotted: a control kinetic curve, which was obtained through the natural degradation of a solution of BC in hexane at room temperature (25.0 °C); and a calibration curve with the standard BC. All the analyses were conducted in triplicate.

### 2.4. Degradation of BC by CSS

To degrade the BC, we used the same method described above. A solution of 7 μM BC in hexane was injected into CSS and this was repeated every 20 min for a period of 9 h, after which the hexane was removed with a rotary evaporator (40 °C) to obtain the sample BC degraded by the *C. sativa* smoke (BCCSS). The entire experiment was conducted at room temperature. Each cigarette consumed generated about 400 mL of smoke.

### 2.5. Analysis and identification of the degraded $\beta$-carotene GC–MS

The BCCSS was analyzed by gas chromatography-mass spectrometry (GC–MS), with an Agilent spectrometer equipped with a silica capillary column (30 m × 0.25 mm × 0.25 μm), operating under the following conditions: column flow of 2.5 mL/min, using helium as the carrier gas (1.5 mL/min); and column temperature program from 60 °C to 320 °C at 3 °C $min^{-1}$. The injector and detector temperatures were both 280 °C.

### 2.6. Antioxidant activity—ABTS$^{\bullet+}$ method [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)]

The antioxidant activity was determined by the ABTS$^{\bullet+}$ free radical sequestration method, as described previously [18]. The sample of BCCSS was obtained as described in paragraph 2.4 and was diluted in ethanol.

### 2.7. Hemolytic assay

The hemolytic assay was performed in a 96-well plate following the method previously described [19]. Each well received 100 μL of a 0.85% NaCl solution containing 10 mM of $CaCl_2$. The first well served as the negative control and contained the vehicle only (10% DMSO). The second well contained 100 μL of the test substance, which was diluted 1:2. The other extracts were tested in concentrations ranging from 15.62 to 2000 μg/mL. The serial dilution continued until the 11th well. The last well received 20 μL of 0.1% Triton X-100 (in 0.85% saline) to obtain 100% hemolysis (positive control). Each well received 100 μL of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM of $CaCl_2$. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed, and the released hemoglobin was measured by spectroscopic absorbance at 450 nm. Extracts with an $EC_{50}$ value lower than 200 μg/mL were considered active, as previously described [20].

### 2.8. Assessment of the toxicity of repeated doses in rats

The testing parameters were those determined by the Brazil National Sanitary Surveillance Agency (ANVISA) and previously reported [21]. Forty animals were allocated to four experimental groups of ten rats each (five males and five females). Each animal received the following daily doses, by orogastric gavage: control group received only the vehicle (corn oil); group BC received 2.5 mg/kg of BC solution; group CSS received 2.5 mg/kg of CSS

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