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Investigation of In Vitro Superoxide Scavenging Capacity of Cape

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Introduction: The antioxidant capacity of CAPE (caffeic acid phenethyl ester) has been investigated in recent years but, so far most of the studies were in vivo studies. In this study we investigated, the in vitro antioxidant capacity of CAPE to scavenge superoxide radicals. CAPE, an active component of propolis from honeybee hives, is known to have anti mitogenic, antioxidant, anticarcinogenic, antiinflammatory and immunomodulatory properties.
Aim of the study: CAPE is an antioxidant molecule in vivo. The aim of this study is to investigate if CAPE under in vitro conditions has the same effect similar to in vivo.
Methods: The method developed by McCord and Fridovich for the determination of SOD activity is modified by replacing SOD with CAPE.
Results: We find that, CAPE has superoxide scavenging capacity in a dose dependent manner. The presence of 50 µmol/L CAPE caused 73.66 % percentage of inhibition in cyt c reduction.

Keywords: CAPE, Superoxide scavenging capacity, Oxidative stress.

Cape'nin Süperoksit Süpürücü Kapasitesinin In Vitro Incelenmesi

Giris:Son yillarda CAPE (Kafeik asit fenil esteri)'nin antioksidan kapasitesi arastirilmistir, ancak su ana kadar yapilmis olan çalismalarin çogu in vivo çalismalardir. Biz bu çalismada CAPE'nin süperoksit radikallerine karsi antioksidan kapasitesini in vitro arastirdik. CAPE bal arisi kovani propolisinin aktif bir bileseni olup antimitojenik, antioksidan, antikarsinojen, antiinflamator ve immünomodülatör özellikleri bilinmektedir.
Amaç: CAPE in vivo antioksidan bir moleküldür. Bu çalismada, CAPE'nin benzer kosullarda ancak in vitro ortamda ayni etkiyi gösterip göstermediginin arastirilmasi amaçlandi.
Metod: McCord ve Fridovich'in SOD aktivitesi tayini için gelistirdikleri yöntem, SOD yerine CAPE kullanilarak modifiye edildi.
Bulgular: CAPE'nin doza bagimli olarak artan bir süperoksit süpürücü kapasiteye sahip oldugunu saptadik. 50 ?mol/L CAPE varliginda cyt c redüksiyonu %73,66 inhibe oldu.

Anahtar Kelimeler: CAPE, Süperoksit Süpürücü Kapasite, Oksidatif Stres.

Electrons, usually paired one electrically charged and rotate upon themselves while inducing a magnetic field called spin. An electron douplet is more stable than two isolated electrons because the pairing of two electrons with opposite spin cancels their reciprocal magnetic fields.[1]

On the other hand, a free radical is a neutral or charged chemical species whose peripheral shell contains an unpaired electron called, singular electron. Free radicals thus contain an odd number of electrons.[2,3]

Free radicals are produced continuously within the cells. The main free radical generators in cells are the mitochondrial electron transport system, autooxidized molecules such as xanthine oxidase, aldehyde oxidase and

microsomal oxidations.[4] Under conditions such as ischemia/reperfusion, inflamation, xenobiotic metabolism and hyperoxy, excessive reactive oxygen species are produced as a result of oxidative stress leading to damage to all biological molecules.[5-7] The oxygen radicals attack nucleic acids and various modified bases in DNA are generated. Among them, 8-oxoguanine is the most abundant, and appears to play critical roles in carcinogenesis and in aging.[8]

When the delicate balance between the intracellular levels of oxidants and antioxidants is perturbed, ROS (reactive oxygen species) are produced in excess and cells are exposed to oxidative stres.[9] The defence mechanisms of the cells against oxidative stress are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), antioxidant vitamins such as vitamin A, E and C, antioksidant molecules such as glutathione, melatonin, bilirubin and elements such as Selenium.[10]

Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) is an active component of propolis from honeybee hives.[11] It has antiviral, antiinflammatory and immunomodulatory properties and has been shown to inhibit the growth of different types of transformed cells. It has been reported that CAPE suppresses lipid peroxidation and displays antioxidant activity.[12-14] On the other hand Laranjinha et al have reported that CAPE is an important free radical scavenger.[15]

In order to investigate antiinflammatory properties of CAPE, Michaluart et al, made a study on rats and they found that, prostoglandine synthesis was suppressed by high doses of CAPE in acut inflammation.[16] The same researchers reported that, CAPE has an inhibitory effect on cyclooxygenase enzyme. On the other hand, Krol et al studied with ethanolic propolis extracts and they showed that, these extracts had scavenging effects on free radicals.[17]

The aim of this work is to construct an in vitro assay in order to demonstrate the superoxide anion radical scavenging capacity of CAPE. The experiment was planned on the basis of the ability of CAPE to inhibit some observing processes. For this reason, the method developed by McCord and Fridovich for the determination of SOD activity is modified by replacing SOD with CAPE.[18]

MATERIAL AND METHODS

Xanthine, xanthine oxidase (XOD, from buttermilk), cytocrome c (cyt c, from bovine heart), NaOH, KH_2PO_4, Na_2HPO_4, EDTA were purchased from Sigma Chemical Company (St. Louise, USA).

Caffeic acid phenethyl ester was synthesized in the Chemistry Laboratory using the method described by Grunberger.[19]

The following solutions were prepared for the assays. And all procedures were done at 37 C. A Shimadzu 1601 UV/VIS spectrophotometer with a connected PC and a Grand LTD 6G thermostability unit adjusted to $37 \pm 0.1°C$ was employed for all spectrophotometric assays.

Solution A: 0,76 mg xanthine in 10 ml of 0,001 N. NaOH solution mixed with 24,8 mg cyt c in 100 ml of 50 mM phosphate buffer (pH:7,8) containing 0,1 mM EDTA.

Solution B: 0,2 U/ml XOD in 0,1 mM EDTA solution.

Determination of the rate of cyt c(Fe^{+3}) reduction in the presence of CAPE: 10 ?l 10% ethanol solution of CAPE (to give a final concentration of 10 ?mol/ml) was added to 750 ?l of solution A. After incubation at 37 C for 30 s. 25 ?l of solution B and 15 ?l of 50 mM PBS (pH:7,8) were added and the absorbance change at 550 nm was observed for 1 min. The assay was repeated with increased amount of CAPE. Each assay was repeated three times and the arithmetic means were calculated and used. A blank was run by substituting CAPE solution with 10% ethanol solution.

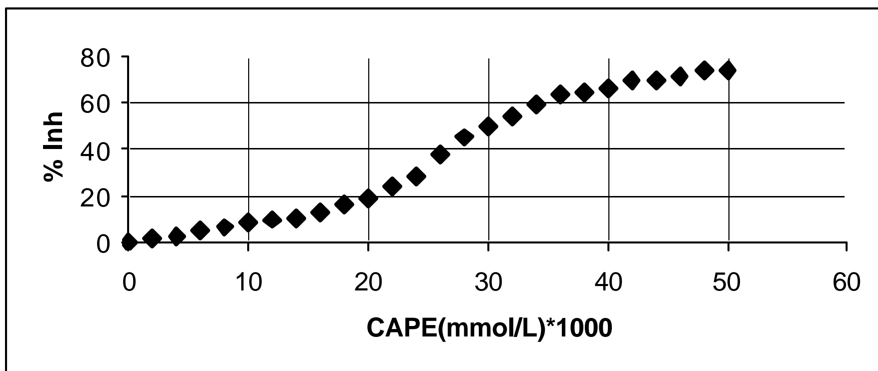
Determination of uric acid absorbance levels: Uric acid has an absorbance peak at 292 nm (at pH> 7), (20). 100 ?l of reaction mix was added to 900 ?l PBS (pH:9,4) at 37 ^{0}C and specific absorbance of uric acid was measured at 292 nm.

Statistical analysis: Results are given as mean values $\pm$ standart deviation. Differences between variables were tested for significance by Independent Samples T-Test, using a level of significance of $p<0,05$. Relationship between percentage of inhibition of cyt c reduction and concentration of CAPE was analyzed by Regression Analyze.

RESULTS

Table I: Percentage of inhibition observed in cyt c reduction with increased CAPE concentration and absorbance of uric acid at 292 nm.

CAPE(?mol/L), n=26	% Inh., n=26	A_{292} without CAPE, n=26	A_{292} with CAPE, n=26
0	0	0,266	0,265
2	1,60	0,264	0,263
4	2,76	0,266	0,267
6	5,13	0,265	0,265
8	6,28	0,264	0,264
10	8,31	0,265	0,264
12	9,88	0,266	0,266
14	10,51	0,265	0,267
16	12,70	0,264	0,263
18	16,61	0,267	0,267
20	19,03	0,269	0,268
22	23,81	0,265	0,267
24	28,45	0,263	0,261
26	37,52	0,266	0,266
28	44,92	0,268	0,266
30	49,34	0,265	0,262
32	54,11	0,263	0,264
34	59,24	0,265	0,266
36	63,78	0,264	0,265
38	64,06	0,269	0,267
40	66,23	0,265	0,265
42	69,68	0,261	0,260
44	69,55	0,264	0,263
46	71,27	0,262	0,260
48	73,63	0,267	0,268
50	73,66	0,264	0,264
?		Mean$\pm$SD: 0,2650$\pm$0,0019	0,2647$\pm$0,0023, ($p>0,05$ and $t=0,526$)



r=0,981;P=0,000; Regression equation, %Inh = -7,8 +1,76[CAPE(?mol/ml)]

Figure I: Relationship between percentage of inhibition of cyt c reduction and concentration of CAPE.

DISCUSSION:

The role of oxidative stress in many diseases including cancer is gaining importance. A wide range of antioxidants have been proposed for use in the treatment of human diseases.

In recent studies, antioxidant properties of many new molecules have been investigated in order to be used as an antioxidant. One of those molecule is CAPE which is derived from honey bee propolis, attracts attention with its antioxidant property. It has been reported in many studies that CAPE has antiviral, antiinflammatory and immunomodulatory properties in addition to its antioxidant property. Masaaki et al, reported that CAPE has showed anti-tumor promotion activity in their study.[21]

Frenkel et al, studied the antioxidant and anticarcinogenic properties of CAPE.[22] In their study, they exposed Sencar mice to a strong cancer promotion, 12-O-tetradecanoylphorbol-13-acetate and then measured oxidized bases 5-hydroxymethyluracil and 8-hydroxyguanine levels in ephidermal tissues of mice. As a result, it was founded that DNA oxidation was blocked by CAPE.

Cengarle et al, researched the antioxidant capacity of propolis extract in poliunsaturated lipidic systems and reported that the antioxidant capacity of propolis extract was better than alpha-tocopherol.[23]

Most of the studies until now performed on CAPE are in vivo and only a few studies exist in vitro.[24-29] Our findings are compatable with the literature. In this study it is aimed to investigate the in vitro effects of CAPE. Our findings clearly exhibited that CAPE inhibed reduction of cyt c by superoxide radicals in a dose- dependent manner. CAPE may possibly exhibited this inhibitor effect by means of two different mechanisms. One of the possible mechanisms is the scavenging effect of CAPE on superoxide radicals produced by xanthine/xanthine oxidase system. The second possible mechanism is inhibitor effect of CAPE on xanthine oxidase enzyme which is responsible in producing superoxide radicals. Thus, superoxide radical production may be decreased.

Xanthine oxidase enzyme produces uric acid together with superoxide radicals. If the amount of uric acid produced by xanthine oxidase is decreased in the presence of CAPE then it may be thought that CAPE has an inhibitor effect on xanthine oxidase. Therefore the spesific absorbance of uric acid was measured at 292 nm. in order to determine whether CAPE has an inhibitor effect on xanthine oxidase or not. And it was observed that in the presence of CAPE there was no significant change at the absorbance levels of uric acid. This result showed that CAPE has no inhibitor effect on xanthine oxidase, so it can be said that CAPE is a strong antioxidant molecule and its antioxidant property comes from its scavenger effect on superoxide radicals.

Antolin et al showed that, melatonin a biomolecule which has free radical scavenging effect like CAPE, increased SOD-mRNA level but, it is unknown that, if CAPE has an effect on mRNA synthesis of antioxidant enzymes like superoxide dismutase, catalase or glutathione peroxidase.[30] Probably, our researches will be focused on that point in the future.

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