

**OXYGEN FREE RADICAL SCAVENGING ABILITIES OF VITAMINS C,
E, β -CAROTENE, PYCNOGENOL, GRAPE SEED
PROANTHOCYANIDIN EXTRACT AND ASTAXANTHINS *IN VITRO***

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SUMMARY

Free radicals have been implicated in more than one hundred disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, aging, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, tumor promotion and carcinogenesis, and AIDS. This wide range of diseases implies that enhanced free radical formation accompanies tissue and cellular injury in most, if not all, human diseases. In most cases, free radicals and their metabolites are increasingly recognized for their contribution to tissue injury leading to both initiation and promotion of multistage carcinogenesis, which ultimately leads to cancer.

Recent studies have demonstrated that these environmental pollutants exhibit the ability to produce enormous amounts of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation of procarcinogens, inhibition of cellular and antioxidant defense systems, depletion of sulfhydryls, altered calcium homeostasis, changes in gene expression and induction of abnormal proteins. Free radical production is also enhanced by carcinogen exposure as well as under the conditions of stress.

Antioxidants/free radical scavengers function as inhibitors at both initiation and promotion/propagation/transformation stage of tumor promotion/carcinogenesis and protect cells against oxidative damage. Antioxidants have been shown to inhibit both initiation and promotion in carcinogenesis and counteract cell immortalization and transformation. There is considerable evidence for a role of antioxidant nutrients including vitamins C and E, and β -carotene, in the maintenance of health in contributing to the decreased incidence of free radical-induced diseases.

The consumption of edible plants, fruits and vegetables has been repeatedly demonstrated to prevent the occurrence of a number of diseases in humans and animals. Vegetables, fruits and their seeds are rich sources of vitamins C, E and β -carotene, and/or protease inhibitors, compounds which might protect the organism against free radical-induced injury and diseases. Several plants have been reported to contain compounds which exhibit chemopreventive properties against neoplasms, such as ellipticine and taxol, indole derivatives, plant phenolics, bioflavonoids, proanthocyanidins, astaxanthins, dithiolthiones, phytoestrogens, etc.

In this project, we have assessed the comparative scavenging abilities of selected antioxidant samples toward biochemically generated oxygen free radicals.

OBJECTIVE

The objective of this project was to assess the oxygen free radical scavenging abilities of selected commercial antioxidant samples as compared to vitamins C, E, and β -carotene. Oxygen free radicals including superoxide anion and hydroxyl radical were generated *in vitro*, and the concentration-dependent oxygen free radical scavenging abilities were determined by chemiluminescence response.

MATERIALS AND METHODS

1. Generation of Oxygen Free Radicals

Oxygen free radicals were generated [Bagchi, D., Das, D.K., Engleman, R.M., Prasad, M.R. and Subramanian, R. Eur. Heart J. 11, 800-813 (1990)] as follows:

- a. Superoxide anion radical: Xanthine (100 μ M) in 5mM Tris-HCl buffer was incubated with 8mU/ml of xanthine oxidase to generate superoxide anion.
- b. Hydroxyl radical: The incubation mixture to generate hydroxyl radical contained in a total volume of 2 ml, 5 mM Tris-HCl, 100 μ M FeCl₃, 100 μ M EDTA, and 100 μ M xanthine. Xanthine oxidase (8mU/ml) was added to initiate the reaction and to produce hydroxyl radicals.

2. Chemicals

See Attached list

3. Chemiluminescence Measurements

Chemiluminescence, as an index of reactive oxygen species production, was measured in a Chronolog Lumivette luminometer (Chronolog Corp., Philadelphia, PA). The assay was conducted in 3 ml glass minivials. The vials were incubated at 37°C prior to measurement and the background chemiluminescence of each vial was checked before use. Samples were pre-incubated at 37°C for 15 min, and 4 μ M luminol was added to enhance chemiluminescence. All additions to the vials as well as chemiluminescence counting procedures were performed under dim lighting conditions. Results were examined as counts/unit time minus background. Chemiluminescence was monitored for 6 min at continuous 30 second intervals [Bagchi, M., Hassoun, E.A., Bagchi, D. and Stohs, S.J., Free Rad. Biol. Med. 14, 149-155 (1993)].

4. Statistical analyses

Significance between pairs of mean values was determined by student's t test. A $p < 0.05$ was considered significant for all analyses. Each value is the mean \pm standard deviation (S.D.) of 4 to 6 replicates.

RESULTS AND DISCUSSION

A. *In vitro* ability of selected antioxidant samples to scavenge biochemically generated superoxide anion and comparison with superoxide dismutase plus catalase

The comparative superoxide anion scavenging abilities of the antioxidant samples are shown in Table 1. Chemiluminescence is a general assay for the production of reactive oxygen species. A combination of SOD (200 μ g/ml) plus catalase (200 μ g/ml) was used as a positive control.

TABLE 1

**BIOCHEMICALLY GENERATED SUPEROXIDE ANION AND COMPARATIVE
SCAVENGING ABILITIES OF VITAMINS C, E AND β -CAROTENE, PYCNOGENOL,
GRAPE SEED PROANTHOCYANIDIN EXTRACT AND ASTAXANTHIN**

	<u>Sample</u>	<u>Chemiluminescence Response</u>
1.	Buffer control	151.3 \pm 26.2
2.	Vitamin C	311.3 \pm 32.4
3.	Vitamin E succinate	398.7 \pm 40.6
4.	β -Carotene (Type I)	211 \pm 27
5.	β -Carotene (Type IV from carrots)	113.3 \pm 26.6
6.	All <i>trans</i> -Retinol	180 \pm 17
7.	Pycnogenol	347.3 \pm 41.8
8.	Vinox	96.7 \pm 14
9.	Astaxanthin	146.7 \pm 37
10.	OR-1 (100 mg/lit)	86.4 \pm 7.5
11.	Xanthine (100 μ M) +XO (8 mU/ml)	9910 \pm 827
12.	X+XO+Vit C (25 μ M or 5 mg/lit)	8821.5 \pm 1174.6
13.	X+XO+Vit C (500 μ M or 100 mg/lit)	8029.5 \pm 981.4
14.	X+XO+Vit E (50 μ M or 26.5 mg/lit)	6144.3 \pm 712
15.	X+XO+Vit E (94.2 μ M or 50 mg/lit)	5646.8 \pm 436
16.	X+XO+Vit E (141.3MM or 75 mg/lit)	5513 \pm 287
17.	X+XO+ β -C Ty I (50 μ M or 26.85 mg/lit)	9315.5 \pm 603
18.	X+XO+ β -C Ty I (100 μ M or 53.7 mg/lit)	9026 \pm 701

19.	X+XO+β-C Ty IV (50 μM or 26.85 mg/lit)	8340.3±429
20.	X+XO+β-C Ty IV (100 μM or 53.7 mg/lit)	7723.5±608
21.	X+XO+β-C Ty IV (100 mg/lit)	7637±319
22.	X+XO+A-tr-R (50 μM or 14.3 mg/lit)	9283±308
23.	X+XO+A-tr-R (100 μM or 28.6 mg/lit)	8864.5±561
24.	X+XO+Asta-X (50 μM or 29.8 mg/lit)	4903±518
25.	X+XO+Asta-X (100 mg/lit)	4063±524
26.	X+XO+Asta-X (100 μM or 59.7 mg/lit)	4010.8±337
27.	X+XO+OR-1 (5 mg/lit)	8657.5±484
28.	X+XO+OR-1 (10 mg/lit)	6917±608
29.	X+XO+OR-1 (25 mg/lit)	4867±422
30.	X+XO+OR-1 (50 mg/lit)	4319.8±502
31.	X+XO+OR-1 (100 mg/lit)	3792.8±415
32.	OR-1 (50 mg/lit)	87±12
33.	OR-1 (100 mg/lit)	86.4±7.5
34.	X+XO+Pycnogenol (50 mg/lit)	3810.3±362
35.	X+XO+ Pycnogenol (100 mg/lit)	3083.8±485
36.	X+XO+Vinox (50 mg/lit)	3245±423
37.	X+XO+Vinox (100 mg/lit)	3244.3±266
38.	X+XO+SOD(200 μg/ml)+Catalase (200 μg/ml)	1290±178

Minimum volume of DMSO was used to dissolve astaxanthin, b-carotene, all-*trans* Retinol, vitamin E succinate samples, and necessary corrections were made in control buffer and water soluble antioxidant samples

B. In vitro ability of selected antioxidant samples to scavenge biochemically generated hydroxyl radicals and comparison with mannitol

The comparative hydroxyl radical scavenging abilities of the antioxidant samples are shown in Table 2. Chemiluminescence is a general assay for the production of reactive oxygen species. Mannitol (1.25 μ M), a potent scavenger of hydroxyl radicals, was used as a positive control.

TABLE 2

BIOCHEMICALLY GENERATED HYDROXYL RADICAL AND COMPARATIVE SCAVENGING ABILITIES OF VITAMINS C, E AND β -CAROTENE, PYCNOGENOL, GRAPE SEED PROANTHOCYANIDIN EXTRACT AND ASTAXANTHIN

	<u>Sample</u>	<u>Chemiluminescence Response</u>
1.	Buffer control	182 \pm 33
2.	Vitamin C	286.3 \pm 47
3.	Vitamin E succinate	343.5 \pm 51
4.	β -Carotene (Type I)	207.8 \pm 24
5.	β -Carotene (Type IV from carrots)	104.8 \pm 11
6.	All <i>trans</i> -Retinol	254 \pm 39
7.	Pycnogenol	416 \pm 47
8.	Vinox	88 \pm 12
9.	Astaxanthin	187.5 \pm 16
10.	OR-1 (100 mg/lit)	96 \pm 11
11.	X+F+E +XO (8 mU/ml)	11998 \pm 1427
12.	OH+Vit C (25 μ M or 5 mg/lit)	11278 \pm 1314
13.	OH+Vit C (500 μ M or 100 mg/lit)	10438.3 \pm 961
14.	OH+Vit E (50 μ M or 26.5 mg/lit)	8087 \pm 380
15.	OH+Vit E (94.2 μ M or 50 mg/lit)	7888 \pm 424

16.	OH+Vit E (141.3 μ M or 75 mg/lit)	7783 \pm 605
17.	OH+ β -C Ty I (50 μ M or 26.85 mg/lit)	11103 \pm 1221
18.	OH+ β -C Ty I (100 μ M or 53.7 mg/lit)	10812 \pm 1065
19.	OH+ β -C Ty IV (50 μ M or 26.85 mg/lit)	9914.8 \pm 1253
20.	OH+ β -C Ty IV (100 μ M or 53.7 mg/lit)	9710.5 \pm 1412
21.	OH+ β -C Ty IV (100 mg/lit)	9644 \pm 867
22.	OH+A-tr-R (50 μ M or 14.3 mg/lit)	10547 \pm 1476
23.	OH+A-tr-R (100 μ M or 28.6 mg/lit)	9672 \pm 1183
24.	OH+Asta-X (50 μ M or 29.8 mg/lit)	7220 \pm 529
25.	OH+Asta-X (100 μ M or 59.7 mg/lit)	6361 \pm 414
26.	OH+Asta-X (100 mg/lit)	6231 \pm 726
27.	OH+OR-1 (5 mg/lit)	9232 \pm 452
28.	OH+OR-1 (10 mg/lit)	7957 \pm 668
29.	OH+OR-1 (25 mg/lit)	6514 \pm 362
30.	OH+OR-1 (50 mg/lit)	6307 \pm 676
31.	OH+OR-1 (100 mg/lit)	4973 \pm 583
32.	OR-1 (50 mg/lit)	100 \pm 14
33.	OR-1 (100 mg/lit)	96 \pm 11
34.	OH+Pycnogenol (50 mg/lit)	4424 \pm 322
35.	OH+Pycnogenol (100 mg/lit)	4150 \pm 467
36.	OH+Vinox (50 mg/lit)	3868 \pm 314
37.	OH+Vinox (100 mg/lit)	3760 \pm 244
38.	OH+Mannitol (1.25 μ M)	1802 \pm 332

X (Xanthine) + XO (xanthine oxidase) + F (FeCl₃) + E (EDTA) was used as the hydroxyl radical (OH) generating system.._Minimum volume of DMSO was used to dissolve astaxanthin, b-carotene, all-*trans* Retinol, vitamin E succinate samples, and necessary corrections were made in control buffer and water soluble antioxidant samples

CONCLUSIONS

This study demonstrates that OR-1 is a potent antioxidant and provided better efficacy as compared to vitamins C, E and β-carotene (natural and synthetic) and all-*trans*-Retinol.

PERSONS INVOLVED IN THIS PROJECT

1. Debasis Bagchi, Ph.D., FACN, Professor, Department of Pharmacy Sciences
2. Archana Chatterjee, M.D., Ph.D., Assistant Professor, Creighton University Health Sciences Center
3. Taharat Yasmin, M.S., Research Assistant, Department of Pharmacy Sciences
4. Sidney J. Stohs, Ph.D., FACN, Professor and Dean, Creightomn University School of Pharmacy & Allied Health Professions

REAGENTS USED IN THIS STUDY

L-ASCORBIC ACID

SIGMA cat. No. A-5960

Minimum 99.0% EC No 200-066-2

CAS (50-81-7)

Lot 30K0246

FW 176.1

XANTHINE

SIGMA cat.no. X-2502

Sodium salt minimum 99%

CAS. (1196-43-6)

Lot 21K1285

FW 174.1

L-ASCORBIC ACID

ALDRICH CHEMICAL COMPANY

Sodium salt, minimum 99% 26,855-0

FW 198.11

PYCNOGENOL

Manufactured by KAL,Inc. Park City, UTAH

Patented Pine Bark Extract

Vitamin C 100mg/tablet

Pycnogenol(pine bark extract) 20mg/tablet

(+)-TOCOPHEROL ACID SUCCINATE

from natural -tocopherol

CAS no. (4345-03-3) EC No. 224-403-8

SIGMA cat no.T-3126
Lot 67H0187
FW 530.8

β -CAROTENE
type 1 synthetic approx. 95%
CAS no. (7235-40-7)
EC No. 230-636-6
SIGMA cat. no C-9750
Lot 110K2519
FW 536.9

POLYPHENOLICS
Vinox Gold
Pin VW7000
Lot 2213-259

ASTAXANTHIN
SIGMA cat.no A-9335
CAS no. (472-61-7)
EC No. 207-451-4
Lot 90K1823
Minimum 98%
FW 596.8

XANTHINE OXIDASE
SIGMA cat. No X-2252
Lot 11K1454
EC No. 232-657-6
CAS No. (9002-17-9)

All trans-RETINOL
SIGMA cat. no R-7632
Minimum 95%
CAS No. (68-26-8)
Lot 110K5001
FW 286.5

β -CAROTENE
Substantially free from α -carotene
Type 1V from Carrots (sealed ampule)
SIGMA cat.no C-0126
Lot 97H7870
FW 536.9

FERRIC CHLORIDE

SIGMA cat. no. F-7134
Lot 20K3681
FW 162.2
Cas No. (7705-08-0)
EC No.231-729-4

EDTA
SIGMA,cat no. EDS
Lot 80K1003
CAS No. (60-00-4)
EC No. 200-449-4
FW 292.2

DMSO
SIGMA cat. no D-2650
Lot 119H2377
CAS No. (67-88-5)