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Research Article

Antioxidant Potential of Bixin, Purpurin and Psoralen Using an Oxygen Radical Absorbance Capacity (ORAC) Assay

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ABSTRACT

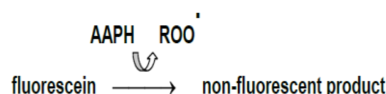
Oxygen radical absorbance capacity (ORAC) is employed to determine the anti-oxidant capacity of a food articles. Usually, antioxidants rich foods showed high ORAC values foods with low antioxidant food. High ORAC value foods stop oxidation of free radicals. The aim of this study was to measure ORAC capacity of bixin, purpurin and psoralen. The ORAC assay measures the fluorescein fluorescence loss progressively as a result of formation of peroxyl-radical by decomposition of AAPH (2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride). Trolox - vitamin E analog, act as a positive control which inhibit fluorescein loss in a dose dependent fashion.The fluorescence recorded kinetically for test drug samples and trolox standard wells once in 60 seconds for 60 min using multimode - plate reader (Perkin Elmer-Enspire) with an excitation wave length of 485 nm and emission wave length of 528 nm subsequently. The antioxidant capacity of test drug samples was measured by using an antioxidant standard curve. The net AUC of several serial diluted compounds bixin, purpurin and psoralen were measured and compared to the net AUC of different concentrations of Trolox. Each curve represents linear regression analysis for comparison to the standard curve of Trolox. The ORAC values of bixin, purpurin and psoralen which were expressed in Trolox equivalents (TE). Bixin and purpurin showed the highest ORAC values (0.814583, 0.65625 µmole TE/kg, respectively) whereas psoralen showed the lowest ORAC value (0.160417 µmole TE/kg).

Keywords: Bixin, Purpurin, Psoralen, Trolox, Multimode-Plate reader, ORAC.

INTRODUCTION

ORAC assay states the mechanism of hydrogen atom transfer which mimics to human biology and *in vivo* antioxidant capacity which gives clinical relevance. Because of its clinical relevance, sensitivity, specificity, ORAC is most approved method to compare as well as standardize the nutritional supplements. In the year 2007, United States department of Agriculture (USDA) has announced list diverse foods commonly available in the US which have high ORAC value which says the significance of ORAC assay. Additionally, the antioxidant capacity of the biological fluids like sera and urine can be determined by using ORAC assay, where it gives direct antioxidant levels of the system.[1] The ORAC assay also a tool for diverse samples include biological fluids and natural products to measure their antioxidant levels.[2]

AAPH (2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride) is decomposed to peroxyl radical (ROO.) at 37 °C. Fluorescein (3', 6'-dihydroxy-spiro [isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one)may be oxidized by peroxyl radical to make a product without any fluorescence. Hydrogen atom transfer mechanism is suppressed this antioxidants which inhibit the fluorescein signals oxidative degradation. This fluorescence signal is measured once in 60 seconds for 60 min using multimode - plate reader (Perkin Elmer-Enspire) with an excitation wave length of 485 nm and emission wave length of 528 nm subsequently. The antioxidant concentrations in the test sample is directly proportional to the fluorescence intensity which is assessed by ORAC assay and compared to standard antioxidant Trolox net area under the curve.[3]



[Antioxidants inhibit the oxidation of fluorescein by hydrogen atom transfer]

Bixin is a carotenoid isolated from the seeds of *bixaorellana* (annatto, or achiote) used as an FDA-approved food colorant and additive, as well as cosmetic and textile colorant. Many orange or yellow manufactured foods are colored with achiote. Natural and processed cheeses, butter and margarine, cakes, cookies, snacks and cereals are all commonly colored with achiote.[4]Previous *in vitro* biochemical assays demonstrated that bixin was able to quench singlet oxygen, a ROS implicated in oxidative lung injury.[5] Consistent with its antioxidant properties, other studies demonstrate that bixin prevents oxidative DNA damage and lipid peroxidation.[6]

Anthraquinones such as purpurin (trihydroxy anthraquinone) is the main bioactive compounds were isolated from root part of *r. cordifolia*. Manjistha (*Rubiacordifolia*) also called as Indian madder belongs to *Rubiaceae* family. Manjistha is an Ayurvedic tonic herb that has been prized for centuries for its long list of benefits and uses. This bitter, astringent herb was traditionally used and valued for its extreme effectiveness in cleansing and purifying the blood, liver and lymphatic

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system.[7]Manjistha is a potent antioxidant, anti-inflammatory and anti-microbial. It enhances circulation, helps to process and remove toxins from the body.[8]

Psoralen (also known as psoralene) is the parent compound in a family of natural products called as fluro coumarins. Psoralen contains in anise seeds, caraway seeds, carrots, celeriac, celery, chervil, cilantro, coriander seeds, cumin seeds, dill, fennel seeds, figs, grapefruit, lemons, limes, lovage, mustard seeds, parsley, parsnips, and root parsley.[9]

MATERIALS AND METHODS

Materials

2,2'-Azobis(2-methylpropionamidine) dihydrochloride, Fluorescein sodium salt; Sisco Research Laboratories Pvt. Ltd., Mumbai: Trolox; Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India: 96 well microtiter plate;Tarsons Products Pvt. Ltd., New Delhi: Bixin; Marven Bio Chem, Hyderabad, India:Purpurin; Sigma Aldrich Chemicals Pvt. Ltd., Bengaluru, India: Psoralen; Yucca Enterprises, Mumbai, India.

Methods

The antioxidant activity of test drugs and standard were determined by the ORAC assay. Different concentrations of the test drug samples were prepared and the assay were carried out in multimode plate reader (Perkin Elmer-Enspire) using fluorescein dye.[10-12]25 µl of standard antioxidant 1 mM Troloxor different concentrations (0.0312, 0.0625, 0.1250, 0.2500, 0.5000, 1 and 2 mM) of test drugs were added by calibrated micropipette into the black 96-well micro plate and 150 µl of the 4 µM fluorescein solution was added to each 96-well micro plate and the 96-well micro plate were shaked properly at maximum intensity for 5seconds to mix fluorecein in wells. The 96 well micro plate were incubated for half an hour at 37°C in multimode - micro plate reader.[13-15]25 µl of 153 mMAAPH was dispensed to all the wells after 30 min incubation. After adding AAPH, the fluorescence of test sample wells and standard sample wells were monitored kinetically once in 60 seconds for 60 min, with an excitation wave length of 485 nm and emission wave length 528 nm subsequently.

0.2 mM stock of standard Trolox was diluted with 0.075 M phosphate buffer for preparing standard curve. Different concentrations of 0-100 µM Trolox were made for building the antioxidant standard curve.[16,17] Trolox dilutions are tabulated in Table.1.

The AUC was determined from the equation 1 and 2:

AUC = 1 + RFU1/RFU0 + RFU2/RFU0 + RFU3/RFU0 +……+ RFU59/RFU0 + RFU60/RFU0 (Eq.1.)

RFU0 = Relative fluorescence value of time point zero.

RFUx= Relative fluorescence value of time points (e.g. RFU60 is relative fluorescence value at minute sixty)

Net AUC was calculated by subtracting the Blank AUC from the AUC of each sample and standard.

Net AUC = AUC (Antioxidant) – AUC (blank) (Eq. 2.)

Tubes	Trolox antioxidant standard (0.2 mM)	Phosphate buffer(0.075 M)	Troloxfinal concentration
1	100 µl	100 µl	100 µM
2	50 µl	150 µl	50 µM
3	40 µl	160 µl	40 µM
4	30 µl	170 µl	30 µM
5	20 µl	180 µl	20 µM
6	10 µl	190 µl	10 µM
7	5 µl	195 µl	5 µM
8	2.5 µl	197.5 µl	2.5 µM
9	0 µl	200 µl	0 µM

Cao and Prior described the determinations of ORAC values.[13]firstly, the standards and samples AUC and the Net AUC of were calculated by taking final assay readings.

Trolox equivalents (TE) of each test sample range were determined by the ratio of the slope (m) of the linear regression analysis of the compound to the slope of the linear regression of Trolox was used:

TE (range of concentrations) = m compound/m Trolox*(Eq. 3.)*

Statistical Analysis

Graph pad prism software, version 6 and MS excel 2007were used to analyze the data.

RESULTS

Figure 1: Plots of Trolox Kinetic Curves

Graph is the normalized fluorescence intensity on the y-axis against time on the x-axis using Graph pad prism software, version 6. Above curve represents fluorescence decay of different concentrations of Trolox0 to100 µM.

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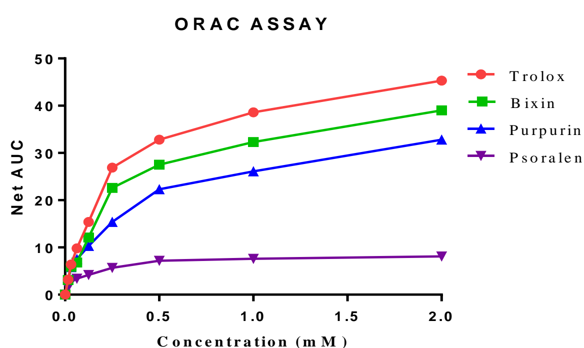

Figure 2: The antioxidant capacity of Bixin, Purpurin, Psoralen

Net AUC is on the y-axis against standard antioxidant Trolox and test drugs different concentration on the x-axis using Graph pad prism software, version 6.

The antioxidant capacity of different test drugs (bixin, purpurin and psoralen) was determined by the ORAC-FL method. As an example, Figure 1 depicts the fluorescence decay curves for Trolox at different concentration (0-100 µM). Antioxidant standard curve was prepared and used to interpolate the antioxidant capacity of test drugs. Figure 2 depicts the net AUC of several serial diluted compounds bixin, purpurin and psoralen were measured and compared to the net AUC of different concentrations of standard antioxidant Trolox. Each curve was subjected to linear regression analysis in order to compare to aantioxidant Trolox standard curve.Table.2 depictsthe oxygen radical absorbing capacity (ORAC) values of bixin, purpurin and psoralen which were expressed in Trolox equivalents (TE).

Bixinand purpurin showed the highest ORAC values (0.814583, 0.65625 µmole TE/kg, respectively) whereas psoralen showed the lowest ORAC value (0.160417 µmole TE/kg).

Table 2: The ORAC values of test drugs

TEST DRUG	ORAC VALUE
Bixin	0.814583 µmole TE/kg
Purpurin	0.65625 µmole TE/kg
Psoralen	0.160417 µmole TE/kg

DISCUSSION

The antioxidant capacity of test drugs can be determined by ORAC assay against peroxyl radicals which is a reactive oxygen species in human body. The main principle of ORAC assay is the transfer of hydrogen atom which mimics to biological system and gives clinical relevance. Antioxidant capacity of foods and dietary supplements can be determined by ORAC assay.[18,19] Bioavailability of the phytochemicals was predicted by *in vitro* antioxidant methods like ORAC assay. Bixin, and purpurin showed the highest ORAC values. Therefore, food additives bixin, and purpurin may be powerful antioxidants against peroxyl radical in human body. In contrast, psoralen showed the low ORAC value. Eventually, psoralen may be less effective against peroxyl radical in human body.

CONCLUSION

The antioxidant potential of food products and natural compounds would be evaluated by ORAC assay. From the results obtained from the ORAC assay, it can be said that the antioxidant capacity of bixin and purpurin would protect cell from oxidative stress caused by free radicals. Oxidative stress is the main cause of chronic diseases like cancer, diabetes, chronic bronchitis etc. Bixin and purpurin may be new promising agents to treat chronic diseases. Further studies are required on Bixin and purpurin to see complete clinical relevance as promising antioxidants.

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