



GESDAV

available at [www.scopemed.org](http://www.scopemed.org)

## Oxidants and Antioxidants in Medical Science

### Brief Report

## Application of oxygen radical absorbance capacity (ORAC) assay in the estimation of antioxidant value of botanicals

Mohamed Rafiq, Mohammed Azeemuddin, Suryakant D. Anturlikar,  
Gollapalle L. Viswanatha, Pralhad S. Patki

R & D Center, The Himalaya Drug Company, Makali, Bangalore, India

Received June 6, 2012

Accepted August 20, 2012

Published Online October 3, 2012

DOI 10.5455/oams.200812.br.002

#### Corresponding Author

Mohamed Rafiq  
Department of Pharmacology,  
R&D Center, The Himalaya Drug  
Company, Makali, Bangalore-562 123,  
Karnataka, India.  
[dr.rafiq@himalayahealthcare.com](mailto:dr.rafiq@himalayahealthcare.com)

#### Key Words

Ayurveda; Botanicals;  
*Embllica officinalis*; Florescein;  
ORAC assay; Poyherbal formulation

#### Abstract

Present study was carried out to standardize the oxygen radical absorption capacity (ORAC) assay for the estimation of antioxidant value of botanicals indigenous to Indian subcontinent by taking Amalaki (*Embllica officinalis*) and an Ayurvedic proprietary polyherbal formulation (PHF) as a representative standard. The ORAC assay for ethanolic and aqueous (Organic Amla-C®) extracts of Amalaki and PHF were carried out using 96-well flat optical bottom clear black plates by the use of sensitive florescent multiple plate reader and fluorescein as a probe, vitamin C was used as a reference standard. The assay was found to be simple, versatile, reproducible, and can be widely used for estimating the total antioxidant activity of the herbs and propriety PHF. The study also highlights Amalaki and Himalaya PHF as rich sources of antioxidants. The antioxidant activities of Amalaki extracts were found to be slightly more than vitamin C and similarly the antioxidant activity of Himalaya PHF was comparable to that of vitamin C (in terms of total ORAC values).

© 2012 GESDAV

### INTRODUCTION

Chemical compounds and reactions capable of generating potential toxic oxygen species/free radicals are referred to as pro-oxidants. In contrast, compounds and reactions that disposing and/or scavenging and/or suppressing the formation and/or opposing the actions of the free radicals are called as anti-oxidants [1]. Oxidative stress can be defined as a disturbance in the pro-oxidant and anti-oxidant balance, in favor of the former leading to potential damage. The role of free radicals in the pathogenesis and progression of diseases and aging process is been well understood with the available literature [2].

The oxygen radical absorption capacity (ORAC) assay is a recent and widely accepted method in US for measuring the total antioxidant activity of samples of different origin [3]. It can be used to measure the antioxidant activities of pharmaceutical products, food products, agricultural products, human and animal serum samples [3, 4]. ORAC assay provides the

measure of the scavenging capacity of antioxidants against the peroxy radical, which is one of the most common reactive oxygen species found in the body [4]. The ORAC assay can be simultaneously used to measure the antioxidant activity of all the antioxidants inherently present in a mixture and also it can be carried out for water insoluble mixtures [3, 4]. These results can be further deduced to measure the fast and slow acting antioxidants for a qualitative as well as quantitative analysis [3-5].

Determining the ORAC value gives both qualitative and quantitative measure of free radical scavenging and antioxidant activity of a test drug. Trolox, a water-soluble vitamin E analogue is used as a calibration standard and the ORAC value is expressed as micromoles of Trolox equivalents per grams of the sample ( $\mu\text{mol TE/g}$ ). ORAC value is the direct measure of antioxidant capacity of the sample or drug. Synonymously, higher ORAC value higher the antioxidant capacity and vice versa [6, 7].

In recent years, the ORAC assay has been widely accepted as a tool for antioxidant assessment, and has been proposed as a method for comparing and standardizing nutritional supplements. In this context we have carried out a series of studies in our laboratory to standardize and validate the ORAC assay for determining antioxidant capacity of herbal products of Indian subcontinent and indigenous patent and proprietary products claiming to have free radical scavenging/antioxidant activity.

With this background, the present study was aimed to standardize and validate the ORAC assay and thereby determine the ORAC values of aqueous (Organic Amla-C®) and ethanolic extracts of Amalaki and a polyherbal formulation (PHF) manufactured by M/s Himalaya Drug Company, Bangalore, India, which is composed of extracts of *Mangifera indica*, *Withania somnifera*, *Daucus carota*, *Glycyrrhiza glabra*, *Vitis vinifera*; powders of *Embllica officinalis*, *Syzygium aromaticum*; Yashad Bhasma and oil of *Triticum sativum*.

## MATERIALS AND METHODS

### Chemicals and instruments

2,2'-Azobis (2-methylpropionamidine dihydrochloride) (AAPH; Across), fluorescein sodium (Fluka), Trolox (Sigma-Aldrich) and other chemicals used were of analytical grade and purchased from standard companies. Corning costar 96-well optical bottom black plates, Synergy™ HT fluorescence multiplate reader (Bio-Tek Instruments) and calibrated fine micro pipettes. The ethanolic and aqueous (Organic Amla-C®) extracts of Amalaki were procured from Phytochemistry Department and Polyherbal formulation (PHF) was obtained from Formulation Development Department, R&D Center, The Himalaya Drug Company, Bangalore, India.

### Sample preparation

**Aqueous samples;** the test drug samples were weighed and dissolved in known quantity of phosphate buffer and centrifuged at 12,000g for 10 min at 4°C to remove any undissolved particulates. The supernatant was collected and diluted with phosphate buffer if required. Certain liquids such as juice extracts may be tested without dilution

**Solid or high protein samples;** solid samples were weighed and homogenized after adding the phosphate buffer (1:2 w/v). The homogenate was centrifuged at 12,000g for 10 min at 4°C. The supernatant containing water soluble fractions, insoluble fraction (residue) was separately recovered and washed with phosphate buffer. The collected supernatant samples were combined and used for the assay either directly or after

diluting with the phosphate buffer. The residue was further extracted with pure acetone (1:4, w/v) at room temperature for 30-60 min. The contents were centrifuged at 12,000g for 10 min at 4°C. The supernatant layer containing acetone extract was collected and used for the assay after diluting with the phosphate buffer.

The total ORAC value is calculated by combining the ORAC values of the water soluble fraction with the ORAC value of acetone extract of residue fraction.

### Assay procedure

The stock solution and dilutions of the test drug were prepared in 0.075 M phosphate buffer (pH 7.4) and the assay was carried out in Synergy™ HT fluorescence multiplate reader (Bio-Tek Instruments) using fluorescein as a probe.

Twenty five microliters (25 µl) of Trolox (1 mM) (standard antioxidant) or test drug solution were dispensed in to the 96-well micro titer plate and 150 µl of the fluorescein (4 µM) solution (1:1000 dilution of 2<sup>nd</sup> stock) was added to each well using dispenser/syringe-1 and mixed thoroughly and the plate was incubated for 30 min at 37°C in Synergy™ HT multi-detection microplate reader. After 30 min incubation 25 µl of AAPH (153 mM) was added to all the wells through dispenser/syringe-2 of the instrument, the contents of the micro plate were mixed thoroughly by shaking the plate at maximum intensity for 5-10 seconds.

Immediately after the addition of AAPH, the fluorescence of sample and standard wells were monitored kinetically at every minute for 60 min using Synergy™ HT multi-detection microplate reader with a excitation and emission wave lengths of 485 nm and 528 nm respectively and The plate reader was controlled by using KC4™ software version 3.4 [4].

**Preparation of antioxidant standard curve;** various concentrations of Trolox (0-50 µM) were prepared by diluting 0.2 mM stock of standard Trolox with 0.075 M phosphate buffer. Dilutions are given in Table.1.

**Table. 1.** Preparation of various dilutions of Trolox

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

**Calculation of ORAC value;** ORAC values were calculated using the area under the curve (AUC) and the net AUC of the standard and the samples, which were determined using KC4™ software (Bio-Tek Instruments, Winooski, VT, USA) or with the help of MS Excel software [4]. The AUC was calculated for standard and test samples using the final assay values and the linear regression formula. ORAC value for the test drug is been calculated by a 6-step procedure given below:

**Step 1:**

$AUC = 0.5 + (R_2/R_1) + (R_3/R_1) + (R_4/R_1) + \dots + 0.5(R_n/R_1)$  (**Equation 1**) where  $R_1$  is the relative fluorescence reading at the initiation of the reaction and  $R_n$  is the last measurement)

**Step 2:**

Net AUC is calculated by subtracting the blank AUC from the AUC of each sample and standard as depicted in Fig.1

**Step 3:**

Net AUC = AUC of test/standard – AUC of blank (**Equation 2**)

**Step 4:**

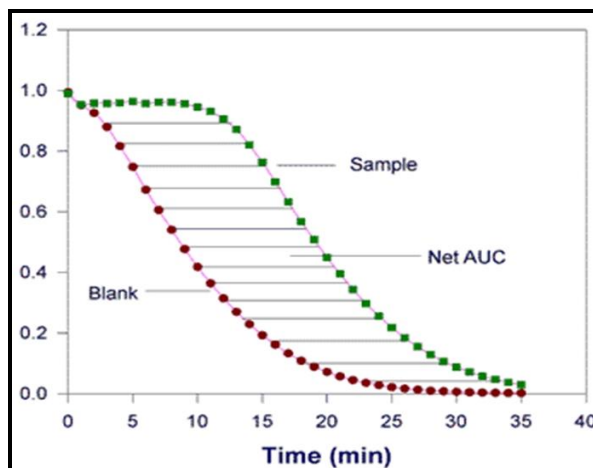
The Net AUC was plotted on the Y-axis against Trolox antioxidant standard concentration on the X-axis

**Step 5:**

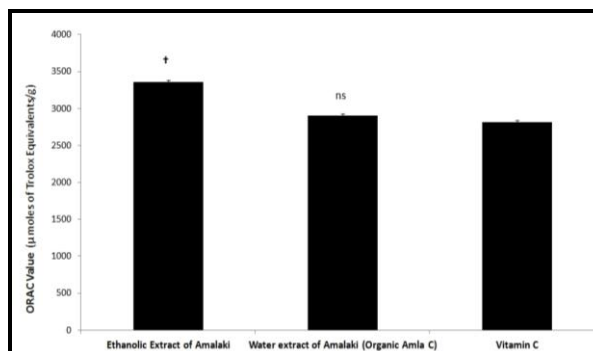
Trolox equivalent (TE) in  $\mu\text{mol}$  of unknown sample was calculated by comparing the standard curve; results (ORAC value) were expressed as TE per gram or liter of sample (TE/g or TE/l)

**Step 6:**

The total ORAC value was calculated by combining the ORAC value of the water-soluble fraction ( $\text{ORAC}_{\text{Hydrophilic}}$ ) and the ORAC value of the acetone extract of the residue fraction ( $\text{ORAC}_{\text{Lipophilic}}$ )



**Figure 1.** Picture depicting the net AUC

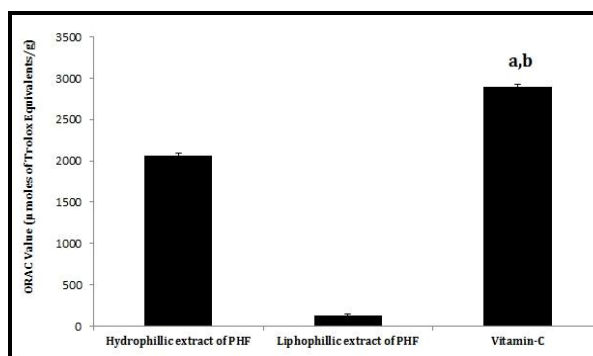


**Figure 2.** Comparative total ORAC values of different extracts of Amalaki. Values are expressed as mean  $\pm$  SEM and compared by ANOVA followed by Dunnet's test;  $^{\dagger}p < 0.01$  compared to Vit.C. [ns, not significant]

## RESULTS

In present study, each test sample was analyzed in triplicate and the ORAC values obtained were compared against the ORAC value of the reference standard, vitamin C. The co-efficient of variation (%CV) or relative standard deviation between the mean values were less than 20. The mean ORAC values were graphically represented (Figs.2&3).

The ethanolic and water extracts of dried Amalaki fruits were tested for their ORAC value and the mean ORAC values were found to be 3360.6 and 2903.2  $\mu\text{mol TE/g}$  respectively. Both ethanolic and water extracts of Amalaki were completely miscible in water and lipophilic fraction ( $\text{ORAC}_{\text{Lipophilic}}$ ) did not yield any ORAC value; therefore the ORAC value of water soluble portion ( $\text{ORAC}_{\text{Hydrophilic}}$ ) was considered as total ORAC value, while vitamin C has yielded an ORAC value of 2819  $\mu\text{mol TE/g}$  which was comparable to water extract of Amalaki. However, ethanolic extract of Amalaki was found to be more potent than vitamin C.



**Figure 3.** ORAC values of polyherbal formulation (PHF). Values are expressed as mean  $\pm$  SEM and compared by ANOVA followed by Dunnet's test:  $p < 0.01$  compared to  $^a$ hydrophilic and  $^b$ lipophilic extract of PHF.

In another set of experiment, a PHF was tested for its ORAC value, it was found that water soluble portion and lipophilic fraction of PHF yielded an ORAC value of 1960.9 and 123.9  $\mu\text{mol TE/g}$ , respectively. Therefore the total ORAC value of PHF was found to be 2084.9  $\mu\text{mol TE/g}$ .

## DISCUSSION

The ORAC assay provides the measure of antioxidant capacity of test drugs against peroxy radicals, a most commonly found reactive oxygen species in the body. Since ORAC assay represents the hydrogen atom transfer mechanism, it closely resembles to human biology and also has biological relevance to the *in vivo* antioxidant efficacy. Because of its sensitivity, specificity and biological relevance with the human body it is most widely accepted method for comparing and standardizing the nutritional supplements. In the year 2007, United States department of Agriculture (USDA) has released list ORAC values of various foods commonly available in the US, endorsing the importance of ORAC assay [8].

Furthermore, ORAC assay can be used as a tool for determining the antioxidant capacity of the biological fluids such as sera and urine, which may give direct measurement of antioxidant status of the system [9], thus ORAC assay can be used for various types of samples including crude drugs and biological fluids for assessing their antioxidant status [10].

In this context, in the present study ORAC values of extracts of Amalaki and PHF were determined to standardize and validate the ORAC assay. The findings of the study showed that the extracts of Amalaki were found to be slightly more potent than vitamin C; while, the antioxidant activity of PHF was found to be comparable with vitamin C (in terms of average ORAC values).

In conclusion, the ORAC assay is relatively recent and simple method for measuring the antioxidant activity of not only the herbs and nutritional supplements but also an important clinical tool for assessing the antioxidant activity of biological fluids such as serum, urine *etc.* In present study, an attempt was made to estimate the antioxidant activity of botanical preparations indigenous to India, and it was found that ORAC assay can be simple and versatile method for estimating the antioxidant activity of these preparations. This study proves the potent antioxidant activity of Himalaya PHF and Amalaki extracts reported elsewhere by other methods.

## ACKNOWLEDGEMENT

Authors are thankful to the M/s The Himalaya Drug Company, Bangalore, for providing all the necessary facilities to carry out the research work.

## COMPETING INTERESTS

Authors declare no conflicts of interest

## REFERENCES

1. Irshad M, Chaudhri PS. Oxidant-antioxidant system: role and significance in human body. *Indian J Exptl Biol* 2002; 40:1233-9.
2. Sies H. Oxidative stress: Introduction. In: Sies H (ed) *Oxidative Stress, Oxidants and Antioxidants*, Academic Press, London, UK, pp xv-xvi, 1991.
3. DeLange RJ, Glazer AN. Phycoerythrin fluorescence-based assay for peroxy radicals: a screen for biologically relevant protective agents. *Analyt Biochem* 1989; 177:300-6.
4. Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic Biol Med* 1993; 14:303-11.
5. Ou B, Hampsch-Woodill M, Flanagan J, Deemer EK, Prior RL, Huang D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J Agric Food Chem* 2002; 50:2772-7.
6. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA* 1993; 90:7915-22.
7. Huang D, Ou B, Prior R. The chemistry behind antioxidant capacity assays *J Agric Food Chem* 2005; 50:4437-44.
8. Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods. U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD, USA, pp 1-34, 2007.
9. McKay DL, Perrone G, Rasmussen H, Dallal G, Hartman W, Cao G, Prior RL, Roubenoff R, Blumberg JB. The effects of a multivitamin/mineral supplement on micronutrient status, antioxidant capacity and cytokine production in healthy older adults consuming a fortified diet. *J Am Coll Nutr* 2000; 19:613-21.
10. Dogra D, Ward N, Croft KD, Mori TA, Barret HR, Hermann SE, Ashlwy BI, Watts GF. Oxidant stress in nephritic syndrome: comparison of F2-isoprostanes and plasma antioxidant potential. *Nephrol Dial Transplant* 2001; 16:1626-30.