

# A Novel and Significant Method for Antioxidant Activity Utilizing Microtitre-plate (Resazurin Reducing Power Assay)

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**Abstract:** There are number of methods used for detection of antioxidant activities. We have developed a new method using a compound (resazurin) which undergoes a visible colour shift by chemical or physical interaction with ascorbate and other antioxidant compounds. This novel method was developed to measure the antioxidant activity using the resazurin dye in microtitre- plate. The experiment protocol, which is rapid and inexpensive, ensures sensitivity and reproducibility in the measure of antioxidant activity of hydrophilic or water soluble antioxidant compounds. This method is able to achieve more accuracy in the determination of the minimum antioxidant concentration (MAC) values of natural products, including crude extract, chromatographic fractions or purified compounds comparing with ascorbic acid and other standard antioxidant. Therefore, in our opinion this procedure can quickly provide useful information on the antioxidant contents of foods and plants extracts using a very small sample quantity.

**Keywords:** Antioxidant, minimum antioxidant concentration, microtitre-plate, resazurin, visible colour shift.

## 1. INTRODUCTION

Antioxidant compounds in food component play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Many synthetic antioxidants such as BHA (Butylated Hydroxy Anisole), BHT (Butylated Hydroxy Toluene) are very effective but they possess certain health risks and toxic properties to human health. Therefore, the search for natural antioxidants of plant origin has gained momentum in recent years [1]. Primary sources of naturally occurring antioxidants are whole grains, fruit and vegetables. The health benefits of citrus fruits have mainly been attributed to the presence of bioactive compounds, such as phenolics, ascorbic acid and carotenoids [2]. Moreover the plant sourced food antioxidants like vitamin C, Vitamin E, carotenes, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Hence most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Antioxidant compounds like phenolic acids, polyphenols and flavanoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxy and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

Hence, studies on antioxidants present in plants and foods have come to be one of the most popular topics of phytochemistry research. Ironically, the biggest problem is the lack of validated assay that can reliably measure the antioxidant capacity of foods and biological samples [3]. Accordingly, many assays for the investigation of antioxidant activ-

ity have been developed and applied. The majority of studies have used assays with spectrophotometry, such as TBA (Thiobarbituric acid assay),  $\beta$  carotene bleaching, conjugated diene, DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), FTC (ferric thiocyanate), FOX (ferrous oxidation-xylenol orange) and FRAP (ferric reducing/antioxidant power) [4]. However, some spectrophotometry assays have problems with substances exhibiting UV wavelengths similar to that the test chemical, overall causing interference of the chemical being tested. Moreover, some other such as MA/HPLC (monoaldehyde/high-performance liquid chromatography) and MA/GC (monoaldehyde/gas chromatography) assays have been developed which are highly specific and selective but costly.

Therefore, the present study was undertaken to evaluate antioxidant activities of plants and their components by developing a new, rapid, inexpensive and reproducible method using resazurin where redox status leading to the two step color change [5]. The 'resazurin reduction test' has been used for about 50 years to monitor bacterial and yeast contamination in milk, and also for assessing semen quality [6]. Recently, the dye has gained popularity as a very simple and versatile way of measuring cell proliferation and cytotoxicity [7]. Resazurin seems to be the original name for Alamar Blue, before it had been used for *in vitro* mammalian cell proliferation and assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis* [8]. There is a direct correlation between the reductions of resazurin and antioxidant activity of the samples.

## 2. MATERIALS AND METHODS

### 2.1. Solution Preparation of Resazurin Dye

The resazurin solution was prepared by dissolving resazurin tablet (270 mg) in 27 ml of sterile distilled water. A homogenous solution was prepared using a vortex mixer [9].

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## 2.2. Preparation of Microtitre Plates

A sterile 96 well plate was taken. A volume of 50 $\mu$ l of test materials was taken from a stock solution of sample and was pipette into the first row of each column. Then, 50 $\mu$ l of normal saline was added in all wells. Serial dilutions were performed using multichannel pipette in such a way that each well had 50 $\mu$ l of the test material in serially descending concentrations. To each well 10 $\mu$ l of resazurin indicator solution was added. Each plate had a set of controls and a column with a standard antioxidant as positive control. In this assay, ascorbic acid was used as positive control. The plates were prepared in triplicate and placed at normal room temperature. The lowest concentration amount of sample at which colour change occurred was taken as the MAC (Minimum antioxidant concentration) value. Results are easily determined visually by reading the change to a stable color from blue to pink. The results can also be determined by spectrophotometry or fluorometry, but the cost of equipment is high and would limit its application in low-resource countries. The average of three values was taken to calculate the MAC for the test material as well as positive control.

## 3. RESULT AND DISCUSSION

When the antioxidant activity of biological material is to be evaluated, one of the major problems is the choice of method of analysis because usually they are specific for one property [10]. Many chemical analyses are based on the ability of inhibiting the oxidation of a target substrate initiated by free radical, for example super oxide anion, hydroxyl and peroxy radicals. A standard protocol was developed to assess the antioxidant activity using the resazurin dye in microtitre plate and ascorbic acid as positive control. Moreover, this new alternative method seem to have the potential to

provide rapid detection of antioxidant sample and do not need any sophisticated equipment, is simple to perform, reduce the time to report first results compared to classical conventional methods. Therefore it could be implemented in laboratories with limited resources. In the summary, this study shows that using this low cost method for rapid detection of antioxidant activity with the high level of agreement.

### 3.1. Principle

The principle of the assay is that resazurin (blue and non fluorescent) is an oxidation-reduction indicator which becomes pink and highly fluorescent when reduced to resorufin. Resorufin is further reduced to dihydroresorufin (un-colored and non fluorescent) (Fig. 1). Moreover, the antioxidant compounds which are able to transfer the hydrogen to resazurin quench the color and produce a decoloration of the solution which is proportional to their amount. This reaction is rapid (less than 12 minutes) and the end point, which is stable, is taken as measure of the antioxidative efficiency. Hence, the present assay is based on the theory that a hydrogen donor is an antioxidant and the mechanisms associated with this assay are the same as those of the reducing power assay (RPA).

### 3.2. Standardization of Method

The preliminary experiments showed that the choice of standard solution and the ratio between the concentration of resazurin and the concentration of the compound are crucial for the effectiveness of the method. To evaluate the sensitivity of the method, the system was tested by using different concentrations of ascorbic acid and resazurin dye. The colour changed from purple to pink and colorless with the increased

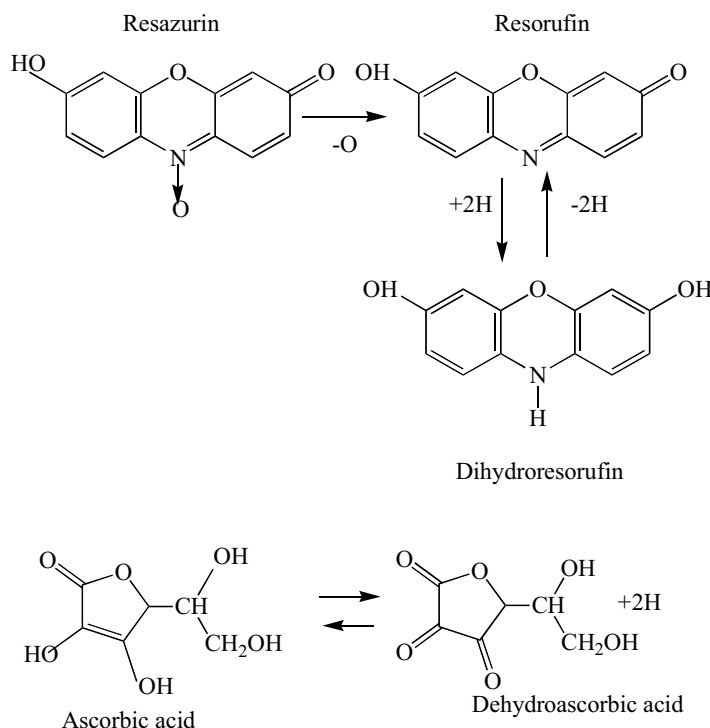
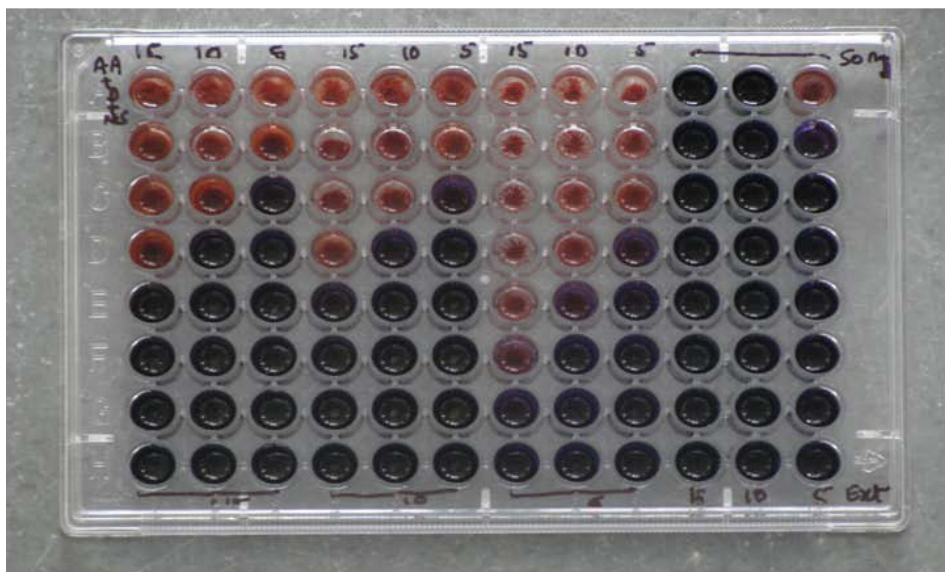
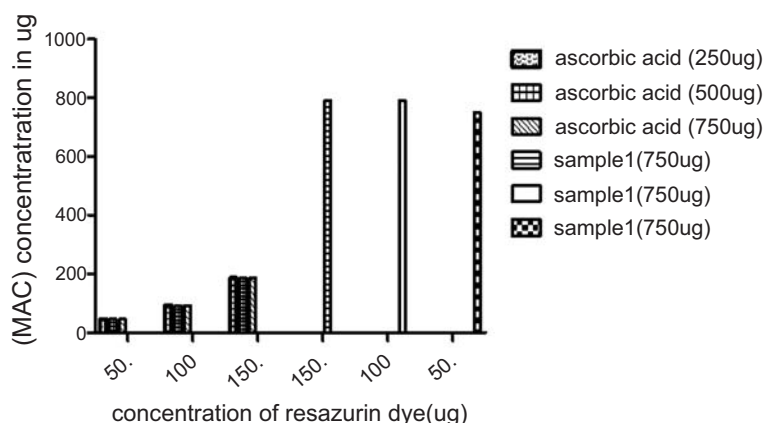


Fig. (1). Structural representation of chemical reaction of Resazurin with ascorbic acid.



**Fig. (2).** Standardization of assay by using different concentration of resazurin and ascorbic acid

Column 1-3 (Asc-750µg, 500µg, 250µg; **Res-150µg**); Column 4-6 (Asc-750µg, 500µg, 250µg; **Res-100µg**); Column 7-9 (Asc-750µg, 500µg, 250 µg; **Res-50µg**); Column 10-12 (Sample-750µg, 750µg, 750µg; **Res-150µg, 100µg, 50µg**)  
Asc- Ascorbic acid; Res- Resazurin dye.



**Fig. (3).** (Minimum antioxidant concentration) MAC of Ascorbic acid in relation to different concentration of resazurin dye

antioxidant activity. The result of critical standardization of concentration of resazurin and ascorbic acid for assay was shown in Fig. (2) which was further explained in Fig. (3). A change in color from blue to pink and then colorless indicated the antioxidant activity of sample and the MAC was interpreted as in the microtitre plate as given in formatted Table 3. Since the concentration of resazurin solution was critical in this assay. By this method the antioxidant activity of small volumes can also be determined by reduced volume of resazurin (5µl). Similarly, this assay was further tested at different ranges of pH by previously standardized concentrations of both resazurin dye and ascorbic acid. At pH range (5-8), resazurin is reduced by ascorbate spontaneously but at low pH the resazurin reduce rapidly even by minimum quantity of ascorbic acid (Fig. 4).

### 3.3. Calculation of MAC

Antioxidant activity has been expressed in various ways including the minimum amount of antioxidant sample used

to reduce the constant amount of fluorescent dye. 'The constant amount' is a minimum amount of resazurin (50µg) recovered after standardization of method which can be used for evaluation of minimum sample volume. An easier way to present antioxidant activity of foods and other extracts would be to reference a common reference standard, known as ascorbic acid. Hence, the antioxidant capacity of an unknown sample was expressed as ascorbic acid equivalence MAC (Minimum antioxidant concentration) by the following equation:

$$\text{MAC}_{\text{Asc/Resaz}} = S$$

S = Minimum amount of unknown sample which shows antioxidant activity in microtitre plate.

Moreover, the antioxidant percentage capacity of an unknown sample was expressed, with respect to ascorbic acid by the:-

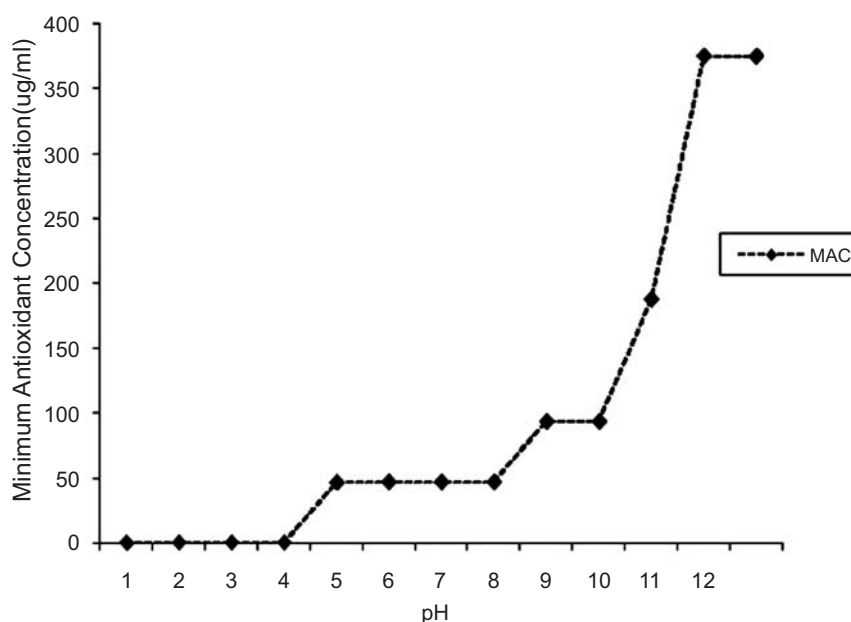


Fig. (4). Effect of various pH ranges on MAC (Minimum antioxidant concentration) of Ascorbic acid.

Table 1. Antioxidant activity in percentage of different fungal endophyte extracts with equivalence to ascorbic Acid (100%).

Name of the Fungal Endophyte	Solvent	Nitric Oxide Radical Scavenging Activity	Reducing Power Assay	Hydroxyl Radical Scavenging Activity	Resazurin Reducing Power Assay
<i>Aspergillus versicolor</i>	Acetone	65.96	49.36	32.37	57.61
	Methanol	44.69	45.63	32.67	50.11
	Aqueous	49.08	44.38	21.08	50.11
<i>Emericella nidulans</i>	Acetone	60.94	46.04	36.72	57.61
	Methanol	49.80	45.83	36.75	50.11
	Aqueous	54.36	36.72	25.88	38.50
<i>Penicillium chrysogenum</i>	Acetone	40.83	7.99	26.9	10.96
	Methanol	66.06	23.42	21.46	21.93
	Aqueous	40.14	17.79	18.89	12.53
<i>Phoma sp.</i>	Acetone	58.58	35.73	21.79	33.50
	Methanol	46.53	35.28	34.84	33.80
	Aqueous	43.37	34.95	22.39	28.82

Percentage Antioxidant capacity/equivalence to ascorbic acid =  $100 \times A / S$

A = Minimum amount of ascorbic acid which shows antioxidant activity in microtitre plate.

Minimum Antioxidant Concentration (MAC) is the lowest concentration of food and plant sample that will change the visible blue color of Resazurin to fluorescent pink or colorless after 10 minutes period of incubation at room temperature. A lower MAC is an indication of a better antioxidant agent. In addition, 'Percentage Antioxidant capacity' is an antioxidant value of sample in percentage with equivalence to ascorbic acid whose antioxidant value considers being 100%.

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### 3.4. Antioxidant Power of Endophytic Fungal Extracts

Plants and their endophytes extracts were widely studied for its antioxidative properties due to their chemical constituents. The various samples extracts of fungal endophytes associated with *Salvadora oleoides* were selected to test the effectiveness of RRP (Resazurin reducing power assay) in comparison to other methods. The 12 sample extracts tested for *in vitro* percentage antioxidant activity equivalence to ascorbic acid (100%) by RRP and other three potential

**Table 2.** Comparison of antioxidant activities of food samples with RRPA and ORAC methods.

Sr no.	pH Value	Food	Antioxidant Activity (Ascorbic Acid /250 µg)	
			RRPA unit	ORAC Value / 5g
1	4.50	Ascorbic acid	30	–
2	4.50	Orange juice	10	37.50
3	8.00	Spinach juice	20	60.50
4	5.30	Tomato juice	30	79.50
5	3.50	Lemon juice	30	94.6
6	8.00	Garlic juice	30	96

RRPA Unit = No. of wells in column in which color of resazurin changed by antioxidant  $\times$  10.  
Standard = Ascorbic acid (250 µg).

**Table 3.** MAC values data based on the changed color of resazurin. (Bold values are the MAC values).

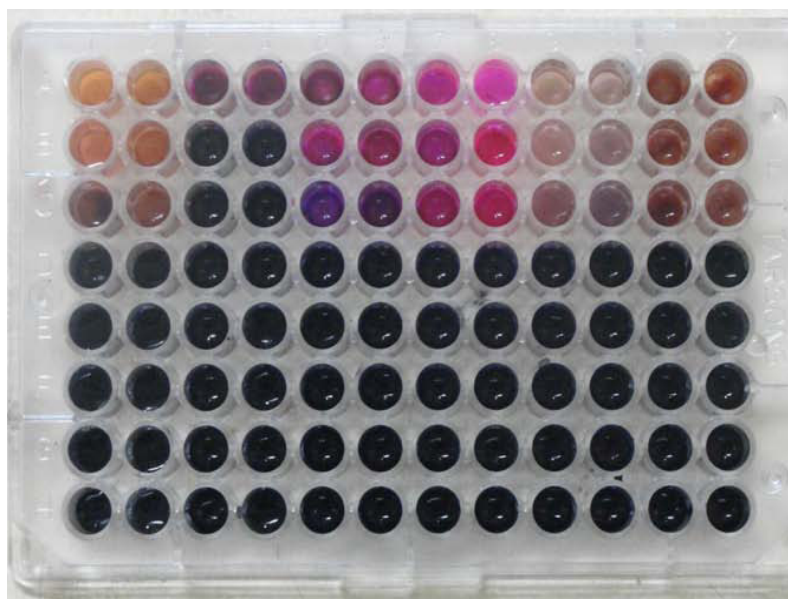
Sr no.	Column no. 1	Column no. 2	Column no. 3
A	Asc-750µg+ Res-150µg	Asc-500µg+ Res-150µg	Asc-250µg+ Res-150µg
B	Asc-375µg+ Res-150µg	Asc-250µg+ Res-150µg	<b>Asc-125µg+ Res-150µg</b>
C	Asc-187.5µg+ Res-150µg	<b>Asc-125µg+ Res-150µg</b>	Asc-62.50µg+ Res-150µg
D	<b>Asc-93.75µg+Res-150µg</b>	Asc-62.50µg+ Res-150µg	Asc-31.25µg+ Res-150µg
E	Asc-46.88µg+ Res-150µg	Asc-31.25µg+ Res-150µg	Asc-15.63µg+ Res-150µg
F	Asc-23.44µg+ Res-150µg	Asc-15.63µg+ Res-150µg	Asc-7.81µg+ Res-150µg
G	Asc-11.72µg+ Res-150µg	Asc-7.81µg+ Res-150µg	Asc-3.90µg+ Res-150µg
H	Asc-5.86µg+ Res-150µg	Asc-3.90µg+ Res-150µg	Asc-1.95µg+ Res-150µg

methods which are indicated in Table 1. The correlation was observed between resazurin reducing power assay and the three other assays which were nitric oxide radical scavenging assay, reducing power assay, hydroxyl radical scavenging assay. Statistically maximum correlation was observed with the reducing power assay. It was  $0.99 \pm 0.07$ ,  $p=0.006$  for the methanol extracts of fungi in reducing power assay. The acetone and the aqueous extract also showed almost significant similar correlation with the reducing power assay i.e.  $0.97 \pm 0.16$  and  $0.97 \pm 0.17$ . However this novel assay showed no significant correlation with the other two assays compared here. For example, the present novel assay showed inappropriate correlation about  $\frac{1}{4}$  with the nitric oxide radical scavenging assay as compared in case of acetone extract of *Penicillium chrysogenum*. The reason may be that acetone extract or others include lot of antioxidant compounds but worked on different principles. Therefore various assays showed insignificant correlation with each other due to dissimilar principles.

### 3.5. Comparison of Analysis of Antioxidant Activity in Food Samples

Food used in regular diet was widely studied for its antioxidant components. The food samples were selected to test the effectiveness of RRPA method on the basis of (ascorbic acid) equivalence and the results have been depicted in Fig

(5). The antioxidant compounds present in food samples are mainly hydrophilic; therefore the antioxidant activities of food samples could be well evaluated by RRPA method. As we know that acids always act as antioxidant because they can easily reduce to other compounds and should be in healthy range of pH for medicinal uses. Therefore acids will exhibit positive results due to principle involved in the present assay. The effect of pH has been shown in Fig. (4). Moreover, it is impractical that the coloured sample influenced the dark blue colour of resazurin but can be slightly alter the light pink or colourless resorufin and dihydroresorufin respectively. The comparison of antioxidant activity tested in six food samples by RRPA and ORAC methods have been presented in Table 2. Together with the ORAC value/5gm measured using the ORAC method. As we know, the ORAC determination is the most popular method for the measurement of antioxidant activity in certain foods, herbs and spices both physiologically and chemically. The main difference between the ORAC determination method and our RRPA method presented here is that the ORAC method requires fluorescence detector and takes a longer time period as compared to our method (RRPA). Moreover, the former method requires more quantity of sample as compared to later method which can determine the activity of even very small amount of sample. There is no evidence that free radicals are involved in this reaction and ORAC values have any



**Fig. (5).** Antioxidant values of Vegetables and fruits juice with RRPA (Resazurin Reducing Power Assay) methods  
Column 1-2 (Ascorbic acid-250 $\mu$ g; **Res-50 $\mu$ g**); Column 3-4 (Orange juice-50 $\mu$ l; **Res-50 $\mu$ g**); Column 5-6 (Spinach juice-50 $\mu$ l; **Res-50 $\mu$ g**);  
Column 7-8 (Tomato juice-50 $\mu$ l; **Res-50 $\mu$ g**); Column 9-10 (Lemon juice-50 $\mu$ l; **Res-50 $\mu$ g**); Column 11-12 (Garlic juice-50 $\mu$ l; **Res-50 $\mu$ g**).  
Res- Resazurin dye.

biological significance following consumption of any food. Moreover, the relationship between ORAC values and a health benefit has not been established. For examples, some plants also contain beneficial compounds with no ORAC value, such as xanthenes, minerals and fibers.

### 3.6. The Advantages and Drawbacks of Method (RRPA)

A number of assays have been described for estimation of total antioxidant status in food, drug and pharmaceutical products which are based on either of the techniques- spectrophotometric, fluorimetric or chemiluminescence. Antioxidant assay based on microtitre plate method is an easy and efficient method because it reduced the time, cost and less volume needed for its detection. Moreover, this microtitre based method can estimate the amount of antioxidant compounds in sample solution by comparing with the known concentration of standard (ascorbic acid and others). The main drawback of the microtitre based present method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants were used.

### 3.7. Application of the Method (RRPA)

The RRPA can quickly provide useful information on the antioxidant contents of foods and plant extracts. In addition, this method can be applied to the measurement of total antioxidant activity (TAA) in orange and Grapefruit juices by carrying out a study of change in TAA during several days of storage. Therefore this method is used to evaluate the TAA of commercial fruit juices, in which ascorbic acid is a principal component with a limit of quantification up to 62.50  $\mu$ g. Moreover, this method can estimate the antioxidant properties of wines which contain the phenolic components. Finally, due to the microplate adaptation of RRPA method described here is cost effective and would be useful

in epidemiological studies, where large number of samples were to be analyzed.

### CONCLUSION

It is reasonable to expect that rich antioxidant foods have greater potential to reduce free radicals in the body compared to low antioxidant foods. Thus it is important to know the antioxidant content of foods, in addition to knowing the basic nutritional information. Hence, in the present study a new method is reported to measure antioxidant power based on the reduction of resazurin. The assay is particularly suitable for a large scale screening of watery food extracts. It is cheaper and less laborious compared to all methods used to measure the antioxidant activity. Moreover, it is the correct assay to assess the antioxidant potential of extracts and compounds which is important for generating high quality data with greatest accuracy, speed and efficiency, enabling the addition of potential new antioxidant compounds and extracts to our armamentarium. However, this microtitre plate-based assay reduces the assay time by analyzing forty-four samples in one hour. It can be concluded that the RRPA is the cost effective method which would be useful in analysis of antioxidant activity where large numbers of samples are to be analyzed in small time with accuracy.

### CONFLICT OF INTEREST

None declared.

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