

# Modification of the time of incubation in colorimetric method for accurate determination of the total antioxidants capacity using 2,2-diphenyl-1-picrylhydrazyl stable free radical

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## ABSTRACT

2,2-diphenyl-1-picrylhydrazyl (DPPH)-based spectrophotometric detection of total antioxidant capacity (TAC) in samples is a common method. However, controversy exists for applying multiple absorption maximum wavelength and final concentration of DPPH used in the reaction mixture to measure TAC in terms of % of inhibition of DPPH (PID). Further, inconsistent spectrophotometric absorption values obtained during the incubation of sample with DPPH are also another drawback of this method. We tried to fix above issues by estimating the TAC in the tissue homogenate of *Heteropneustes fossilis*. Ascorbic acid was used as standard antioxidant in this study, and depending on tissues, the time of incubation of tissue extracts with DPPH (1.35  $\mu$ M optimum concentration) to obtain optimal consistent result was found to be 20–30 min. The absorption maximum of DPPH was found to be 516 nm as compared to the used wavelength ranging from 515 to 546 nm. The order of TAC in tissues of the fish was muscle (76% PID, 20–60 min incubation), gill (71% PID, 30 min incubation), and liver (49.9% PID, 30 min incubation). This report suggests that the incubation time of tissue extracts with DPPH is important and needs to be determined accurately to get consistent results.

## 1. INTRODUCTION

The major cytotoxic by-products in organisms during the oxidative metabolism are the free radicals. The reactive oxygen species (ROS) such as superoxide radical, hydroxyl ion radical, and hydrogen peroxide are some of the examples of the oxidants or free radicals produced in cells. They have deleterious effects as they oxidize all biomolecules present in their vicinity and cause oxidative stress (OS). In general, ROS oxidize lipids, proteins, and nucleic acids to lipid peroxides, protein carbonyls, and nucleic acid adduct, respectively. When the levels of the above compounds are elevated, it leads to cellular disturbances under OS condition. Elevated OS leads to tissue damage, protein misfolding, diseases susceptibility, and aging [1]. As a result of oxidation of the above biomolecules, the reduced efficiency of enzymatic and other functions of proteins, loss of membrane fluidity, unwanted modulation in gene expression, and complete or partial arrest in several anabolic processes occur in cells [2]. To counteract OS, antioxidants can donate an electron to ROS to make them chemically stable and inert. Antioxidants may be enzymatic

(superoxide dismutase, catalase, glutathione peroxidase, etc.) or non-enzymatic (Vitamins C and A, flavonoids, carotene, etc.) in nature [1]. Thus, the activities of the antioxidants along with OS parameters serve as biomarkers of OS physiology.

The total free radicals or the ROS scavenging activity by the antioxidant defense system are considered as a measure of the total antioxidant capacity (TAC) of tissues. Various analytical methods are employed to estimate the TAC potency and are classified into two categories: (i) Hydrogen atom transfer-based assays and (ii) single electron transfer-based assays [Table 1], [3-7]. In addition to the above assays, fluorimetric, electrochemical techniques, and chromatography techniques are also employed to estimate the TAC level. Molecules or probes such as 2', 7'-dichlorodihydrofluorescein diacetate, 1, 3-diphenylisobenzofuran, and dihydroethidium are used to estimate total ROS scavenging activity or TAC in cells [8]. However, the simplest, economic, and rapid result providing method among above is the 2,2-diphenyl-1-picrylhydrazyl (DPPH, C<sub>18</sub>H<sub>12</sub>N<sub>5</sub>O<sub>6</sub>, 394.33 g mol<sup>-1</sup>) scavenging assay which was first developed by Blois in 1958. To determine the TAC, the stable free radical DPPH is used. Its paramagnetic structure enables it to accept an unpaired electron or a free radical to become a stable diamagnetic structure. DPPH shows a strong absorption band at 517 nm due to its odd electron and the solution in alcohol appears a deep violet color but the absorption vanishes as the electron pairs off. The resulting decolorization is stoichiometric based on the number of electrons taken

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up in the reaction. About 0.5 mM alcoholic solution of DPPH is densely colored, and at this concentration, the Lambert-Beer law is obeyed over the useful range of absorption [9]. The unpaired free electron on the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants. Due to obtaining erroneous results, the original Blois method has been modified to bring out accuracy. It has been recommended to use the plastic cuvettes as it does not interfere with the methanolic or ethanolic extracts of the sample [10]. The initial DPPH concentration (50–100  $\mu$ M) should give absorbance values <1.0. The stock solution of DPPH slowly deteriorates; thus, using an automatic burette in a nitrogen atmosphere is recommended for minimizing the loss of free radical activity [9].

In addition, a variable range of absorption maximum such as 515 nm [11,12], 516 nm [13], 517 nm [14,15], 518 nm [16], 520 nm [17], and 546 nm for determining TAC using DPPH has been noticed [18]. The other improvement in the method includes an incubation time because DPPH takes time to interact with the weak antioxidants. The incubation time of DPPH with samples depends on the type of samples used as the later have varied range of antioxidants that may show different rate of reaction [19]. Besides the above modifications, still pitfalls exist related to instability and inaccuracy in estimating the TAC using DPPH [20]. First, the problem regarding the assay is with absorption maximum wavelength and the second problem is in getting stable and accurate replication values. It is because the absorbance increases with time and sometimes a negative absorbance is also obtained [20]. This problem could be with the sample incubation time (at 515–546 nm) with DPPH solution with different concentration (25–100 ppm) as proposed in literature [11-18]. We tried to modify the existing method of DPPH assay to solve above issues basically by

modifying the concentration of DPPH, time of incubation of DPPH with sample after determining its absorption maximum.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

The reagents such as DPPH and ethylenediaminetetraacetic acid (EDTA) were obtained from HiMedia, Mumbai. The reagents  $K_2HPO_4$ ,  $KH_2PO_4$ , and methanol were procured from Merck, Germany. All other chemicals of analytical grade were locally purchased. All the absorbance readings and spectral analysis were recorded at room temperature with a dual-beam UV-VIS spectrophotometer (PerkinElmer, UV/VIS – Lambda 365) using a 1 cm quartz cuvette.

### 2.2. DPPH Stock Solution and Tissue Extraction

The DPPH stock solution was prepared in methanol at a concentration of  $7.5 \times 10^{-2}$  M. The wavelength was scanned within a range of 400–800 nm to find out the absorption maxima and it was determined to be 516 nm [Figure 1]. To validate the TAC, ascorbic acid (100  $\mu$ M) was used as a positive control against the tissue homogenates. The fish *Heteropneustes fossilis* was procured from the local markets of Bhubaneswar, Odisha, India, for this experiment. The muscle, gill, liver, and accessory respiratory organ of the fish were dissected out immediately after the fishes were sacrificed using the protocols of Institutional Ethics Committee.

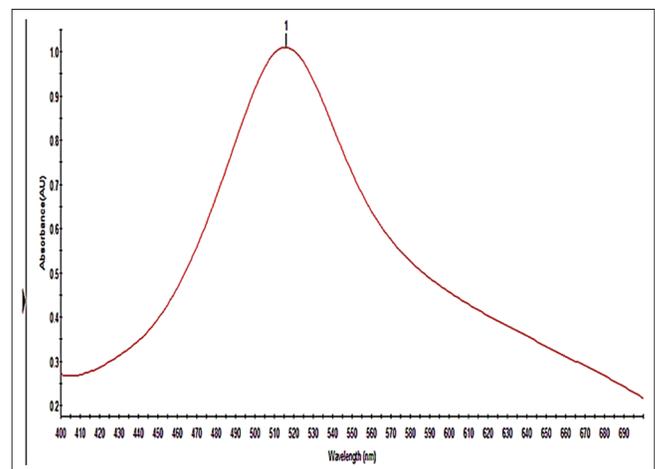
A 10% tissue homogenates of muscle, gill, liver, and accessory respiratory organ were prepared in 50 mM phosphate buffer containing 2 mM EDTA, pH-7.4 using pre-chilled mortar and pestle. The tissue homogenate obtained was collected as post-nuclear fraction [21]. The homogenates were then centrifuged at  $\times 1000$  g for 5 min at 4°C to obtain the final tissue supernatant extract that was used for the determination of TAC of tissues. The reaction mixture contained 1.6 mL of methanol with 200  $\mu$ L of freshly prepared DPPH solution and tissue supernatant. About 200  $\mu$ L of the obtained supernatant was incubated with 1.8 mL of  $1.35 \times 10^{-3}$  M ( $7.5 \times 10^{-2}$  M of 200  $\mu$ L in 1.6 mL methanol) DPPH in methanol. The final concentration of DPPH in the 2 mL of the reaction mixture was  $1.5 \times 10^{-3}$  M.

The reaction was initiated after adding 200  $\mu$ L of the tissue sample. The mixture was centrifuged (model 5430R Eppendorf, Germany) at 6000 rpm for 5 min at 4°C to pellet down the debris. DPPH-treated

**Table 1:** Assay methods employed to measure reactive oxygen species or oxidative stress status.

S. No.	Assays/methods	Mode of action	End-product determination
1.	Oxygen radical absorbance capacity ORAC	HAT	Loss of fluorescence of fluorescein
2.	Lipid peroxidation inhibition capacity	HAT	Colorimetry measuring TBARS
3.	Total radical trapping antioxidant parameter	HAT	Chemiluminescence quenching
4.	Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)	HAT	Loss of fluorescence of fluorescein
5.	Scavenging of H <sub>2</sub> O <sub>2</sub> radicals	HAT	Loss of fluorescence of fluorescein
8.	ABTS radical scavenging	HAT	Colorimetry
9.	Scavenging of superoxide radical formation by alkaline	HAT	Colorimetry
10.	Trolox equivalent antioxidant capacity	ET	Colorimetry
11.	Ferric reducing antioxidant power	ET	Colorimetry
12.	DPPH free radical scavenging	ET	Colorimetry
13.	Copper (II) reduction capacity	ET	Colorimetry
14.	Total phenols by Folin–Ciocalteu	ET	Colorimetry

HAT: Hydrogen atom transfer



**Figure 1:** Wavelength scan of 2,2-diphenyl-1-picrylhydrazyl showing the absorption maxima (Amax) at 516 nm.

tissue samples were incubated (the incubation hereafter is defined as the incubation of DPPH with ascorbic acid as positive control or incubation of DPPH with tissue extract) for 5, 10, 20, 30, 60, and 120 min in dark at room temperature and the absorbance of the samples was recorded after the above time intervals. The supernatant was collected and incubated for 20 min at room temperature and absorbance was recorded at 516 nm. The activity was then expressed as a percent of inhibition of absorbance, that is,  $(\text{Control OD} - \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}}) \times 100$  [22]. Data ( $n = 3$ )

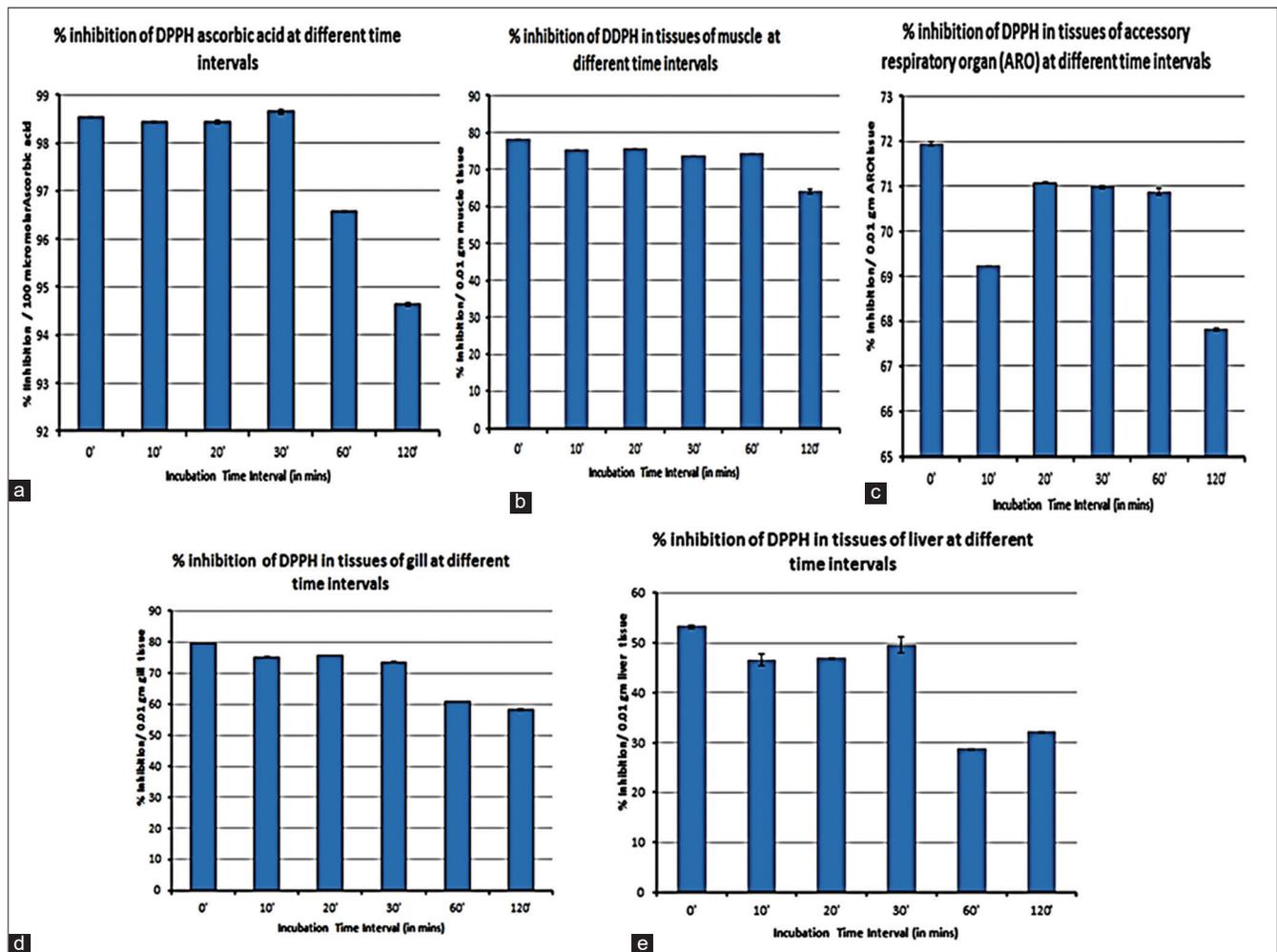
were presented as mean  $\pm$  SEM and subjected to ANOVA followed by Duncan new multiple range test to accept the statistical significance level at  $P < 0.05$ .

### 3. RESULTS AND DISCUSSION

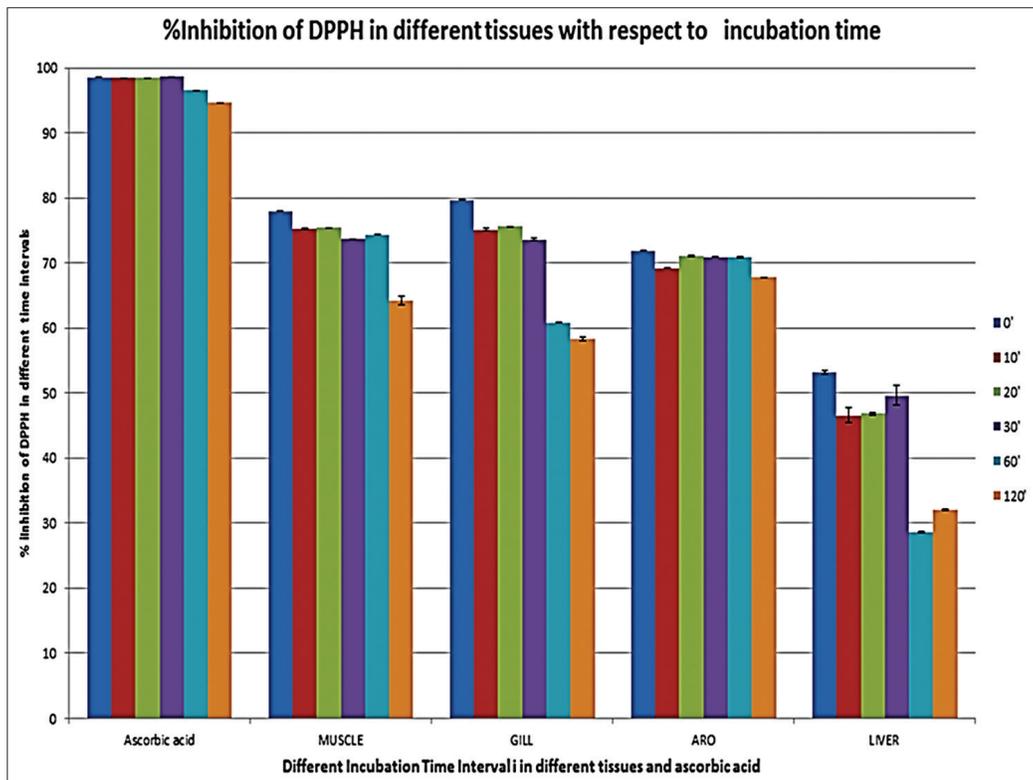
The absorbance of 75  $\mu\text{M}$  DPPH in methanol was  $\sim 1$  (1.0672) at 516 nm. It was obtained after scanning it within the visible range, that is, from 400 to 800 nm [Figure 1]. The result of the present study reflects that ascorbic acid

when used as positive control with DPPH, an increase in inhibition activity for TAC, that is, PID was noticed till 30 min, that is, 98.8% inhibitory effect by ascorbic acid. Therefore, the working condition of the assay system was confirmed. Incubation of DPPH with ascorbic acid for 60 and 120 min was resulted in significant decline of absorbance up to 96.6% and 94.8%, respectively [Figure 2a]. Ascorbic acid had also showed inhibition of 98.5% DPPH level at the initial time period of incubation (10 min) which was further increased to 98.8% at 30 min incubation time period.

The DPPH inhibitory effect in muscle tissue extract was observed up to 60 min incubation time period and 76% inhibition efficiency was observed by the tissue. The inhibition activity was alleviated after 60 min and was 64% at 120 min incubation time [Figure 2b]. The effective time of incubation for the muscle tissue was at 20 min [Figure 3]. About 71% DPPH inhibitory efficiency of accessory respiratory organ was noticed after 20 min of incubation that was retained until 30 min incubation time. At 60 min, it starts to decline and finally reached to 67.9 % inhibition PID value as compared to about 71% PID value obtained at 30 min incubation time [Figure 2c].



**Figure 2:** The radical scavenging activity of ascorbic acid and different tissues plotted in Y-axis against time of incubation in X-axis. Data are presented as mean  $\pm$  S.E.M ( $n = 10$ ). (a) Percentage inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by ascorbic acid at different incubation time intervals, (b) percentage inhibition of DPPH by methanolic extracts of muscle tissues at different incubation time intervals, (c) percentage inhibition of DPPH by methanolic extracts of accessory respiratory organ tissues at different incubation time intervals, (d) percentage inhibition of DPPH by methanolic extracts of muscle tissues at different incubation time intervals, (e) percentage inhibition of DPPH by methanolic extracts of liver tissues at different incubation time intervals. Data ( $n = 3$ ) were presented as mean  $\pm$  SEM and subjected to ANOVA followed by Duncan new multiple range test to accept the statistical significance level at  $P < 0.05$ .



**Figure 3:** Comparative radical scavenging activity of all tissues of *Heteropneustes fossilis* plotted at different incubation time. Red arrow mark shows the effective time of incubation which exhibits maximal inhibitory effect for different tissues, that is, 20 min for muscle along with gill tissues and 30 min for accessory respiratory organ and liver tissues. Data ( $n = 3$ ) were presented as mean  $\pm$  SEM and subjected to ANOVA followed by Duncan new multiple range test to accept the statistical significance level at  $P < 0.05$ .

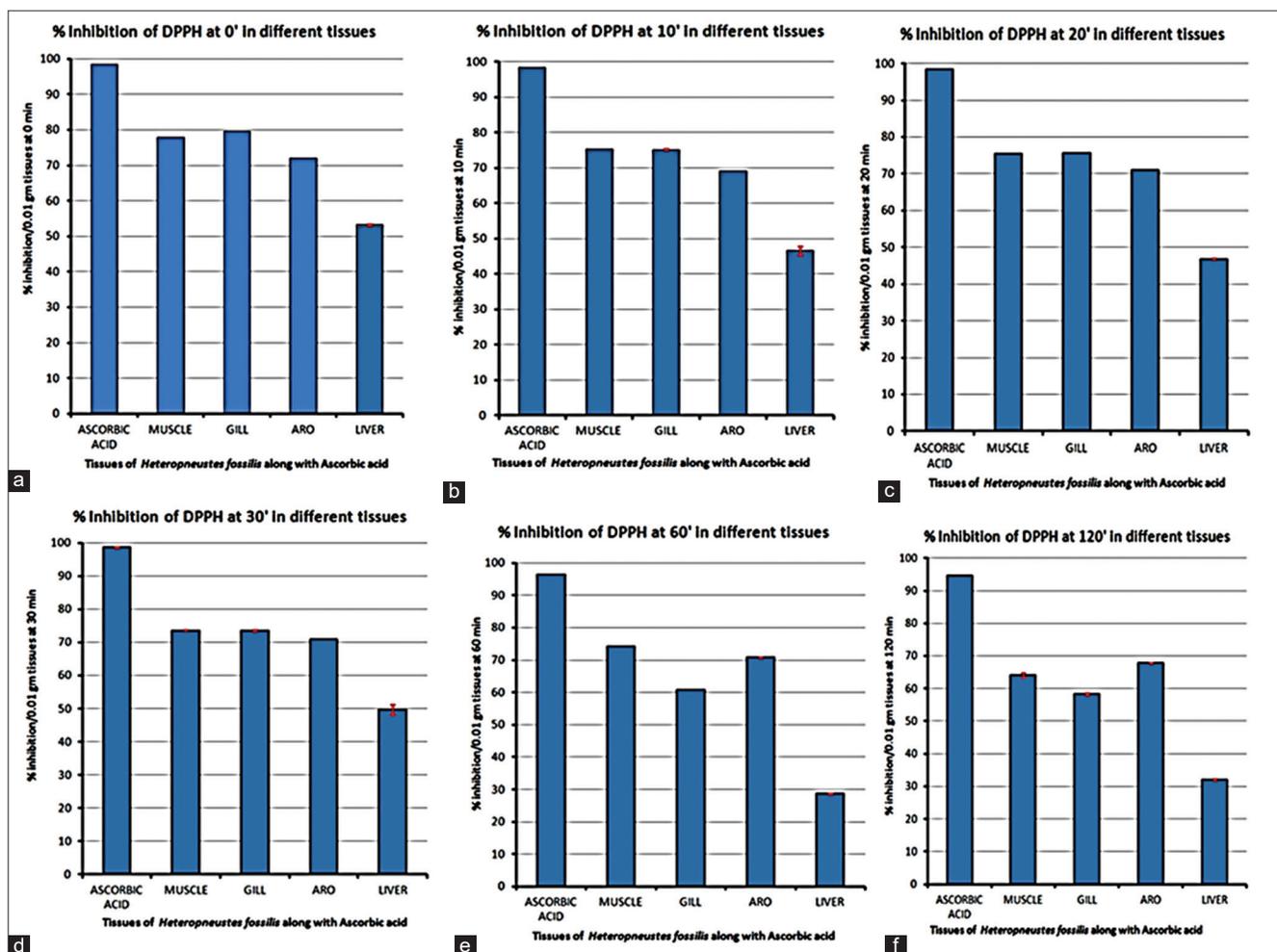
Therefore, effective time of incubation for the accessory respiratory organ was 30 min [Figure 3]. The DPPH scavenging activity by the gill tissue extracts exhibited 75% PID value up to 20 min incubation. The inhibitory action starts to decrease from 30 min and declines to 59% at 120 min incubation time [Figure 2d]. From the time line incubation for the gill tissue, the effective radical scavenging activity was observed at 20 min [Figure 3].

Liver extract showed a DPPH PID value 49.9% at 10 min incubation and the value was remained unchanged till 30 min incubation [Figure 2e]. The highest inhibitory effects were exhibited by muscle tissue extract and lowest by liver tissue among all tissues considered, that is, accessory respiratory organ, gill, liver, and muscle [Figure 3a-f]. The results infer that the muscle possesses more radical scavenging potentials than other tissues or due to the pale red color of the liver tissue extract its PID value was low. The most stable and effective inhibitory effect was observed in a range of incubation time of 10 min, 20 min, and 30 min in different tissues [Figure 4b-d]. After 30 min of incubation, all the tissues showed variable significant decline in inhibitory activity recorded up to 60–120 min incubation [Figure 4e-f]. The incubation time period allows the small antioxidant molecules to bind and reduce the DPPH radical.

*H. fossilis* is a fish of high nutritional value recommended to patients [23-26]. This is a hardy fish and therefore can be used as for various eco-physiological studies including OS physiology [2,27-29]. TAC is an important parameter in the above studies. On the other

hand, incomplete oxidation of oxygen and nutrients due to stress may result in deteriorating the nutritional quality of the fish. As a protective shield, antioxidants act against the free radicals generated due to auto-oxidation by intervening one of the three steps followed by free radicals, that is, initiation, propagation, and termination [30]. Therefore, estimating the accurate value of TAC or PID using DPPH as stable free radical in animals in general and in the fish *H. fossilis* in particular is important.

To quantify the TAC, many methods are proposed with their merits and demerits. Therefore, still problems exist in determining the accurate value of TAC using DPPH method. A lot of queries and pitfalls have been reported regarding the assay method involving DPPH [20]. Determining TAC using DPPH is a simple, robust, and inexpensive method to determine the antioxidant activity by spectrophotometric method. The fluctuations in getting the absorbance in the assay have been considered as major drawback. Therefore, an attempt was made to get a stable and modified method for determining the activity consistently. In addition, it was also noticed that different tissues needed variable time of incubation with DPPH to give the accurate results as they possess different types and quantity of radical scavenging molecules [2,26-29]. The advantage of this method is that DPPH is allowed to react slowly with the weak antioxidants by giving an incubation time, and in the current study, the incubation time was standardized to be 30 min at DPPH concentration of 75  $\mu$ M in 2 mL reaction volume.



**Figure 4:** The radical scavenging activity in terms of percentage inhibition of 2,2-diphenyl-1-picrylhydrazyl for different tissues with respect to ascorbic acid at different time intervals (a) 0 min, (b) 10 min, (c) 20 min, (d) 30 min, (e) 60 min, and (f) 120 min. Data ( $n = 3$ ) were presented as mean  $\pm$  SEM and subjected to ANOVA followed by Duncan new multiple range test to accept the statistical significance level at  $P < 0.05$ .

#### 4. CONCLUSION

Results of the present study indicate that the absorption maximum of DPPH was 516 nm as compared to the suggested absorption maxima ranging from 515 to 546 nm. The optimum final concentration of DPPH in 2 mL reaction volume with 200  $\mu$ L tissue extract was suggested to be 1.35  $\mu$ M. The incubation time of DPPH with tissue extracts was tissue specific in *H. fossilis* and was ranged from 20 to 30 min. Following the standardized method in the current work, muscle had 76% PID value at 20–60 min incubation followed by gill (71% PID, 30 min incubation) and liver (49.9% PID, 30 min incubation) tissues. Results of the present study suggest that determination of absorption maxima, the incubation time of tissue extracts with particular concentration of DPPH are important and need to be determined accurately to get stable results.

#### 5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as

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The authors report no financial or any other conflicts of interest in this work.

#### 8. ETHICAL APPROVALS

Not applicable.

#### 9. PUBLISHER'S NOTE

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