

## Antioxidant Edible Mushrooms: A Green and Rapid Electrochemical Study with the Aqueous Extracts

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### Abstract

A greener and rapid electrochemical technique was performed by indium doped tin oxide (ITO) glasses as working electrode in cyclic and differential pulse voltammetry to screen the antioxidant activities of the aqueous extracts of different species of edible mushrooms *Plureotus florida*, *Calocybe indica* and *Tricholoma giganteum* found in West Bengal. The ITO glasses as electrode was very sensitive for the detection of electrical potentiality with 10  $\mu$ l of 20  $\mu$ g/ml (volume and concentration) concentration in phosphate buffer (pH 7.0) for the isolated aqueous extracts of mushrooms along with ascorbic acid and gallic acid as standards. In addition to the electrochemical study, the spectrophotometric assays were used to evaluate the antioxidant constituents, their reducing power and free radical scavenging ability of the extracts. The results obtained from both electrochemical and spectrophotometrical assays were in good agreement to each other for the detectable and significant range of antioxidant potentiality of these extracts. The present electrochemical ITO electrode method is a greener, efficient, cost effective, recyclable, and less cumbersome for detection of antioxidant properties of mushrooms compared to the other available electrochemical techniques.

**Keywords:** Anti-oxidant; Electrochemical technique; Voltammetry; *Plureotus florida*; *Calocybe indica*; *Tricholoma giganteum*

### Introduction

The disorder of oxidative metabolism in humans encourages the attention of biochemists to explore and explain the cause of this medical problem with a quantitative estimation throughout the world. The excess of oxygen and its reactive species perform the radical-chain oxidation processes in cellular tissues which are the causes for early aging, cell destruction and seeding for few incurable diseases [1]. The presence of antioxidants in food, additives, cosmetics, and medicines, is a better and excellent approach to prevent, depress or partially inactive the unhealthy facts of oxygen metabolism. Edible mushrooms, in Indian subcontinent and Asia, are the richest source of antioxidants. The mushrooms contain many biological active ingredients which are beneficial for the health, the vigour and the protection against degenerative diseases [2]. *Plureotus florida*, *Tricholoma giganteum* and *Calocybe indica* are the most widely cultivated and consumed mushroom species of West Bengal in India. Along with that these three mushroom species are also available in other mushroom cultivating area of world. Different assays have been used to evaluate the antioxidant activity of natural products by spectrophotometry, chemiluminescence, and electrochemical analysis. Methanolic extracts of commercially obtained mushrooms *Agaricus bisporus*, *Hypsizygus ulmarius*, and *Calocybe indica* were analyzed for their antioxidant activity in different chemical systems including reducing power, free radical scavenging, ferric reducing antioxidant power (FRAP), superoxide scavenging, peroxide scavenging, metal chelating activities [3]. But, a comparison of the results is complicated because each method has different standard compounds for comparative study.

Antioxidant activities of pure compounds and plant extracts obtained from different solvent (aqueous, ethanol, methanol ethyl acetate etc.) are traditionally determined, by spectrophotometry e.g., ABTS+• and DPPH• for determination of the free radical scavenging activities [3,4]. These spectral methodologies inherently have few difficulties since they require the use of specific reagents e.g., Butylated hydroxyanisole (BHA),  $\alpha$ -tocopherol [5,6]. TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analog of vitamin E sold by Hoffman-LaRoche, used in biological or biochemical applications to reduce oxidative stress or damage. Trolox equivalent antioxidant capacity (TEAC) is a measurement of antioxidant strength based on Trolox, measured in units called Trolox Equivalents (TE). Trolox was used as standards for DPPH free radical scavenging activities, whereas ascorbic acid and gallic acid were used as standards for the iron-reducing property and for phenolic estimations respectively. Moreover, to get a preliminary insight about the antioxidants properties of any natural products extract by spectrophotometry it is necessary to calculate the results using a standard mathematical technique, e.g.,

$$\text{Scavenging effect (\%)} = \left[ \frac{(\text{control absorbance} - \text{sample absorbance})}{(\text{control absorbance})} \right] \times 100$$

Still, all spectrophotometric analyses are executed and determined with the help of standard or reference molecules whereas electrochemical analysis does not depend on reference molecule. Electrochemical measurements have advantages in determining the antioxidant activity as it can be used as a rapid tool for natural extract [7]. The inherent oxidation potentials were measured by Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV). These were used to compare the antioxidant effectiveness of compounds

(phenolic acids, flavonoids, cinnamic acids, etc.) in either pure form or in natural extract [8,9]. Electrochemical analysis depends on the nature of electrodes. The selection of electrode plays an important role. Thus, the aim of the present work is to develop a rapid, low cost, easy-operating, and highly-sensitive electro analytical technique for the evaluation of antioxidant properties of the mushroom samples by an optically transparent electrode (OTE) using indium doped tin oxide (ITO) glass. Moreover, the use of ITO glasses is less expensive. The present electrochemical technique is applied directly for the estimation of oxidation potential of the natural aqueous extract by just maintaining the pH with phosphate buffer using Cyclic Voltammetry and Differential Pulse Voltammetry. This makes an important contribution to the determination of the antioxidant properties of mushroom aqueous extracts.

## Materials and Methods

**Reagents:** Methanol, Whatman filter paper, potassium ferricyanide, sodium phosphate buffersalts, Ferric chloride, Sodium carbonate(SRL), 2,2'-diphenyl-1-picrylhydrazyl (DPPH, Sigma), Folin-Ciocalteureagent, Sulphuric acid (Qualigens), Trichloroacetic acid, Phenol(Merck).

**Instruments:** Spectrophotometer-JASCO R-630, cyclic voltammetry: Autolab type III FRA2 potentiostat/galvanostat, Centrifuge: REMI 1200.

### Preparation of aqueous extract from mushrooms

All mushroom species were collected from a mushroom farm of Jalpaiguri, West Bengal (26.6835°N, 88.7689°E), India, in dried condition and grinded to produce the mushroom powder which was used as the source material for water extraction. The mushroom powder (5 gm) of each species was separately extracted with 150 ml of water after soaking for overnight at room temperature, followed by extraction for 5 hr at 50°C. The extract of each was centrifuged at 1.957 × g (or 5000) rpm for 20 mins and the supernatant was collected by filtration through linen cloth, which was used as the working sample for all electrochemical experiments. The supernatant was freeze dried for further use. All the dried extracts were completely soluble in water.

**Total phenol estimation:** The extracts (0.15 ml of different dilutions) were mixed with Folin-Ciocalteu reagent (300 µl, diluted to 10%, with distilled water) for 5 min and aqueous Na<sub>2</sub>CO<sub>3</sub> (0.7 M, 1.2 ml per reaction mixture) were then added. The mixture was allowed to stand for an hour of incubation and the phenols were determined by UV-Vis spectrophotometry at 765 nm. [Gallic acid was used as a standard and total phenol values were expressed in terms of gallic acid equivalent (in mM) [10].

### Determination of antioxidant properties by chemical method

**DPPH radical scavenging activity assay:** The free radical scavenging activity of the extract was measured *in vitro* by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay with some modification [11]. A 3 ml (6 × 10<sup>-5</sup> mM) aliquot of DPPH solution was mixed with 100 µl of the sample at various concentrations (0.1-0.3 mg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. Ascorbic acid used as standards [12]. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

Scavenging effect (%)=[(control absorbance-sample absorbance)/(control absorbance)] × 100.

**Iron reducing power determination:** The reducing power was measured by the method of Oyaizu [13] with slight modification; various concentrations of mushroom water extracts (2.5 ml) were mixed with sodium phosphate buffer (pH 6.6, 200 mM, 2.5 ml) and potassium ferricyanide (1% w/v, 2.5 ml). The mixture was incubated at 50°C for 2 min. Trichloroacetic acid (10%, 2.5 ml) was added, and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (5 ml) was mixed with deionised water (5 ml) and ferric chloride (0.1%, 1 ml), and the absorbance was measured by spectrophotometer at 700 nm. Higher absorbance indicates better reducing power. Ascorbic acid was used as standard.

## Electrochemical analysis

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed on Autolabpotentiostat/galvanostat (FRA2) using a closed standard three electrode cells. An indium doped tin-oxide (ITO) (4 cm × 1 cm) resistance 8-12 Ω/sq was used as the working electrode and platinum as the counter electrode. All potentials are referred to an Ag/AgCl 3 M KCl as reference electrodes, the working electrode was washed with milli-Q water followed by sonication then with methanol. The background cyclic voltamograms was taken in the phosphate buffer solution. The voltamograms were analyzed for peak potentials and currents.

All the mushroom aqueous extracts and standard compounds were studied in phosphate buffer (0.1 M, pH 7.0), Ascorbic acid and Gallic acid, were taken as standard, the concentrations were set between 0.1 to 1 mM, while for mushroom extracts; the concentrations were changed from 20 to 80 µg/ml. All solutions were analyzed immediately after preparation in phosphate buffer and the electrochemical responses were recorded after the immersion of ITO electrode into the required solution. Cyclic voltammetry was used to characterization the electrochemical responses between 0 and +1.2 V, at 0.1 Vs<sup>-1</sup>. DPV were used for characterizing electrochemical responses as a linear function of current generated with concentrations. The antioxidant power for a range of concentrations was evaluated by DPV, at 60 mV pulse amplitude and 0.030 Vs<sup>-1</sup> as scan rate. For the standards and each extract, the current density was plotted as a function of corresponding concentration.

## Results and Discussion

### Estimation of phenolic content

The phenolic moieties are the most important antioxidant parameters for the edible mushrooms. In the present study, the antioxidant potentiality of any edible source was found to be directly proportional to the amount of phenol content. The spectrometric measurement showed the total phenolic content in the order *P. florida* > *T. giganteum* > *C. indica* (Table 1).

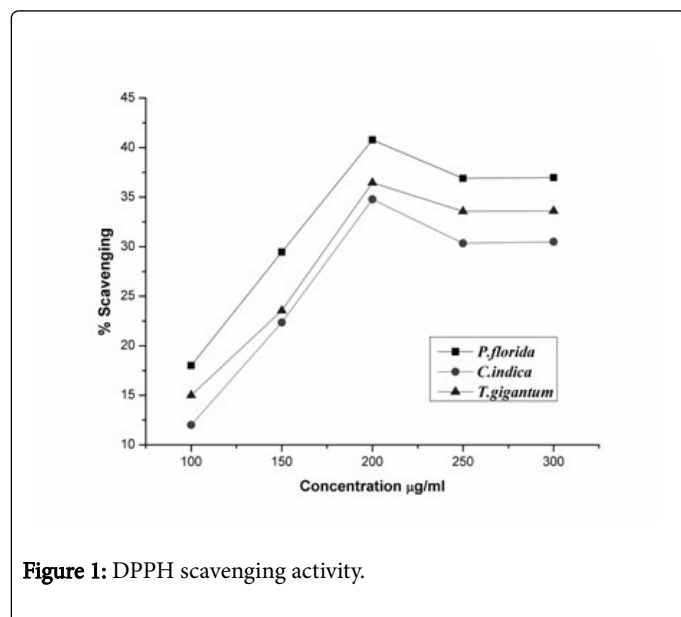
Mushroom	Total Phenol (mg/mg) ± SD
<i>Pluerotus florida</i>	3.43 ± 0.0503
<i>Tricholoma giganteum</i>	3.19 ± 0.070

<i>Calocybe indica</i>	2.08 ± 0.0803
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**Table 1:** Comparative data of total phenol content in water extract of mushrooms.

### Evaluation of antioxidant properties by DPPH spectrophotometric assay

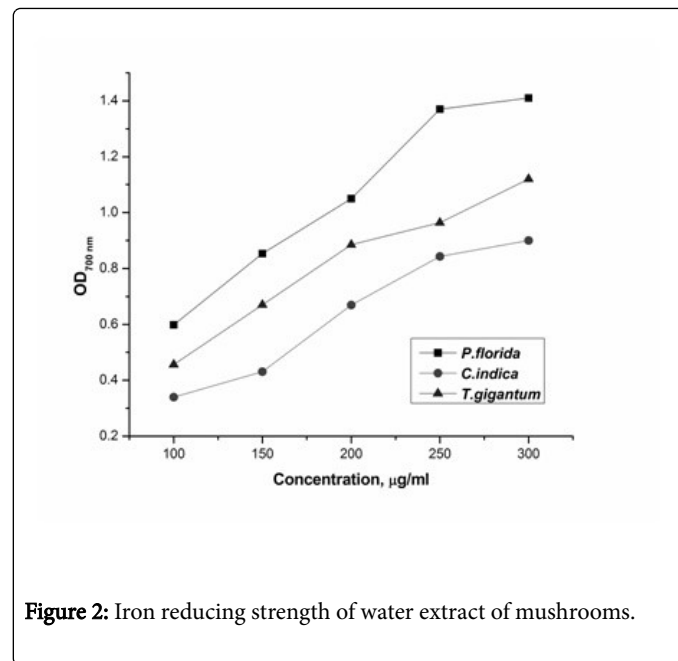
DPPH method has been widely applied for estimating antioxidant activity, however, its applications should be carried out on the basis of the method, and the need wherever possible to establish the stoichiometry for the quenching reaction, so that the antioxidant activity may be related to the structure of the substrate molecule. The method offers advantages of being rapid, simple and inexpensive and provides first hand information on the overall antioxidant capacity of the test system. The trend in antioxidant activity obtained by using the DPPH method is comparable to trends found using other methods [14]. Spectrophotometric assay with DPPH was performed to screen the antioxidant potentiality for scavenging activity. All the species proved to have antioxidant activity through radical scavenging activity. The scavenging activity was ranged from 30-42.5% even at low concentration of 100 µg/ml. All the species showed a maximum free radical scavenging activity at 200 µg/ml and *C. indica* showed a lowest scavenging of 30% and *T. giganteum* 36% and *P. florida* with highest 42.5% (Figure 1).



**Figure 1:** DPPH scavenging activity.

**Iron reducing power:** Reducing power of the aqueous extracts were measured by spectrophotometer at 700 nm which increased steadily with increased concentration. The ranges for iron reducing capacity (100 µg/ml) were 0.33-1.4 for three mushroom samples. The reducing power of ascorbic acid at 300 µg/ml was 1.6. The iron reducing power

of *P. florida* was better than the other two mushrooms; *C. indica* and *T. giganteum*. For example, *P. florida* showed a maximum iron reducing activity of 1.4 at 300 µg/ml whereas, *T. giganteum* and *C. indica* showed 0.9 and 1.1 respectively at the same concentration (Figure 2).



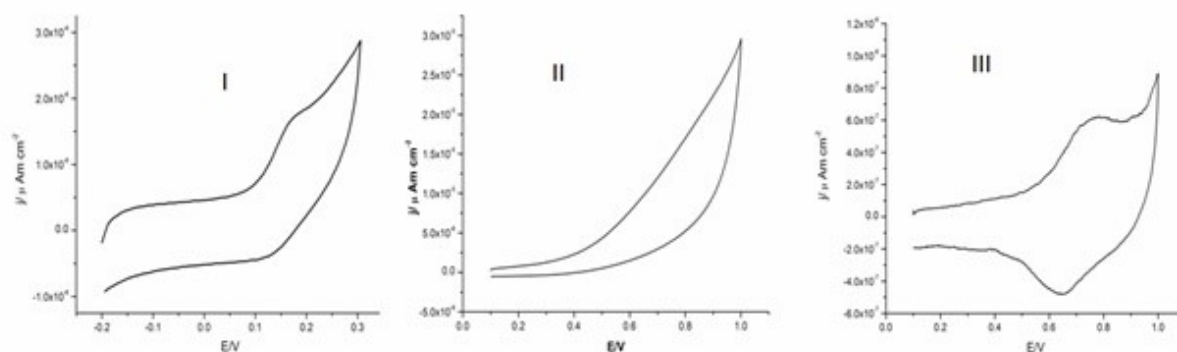
**Figure 2:** Iron reducing strength of water extract of mushrooms.

### Evaluation of antioxidant properties by electrochemical techniques:

Cyclic Voltammetry (CV) is the most commonly used technique for the characterization of redox system along with the number of stable redox states of molecules. CV can also be identified as the widely used tools for the qualitative and quantitative estimation of antioxidant capacity of biomolecules (polyphenol, flavonoids, etc.). In the CV analysis the surface of working electrode is the place where oxidation of the biomolecules takes place during the scanning of the voltage of a working electrode. The amount of biomolecules with antioxidant capacity is proportional to the current produced. The redox chemistry of the biomolecules is observed in CV analysis when ITO electrode used as sensitive working electrode.

The cyclic voltammograms of the standards gallic and ascorbic acids, showed typical irreversible oxidation processes (Figures 3(I) and 3(II)) when the experiments setup with pulse amplitude at 60 mV and 0.030 Vs<sup>-1</sup> as scan rate.

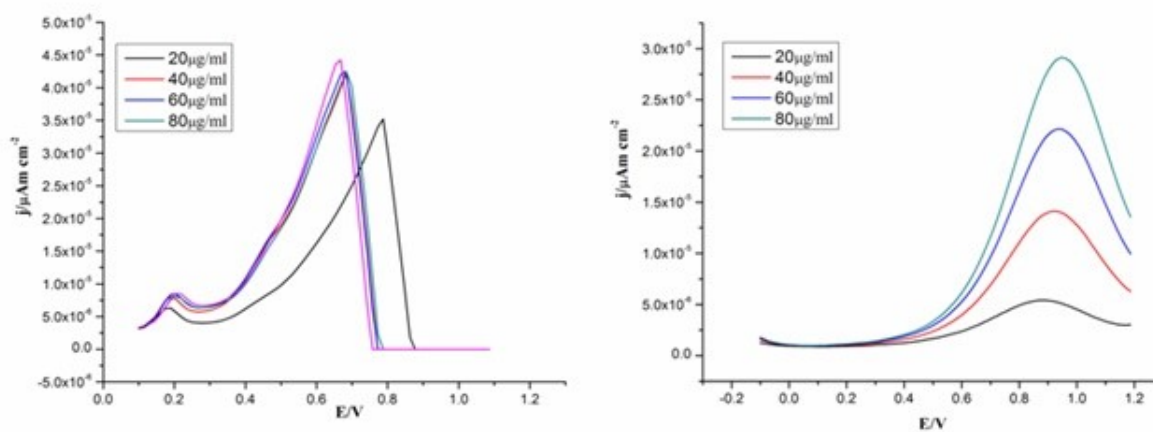
The similar irreversible electrochemical behavior was observed for the extracts of *P. florida* although with oxidation potentials around +0.8 V (E/V=+0.78 V) (Figure 3(III)). These results showed that both standards were oxidized at less positive potentials than the compounds responsible for the electrochemical responses of the mushroom extracts. The electrochemical behaviors in the oxidation potential between all the species indicated that the different extracts may have an analogous composition with respect to the electroactive species.



**Figure 3:** Cyclic Voltamogram of Gallic acid(I), Ascorbic acid(II) and *Plureotos florida*(III) in 0.1 M Phosphate buffer (pH 7.0) at 0.3 Vs<sup>-1</sup>.

Differential pulse voltammetry (DPV) is a selective and sensitive tool for the recording of changing of potential with time by the potential pulses of the amplitude between 10 and 100 mV for several milliseconds. The difference between the observed currents employed immediately before the pulse application and at the end is recorded. Dependence of the difference between these two currents on the applied potential goes through maximum, so it has a peak shape. The position of the peak on the potential axis is given by the quality of analyte, and its height depends on the concentration of the analyte.

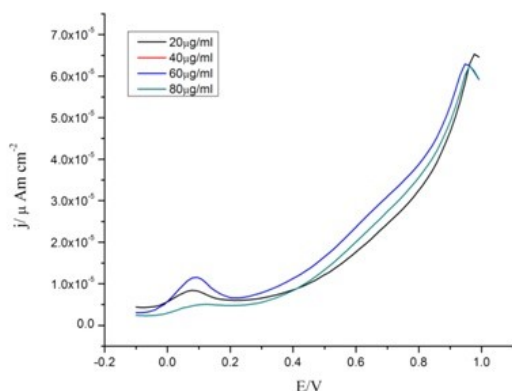
The differential pulse voltammograms, at several concentrations of gallic acid and ascorbic acid showed there was an increase in peak current with the gradual increase in concentration which leads to a linear relation between these two parameters. Where gallic acid consist of two major peaks on around +0.21 V and +0.67 V, with  $7.7 \times 10^{-6} \mu\text{Amcm}^{-2}$  and  $4.19 \times 10^{-5} \mu\text{Amcm}^{-2}$  whereas ascorbic acid showed only a single peak at +0.95 V,  $2.89 \times 10^{-5} \mu\text{Amcm}^{-2}$  (Figures 4(I) and (II)). At concentration 20  $\mu\text{g/ml}$  of gallic acid, the peak current deviated with rest of the other concentrations.



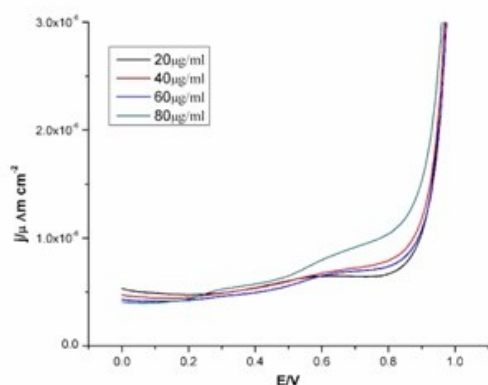
**Figure 4:** Differential pulse voltammograms of gallic acid (I) and ascorbic acid (II) in 0.1 M Phosphate buffer (pH 7.0) of 20-80  $\mu\text{g/ml}$  concentration.

The extract of *T. giganteum* showed (Figure 5) a potential (E/V= +0.0935 V,  $1.15 \times 10^{-5} \mu\text{Amcm}^{-2}$ ) with 20  $\mu\text{g/ml}$  concentration which was saturated at 80  $\mu\text{g/ml}$  concentration. There was no prominent peak for the extract of *C. indica*, but a broaden area was observed (Figure 6) at +0.68 V,  $1.10 \times 10^{-6} \mu\text{Amcm}^{-2}$  with concentration of 20-80  $\mu\text{g/ml}$ . The extract of *P. florida* showed (Figure 7) a significant peak at +0.48 V, with  $1.06 \times 10^{-6} \mu\text{Amcm}^{-2}$  at 80  $\mu\text{g/ml}$  concentration, the peak showed

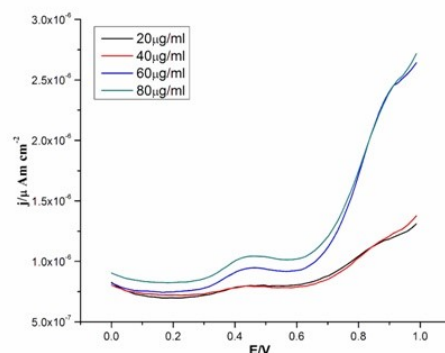
broadening for the lower concentrations (20-60  $\mu\text{g/ml}$ ). The concentration upto 120  $\mu\text{g/ml}$  (data were not shown) were capable of the efficient performance of the electrode used and the higher concentration (>120  $\mu\text{g/ml}$ ) was anomalous because of the accumulation of organic moieties on the surface of electrode. Thus, electrode lost its activity. The electrode was reused by thorough washing and sonication (Figure 8).



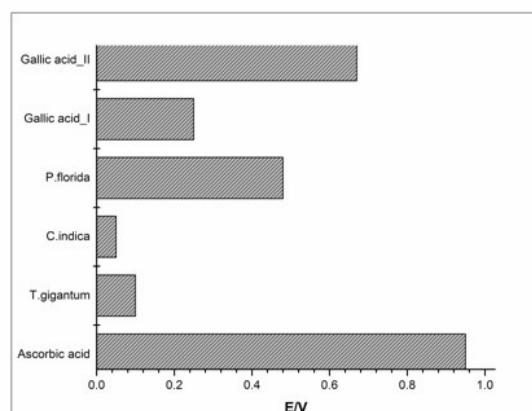
**Figure 5:** Differential pulse voltammograms of *Tricholoma giganteum* water extract in 0.1 M Phosphate buffer (pH 7.0) of 20-80 µg/ml concentration.



**Figure 6:** Differential pulse voltammograms of *Calocybe indica* water extract in 0.1 M Phosphate buffer (pH 7.0) of 20-80 µg/ml concentration.

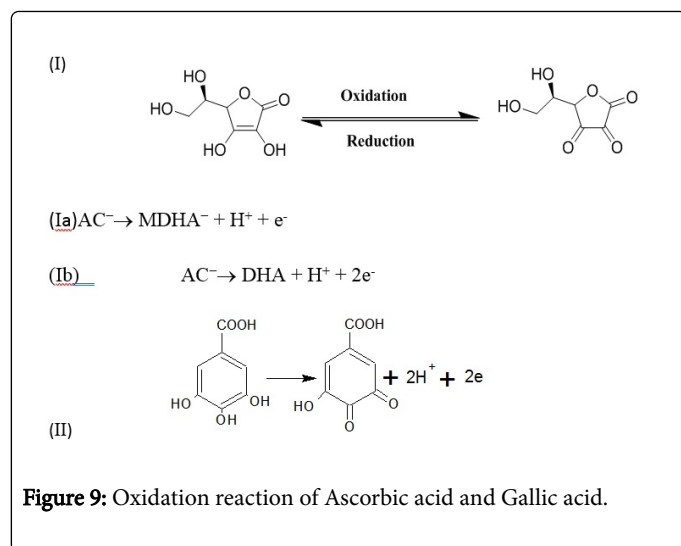


**Figure 7:** Differential pulse voltammograms of *Plureotus florida* water extract in 0.1 M Phosphate buffer (pH 7.0) of 20-80 µg/ml concentration.



**Figure 8:** Comparative values (single point experiment) of E/V in DPV of all mushroom extract on ITO electrode.

The study was conducted on the extract of mushrooms for the electrochemical analysis. The electrochemical properties were responsible for the antioxidant potentiality. At neutral pH ascorbic acid (AC) is a powerful antioxidant, which scavenges free radicals. Ascorbic acid oxidizes either a single-electron oxidation (dehydroascorbate radical anion, MDHA-) or a two-electron oxidation (dehydroascorbate, DHA) according to the following reaction (I, Ia, Ib). Gallic acid oxidized accordingly following the reaction II (Figure 9).



The cyclic voltammetry method is recommended as it represents a relatively clean chemical system, easy to control, rapid, cost effective and it is not affected by turbid solutions of the extracts. All the extracts showed a significant amount of peak current generation with detectable range started as low as 20  $\mu\text{g/ml}$ , whereas the study carried out up to 80  $\mu\text{g/ml}$  with a regular interval. Antioxidant compounds acted as reducing agents and, insolutions, they were easily oxidized at the electrodes.

However, there were greater chances of oxidation on the electrode surface in presence of organic substrates. A few papers established an interesting relationship of electrochemical properties and antioxidant compounds. It was stated before that ascorbic acid and phenolic compounds are common antioxidants in mushrooms. On this basis, electrochemical measurements corresponded to the oxidation of content of these compounds (as eq I and II) and their electrochemical behavior, although the peak current density versus extract concentration was not the same. For this technique the peak current density depended not only on the concentration, but also on the electron transfer kinetics and the diffusion co-efficient of the electroactive species, preventing the direct comparison between the standards and samples data [2,14,15]. In Figure 8, the comparative of E/V values for the water extract of all mushroom species, where the *P. florida* showed highest value and *C. indica* showed lowest E/V values was shown. The extract which was rich in phenol content shows a very prompt in electrochemical analysis with prominent peak. On the other hand, the extracts that have non-phenolic content like sugar showed broaden peak in increasing order of sugar content *C. indica* > *T. giganteum* > *P. florida*. Therefore, the electrochemical response of the extracts showed in following order *P. florida* > *T. giganteum* > *C. indica* (Figure 8).

Our newly developed techniques also fulfilled the basic standard of the electrochemical technique as per the Analytical Methods Committee [16] as the technique was more advantageous over their existing counterpart as determination of the analyte in high ionic strength media without matrix and the removal of matrix for the present technique with the higher range of the buffer strength, 100 mM upto 900 mM was possible. There was no limitation of choice for the buffer from any molar strength to any compositions from phosphate buffer, HEPES or Tris, etc. Excellent range of detection that was adjustable by changing deposition (pre-concentration) time (from

minutes to seconds) was applied. The range of detection for analyte was started from 20  $\mu\text{g/ml}$ . However, the detection was also performed using 5-10  $\mu\text{g/ml}$  which was sufficient and efficient to identify and determine the antioxidant properties of the analyte in buffer solutions. There was a clear indication of the linearity of the sample analysis with regular interval of increment or decrement (after saturation) of sample concentration with total peak current generation ( $J/\mu\text{Am cm}^{-2}$ ). This was an on-line or in situ application for near-real time determination of analytes where no need of prolonged incubation time period (only 2 sec) was required for acquiring data by cyclic voltammeter. This method was applied for the direct determination of operationally assumed groups responsible for antioxidant activities, without prior sample treatment and there was no sample pretreatment phase in this procedure. It was possible to perform the assay with direct addition of the analyte to the system with pre-defined concentration. The low cost of the electrodes which were used in this technique was easily recyclable and it was a greener method of recycling. The washing and cleaning of the electrochemical deposition on electrodes were very simple with sonication followed by rinsing in any polar or nonpolar solvent of interest. This recent study also fulfilled the assigned parameter for the electrochemical analysis and used for the rapid identification and determination of the antioxidant properties of the aqueous extracts of edible mushrooms inhabitant of West Bengal.

## Conclusion

The present study was successfully executed to sensitive detection of antioxidant potentiality of edible mushroom extracts in aqueous phase using cyclic and differential pulse voltammetry, where indium doped tin oxide (ITO) glasses used as electrode. The method of extraction is greener as there were no use of organic solvent and the analyte detection background was only phosphate-buffer based. The analysis for the response of antioxidant potentiality was more sensitive than spectrophotometric measurement. There was no sample pretreatment phase in this procedure. It was possible to perform the assay with direct addition of the analyte to the system with pre-defined concentration. The required amount of analyte is lesser for the electrochemical measurement and it was rapid, cost-effective and greener. Considering the simplicity and merits of this method, it will be helpful for food researcher to evaluate a fast hand screening of any food or food derived product for its antioxidant properties. Though further method optimization and validation is required for best industrial practice.

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