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TPC, TFC & Antioxidant activities of *Crepidium acuminatum* (D. Don) Szlach

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Abstract
Crepidium acuminatum D. Don Szlach is an important medicinal plant belonging to family Orchidaceae. This plant is an important part of Astavarga used in nutritional preparation. Only a few papers have been reported in scientific database. We focussed on estimation of total phenolic content, total flavonoids content and *in vitro* antioxidant potentials by three methods i.e. reducing power method, Total antioxidant activity by Ammonium molybdate method and Ferric Reducing Antioxidant Powder method. The phenolic content with respect to gallic acid was found to be 70.9 ± 0.53 (µg gallic acid equivalent/ml of extract) for methanol extract. The flavonoid content was found to be as: 34.27 ±0.61 (µg quercetin equivalents/ml of extract) in methanol extract Three types of extracts were used for anti-oxidant studies: aqueous, hydroalcohlic and alcoholic. It was concluded that aqueous extract has more potential as antioxidant as compared to hydroalcolic and alcoholic extracts.

Keywords: TPC (Total Phenolic content), TFC (Total flavonoids Content), *Crepidium acuminatum*, antioxidant, antioxidant activity, ROS (Reactive oxygen species)

1. Introduction
Modern life style, Fast food habits and consequence effects on health are gaining attention these days [1].Some of the characteristics of modern life style are very busy life, high work load, tight competitions and deadlines to be met, eating while doing, fast food etc. These kinds of life styles are sensitive to stress [2]. Chronic psychological stress accelerate biological aging and result in oxidative damage [3] there are number of studies supporting the link between life, stress, illness behaviour and chronic disorders. Concepts regarding stress and coronary heart disease have found a positive correlation in all over the world [4]. As a result the field of free radical chemistry is gaining more importance these days. Free radicals are reactive oxygen species (ROS) produced during metabolism in the body. Uncontrolled generation of these free radicals leads to attack on membrane lipids, proteins, enzymes and DNA causing oxidative stress and ultimately cell Senescence. These ROS are responsible for many degenerative diseases like diabetes mellitus, cancer, Parkinson's disease, Alzheimer's disease atherosclerosis, neurodegenerative disorders, ageing and many more inflammatory diseases [5]
Reactive oxygen species (ROS) are superoxide anion (O^{2-}), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2) are by-product molecules generated during aerobic cell metabolism. The presence of these molecules is crucial as they alter physiological signalling and homeostasis. As a result, overproduction of ROS results in oxidative stress and contributes to the development of various degenerative, cardiovascular disease and neurodegenerative diseases [6].
Reactive oxygen species (ROS) and free radicals are generated in the human body through varied endogenous systems, exposure to various physiochemical conditions, and pathological situations. For ideal physiological condition antioxidant balance is regulated by endogenous antioxidant defense mechanisms. If this protection does not adequately remove dangerous radicals oxidative stress proceeds. An external source of antioxidants can be used to overcome oxidative stress. Many such natural antioxidants occur in various plant sources such as polyphenols, flavonoids, and condensed tannins [7]
An antioxidant can be defined as any substance when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate. The role of antioxidants is to avert damage to cellular machinery happening as a result of chemical reactions involving free radical [8].
Recently synthetic antioxidants are not preferred because of harmful effects observed such as human toxicity and environmental pollution and due to carcinogenicity.

From this point of view, an increasing tendency towards the use of natural products instead of synthetic has been observed in a high demand for food [9, 10].

Phenolic compounds are constituents of many plants and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants These phenolic compounds exhibit promising free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents and metal ion chelating properties [1]

The basic endeavour of the present research was to determine the total phenolic and flavonoids content in various extracts of the species *Crepidium acuminatum* D. Don Szlach using spectrophotometric methods, as well as to look at antioxidant potential of plant extracts using *in vitro* model system.

Material and Methods

Chemicals and Instruments required:

For TPC: Folin Ciocalteu reagent, sodium carbonate, Gallic acid (Standard), Ethanol and distilled water;For TFC: Aluminium chloride, Quercetin (standard), Ethanol, Methanol, sodium acetate and distilled water;For antioxidant studies (a) For reducing power assay: BHT (standard) phosphate buffer, potassium ferricyanide, Trichloroacetic acid, $FeCl_3$ and distilled water.(b) For Total antioxidant Capacity (TAC): phosphomolybdate reagent solution (0.6 M sulphuric acid, 28 µM sodium phosphate and 4 µM ammonium molybdate). Ascorbic acid (standard) (c) For Ferric Reducing Antioxidant Power (FRAP) Assay: TPTZ [2, 4, 6-tri (2-pyridyl)-s-triazine] FRAP reagent (sodium acetate, TPTZ $FeCl_3.6H_2O$), $FeSO_4$, Ascorbic acid (standard)

Instruments required: UV-Double beam spectrophotometer, incubator, pH meter.

Plant Material

The plant consists of fresh pseudobulbs of *Crepidium acuminatum D.Don* belonging to family Orchidaceae.

Collection and Authentication

The pseudobulbs part of the plant *Crepidium acuminatum D.Don*, and family Orchidaceae was purchased from "Chandigarh" in the month of February, was dried at 40°C in hot air oven, powdered and stored in air tight container. It was authenticated and identified as *Crepidium acuminatum D.Don Szlach* from National Institute of Science Communication and Information Resources (NISCAIR), New Delhi.

Extraction procedure

About 4.0 gm of coarsely powdered air dried material accurately weighed and macerated with 100 ml of the solvent (methanol, Ethanol,80% ethanol v/v, aqueous) separately in a glass stopper closed flask for 24 h with frequent shaking during the first 6 h and allowed to stand for 18 h. It was filtered rapidly taking care not to lose any solvent and then 25 ml of the filtrate was transferred in to a tarred flat bottom dish and evaporated to dryness on a water bath. Dried at 105°C for 6 h and cooled in a vacuum desiccators for 30 min. then weighed without delay. Calculated the content of extractable matter in mg/g of air dried drug. [1] and labelled as Methanol extract (ME), Ethanol Extract (EE), Hydroalcoholic (80% Ethanol) and Distilled water extract as (AE)

Total Phenolic Content

Quantification of total phenolic content (TPC) was carried out by Folin Ciocalteu method. [13, 14] with slight modifications**.** The FCR reagent oxidizes phenols in plant extract and changes in the dark blue colour (k = 765 nm) which is monitored by UV-Visible spectrophotometer. Briefly 5 ml of 10% (FC reagent in distilled water) Folin Ciocalteu reagent was mixed with 1 ml of aliquot (1mg/ml). The test tube was shaken up to mix the solutions thoroughly, followed by addition of 4 ml Na_2CO_3 (1M). The solutions were mixed again and left at room temperature for15 min. The phenolic content was expressed as Gallic acid equivalents using the following linear equation based on the calibration curve: $A=0.01C+0.046$, $R^2=0.99$ Where A is the absorbance and C is concentration as Gallic acid equivalents (µg /ml). The standard curve was prepared by using 5µg/ml to 40µg/ml solutions of gallic acid (standard phenolic compound) in ethanol.

Total Flavonoids Content: Aluminium chloride colorimetric method was used for flavonoids determination [1]. Each plant extracts (0.5 ml of 1:1 mg ml-1) in methanol were separately mixed with 1.5 ml of ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M sodium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solutions at concentrations (standard) of 10, 20, 30 to 80 µg/ml in Ethanol. Flavonoids content in drug was calculated by using standard calibration curve**.** The flavonoids content was expressed as quercetin equivalents using the following linear equation based on the calibration curve: $A=0.003C-0.029$, $R^2=0.994$ Where A is the absorbance and C is concentration as quercetin equivalents (µg /ml)

In-Vitro **Antioxidantassay:** The *in-vitro* antioxidant assays were done with ethanol, 80% ethanol (hydro-alcoholic) and aqueous extracts of *Crepidium acuminatum D.Don* with the help of different models discussed below:

Reducing power Assay
TAC by phosphomolybdenum assay
Ferric Reducing Antioxidant Powder assay
Reducing power Assay

Reducing power is associated with antioxidant activity and serves as a significant reflection of antioxidant activity **[16]** Compounds with reducing power indicate that they are electron donors and reduced the oxidized intermediates of lipid peroxidation processes; so that they can act as primary and secondary antioxidants. The reducing power was determined by the method of **[17]**

1 ml of extract was mixed with 2.5 ml of phosphate buffer (200 µM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 µM) at 50°C for 20 min. Thereafter 2.5 ml of Trichloroacetic acid (600µM) was added to the reaction mixture, centrifuged for 10 min at 3000rpm. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6µM) and absorbance was measured at 700nm. Butylated hydroxytoluene (BHT) was used as positive control.

Total antioxidant Capacity (TAC) by Phosphomolybdenum Assay:

The total antioxidant capacity of the extracts was determined by phosphomolybdate method using Ascorbic acid as the standard. Suitable working standards (50-250 mg/ml) of the plant residues were prepared by dissolving the extracts in water. Aliquots (0.1 ml) of the sample were mixed with 1 ml

of the reagent solution (0.6 M sulphuric acid, 28 µM sodium phosphate and 4 µM ammonium molybdate). The tubes were capped with silver foil and incubated at 95°C for 90 min [18] The tubes were cooled to room temperature and the absorbance was measured at 695 nm with UV double beam spectrophotometer (Schimadzu 1800) against a blank. Ascorbic acid was used as a standard.. The total antioxidant capacity was expressed as µg equivalents of Ascorbic acid by using the standard Ascorbic acid graph.

Ferric Reducing Antioxidant Power (FRAP) Assay:
FRAP assay depends on the capacity of antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ [2, 4, 6-tri (2-pyridyl)-s-triazine] forming a deep blue complex with an absorption maximum at 593 nm at particular pH (optimum pH 3.6) [19]. 0.2 ml(1mg/ml) of the aliquot is added to 3.8 ml of FRAP reagent which is generated by mixing 10 parts of 300µM sodium acetate at pH 3.6, 1 part of 10.0µM TPTZ solution and 1 part of 20.0 µM $FeCl_3.6H_2O$ (10:1:1;v/v/v)solution. The reaction mixture is incubated at 37°C for half an hour and the increase in absorbance at 593 nm with UV (Schimadzu 1800, Japan) was measured. Fresh solution of $FeSO_4$ was used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as Mmol $FeSO_4$ equivalents per gram of sample. The results being expressed as Ascorbic Acid Equivalent Antioxidant Capacity, AEAC)

Statistical Analysis
All data were expressed in mean ± SEM. Statistical analysis was performed using SPSS software.

Results
Total Phenolic Content
The greater amount signifies the presence of different constituents having phenolic moiety in their structures. The phenolic content with respect to gallic acid was found to be 70.9 ± 0.53 (µg gallic acid equivalent/ml of extract) for methanol extract. The standard plot is shown in figure 1

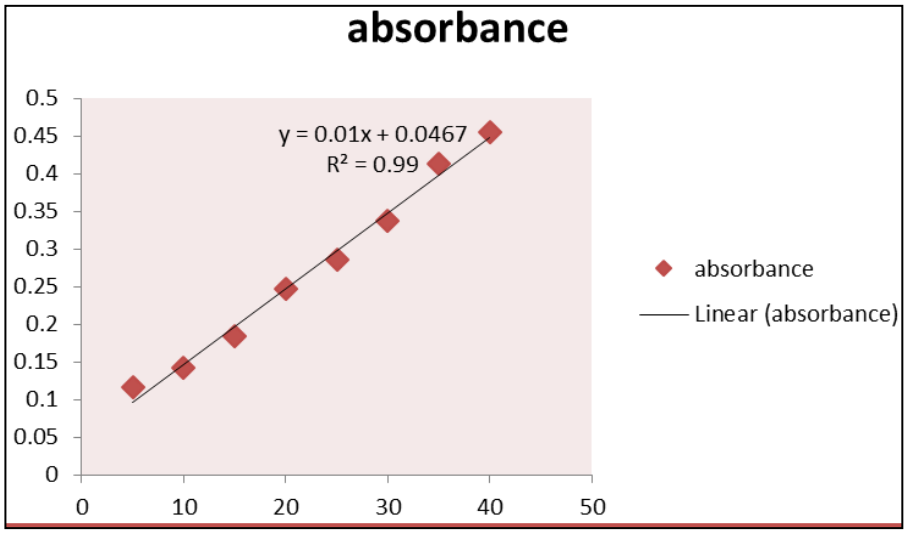

Fig 1: Standard plot of gallic acid

Total flavonoids content:
The total flavonoids content of the methanol extract was estimated taking quercetin as standard. The standard curve of quercetin was plotted and all the findings were made in comparison to quercetin. The greater amount signifies the presence of more flavonoids moieties in the constituents. The flavonoid content was found to be as: 34.27 ±0.61 (µg quercetin equivalents/ml of extract) in methanol extract. The standard plot for Quercetin is shown in Fig 2.

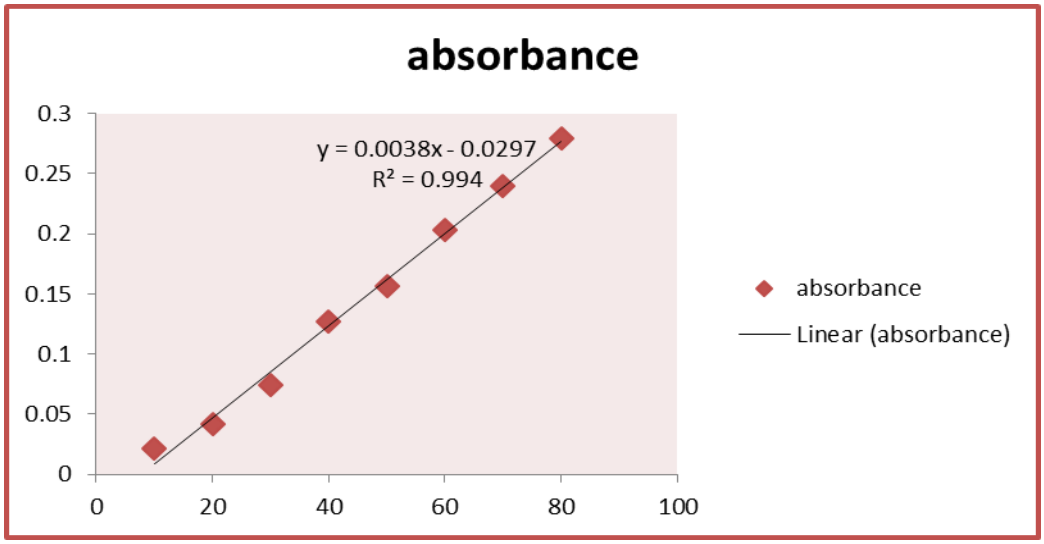

Fig 2: Standard plot of Quercetin

***In-Vitro* Antioxidant Studies**

In the present study three extracts viz. Ethanol,80 % ethanol and aqueous (EE,80% EE, AE) were investigated for antioxidant potential by using three different methods. All the extracts showed antioxidant activity to varying extent. out of EE.80 % EE, AE showed more antioxidant activity by three different methods.

Reducing power of various extracts

In the reducing power assay, the more antioxidant composites change the ferric (Fe^{+3}) ions to ferrous (Fe^{+2}) ions. The reducing activity of various extracts of *Crepidium acuminatum D.Don* extracts and standard BHT (butylated hydroxytoluene) at various concentrations has been represented in Table 1 and Figure 3. Moieties with reducing power shows that they are electron donors and can reduce the oxidized intermediates so that they can acts as antioxidants.

Table 1: Reducing power of Different extracts of *Crepidium acuminatum D.Don* pseudobulb samples and BHT (Standard)

Conc. (µg/ml)	BHT	EE	AE	80% EE
0	0	0	0	0
50	0.1211±0.39	0.0223±0.52	0.0491±0.63	0.0297±0.29
100	0.2321±0.42	0.0552±0.71	0.1199±0.57	0.0677±0.21
150	0.2979±0.74	0.0849±0.55	0.1974 ±0.32	0.1289±0.69
200	0.3298±0.23	0.1099±0.81	0.2428±0.36	0.1854±0.29
250	0.3609±0.44	0.1450±0.53	0.2899±0.67	0.2035±0.83

Values are mean ± SE (n = 3)

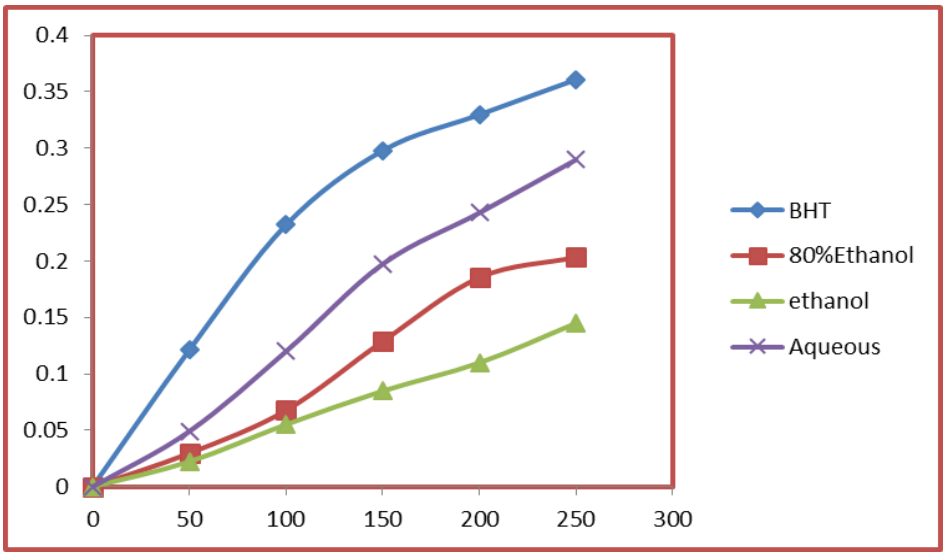

Fig 3: Reducing powers of different extracts of sample

The result of the study shows that the reducing power of the aqueous extract was more active and shows the intense absorption than ethanol and 80% ethanol extracts but lesser active than BHT. In an overall reducing power analysis the test can be arranged as:

Ascorbic acid> Aqueous extract> 80% Ethanol> Ethanol

Total antioxidant activity by Phosphomolybdenum method

The phosphomolybdenum method is routinely routinely to evaluate the total antioxidant capacity of plant extracts.I In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green colored phosphomolybdenum V complex, which shows a maximum absorbance at 700 nm (20). Figure 4 and table 2 shows that the antioxidant capacity of the extracts of *C. acumintum pseudobulbs* with different solvents can be ranked in the order From the results obtained, it can be seen that aqueous extract possessed significant antioxidant activity which increase in a concentration – dependent manner.

Table 2: Total antioxidant activity of ascorbic acid (Standard) and various extracts

Conc. (µg/ml)	Ascorbic acid	AE	EE	80%EE
0	0	0	0	0
50	0.4360±0.24	0.1873±0.35	0.0522±0.55	0.1873±0.63
100	0.6621±0.71	0.2289±0.38	0.0884±0.27	0.2289±0.46
150	0.7842±0.49	0.2571±0.45	0.1088±0.53	0.2570±0.67
200	0.8923±0.37	0.2819±0.28	0.1161±0.58	0.2819±0.72
250	0.9971±0.21	0.3380±0.19	0.2589±0.58	0.3381±0.47

Values are mean ± SE (n = 3)

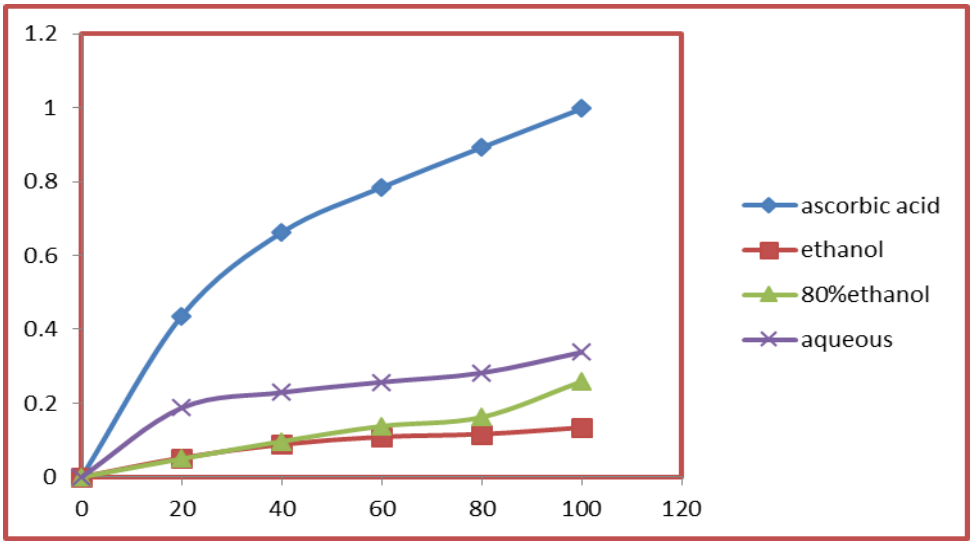

Fig 4: Total antioxidant activities of different extracts of sample

The result of the study shows that the total antioxidant activity of the aqueous extract was more active and shows the intense absorption than ethanol and 80% ethanol extracts but lesser active than Ascorbic acid. In an overall total antioxidant activity analysis the test can be arranged as:

Ascorbic acid> Aqueous extract> 80% Ethanol> Ethanol

Ferric reducing antioxidant powers of various extracts of *Crepidium acuminatum D.Don* pseudobulb samples:

FRAP assay is based on the ability of antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+} - TPTZ complex with an absorption maximum at 593 nm.

Table 3: Ferric reducing antioxidant powers of ascorbic acid (Standard) and various extracts of *Crepidium acuminatum D.Don* pseudobulb samples

Conc. (µg/ml)	Ascorbic acid	AE	EE	80%EE
0	0	0	0	0
50	0.4080±0.32	0.2331±0.46	0.0384±0.37	0.0302±0.24
100	0.5821±0.45	0.3983±0.64	0.0444±0.73	0.0667±0.28
150	0.6912±0.39	0.4733±0.65	0.0593±0.46	0.0884±0.26
200	0.7822±0.58	0.5745±0.61	0.0782±0.33	0.1427±0.52
250	0.8995±0.92	0.6632±0.56	0.0929±0.64	0.1902±0.57

Values are mean ± SE (n = 3)

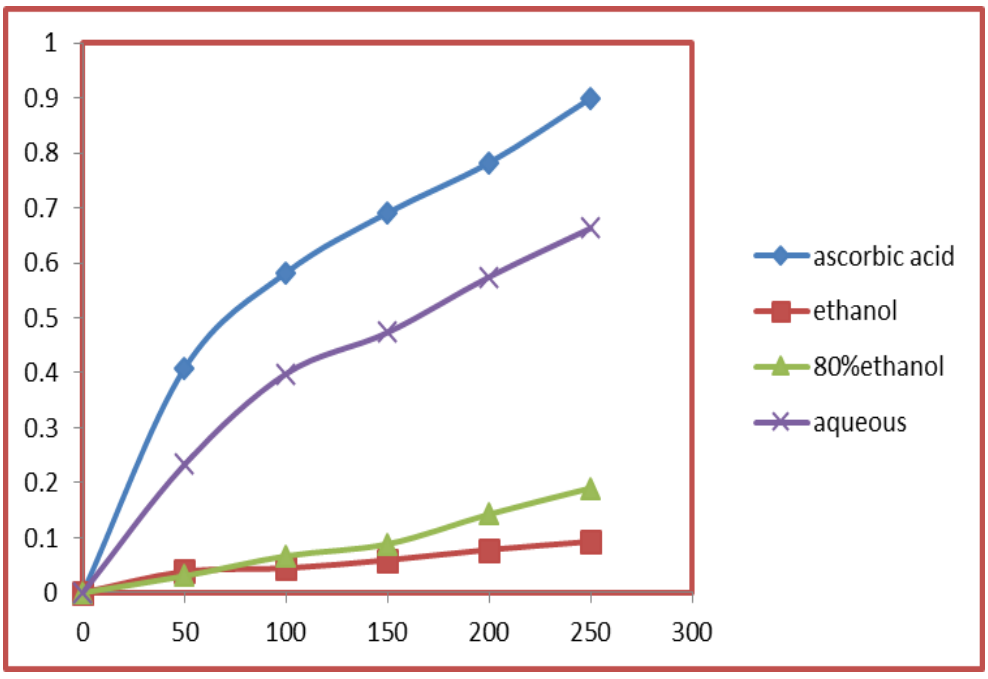

Fig 5: Ferric reducing antioxidant powers of different extracts of samples

The result of the study shows that the Ferric reducing antioxidant powers of the aqueous extract was more active and shows the intense absorption than ethanol and 80% ethanol extracts but lesser active than Ascorbic acid. In an overall the Ferric reducing antioxidant powers analysis the test can be arranged as:

Ascorbic acid> Aqueous extract> 80% Ethanol> Ethanol
All the above tests shows the similar pattern of the results which indicate aqueous extracts of pseudobulb powder of *Crepidium acuminatum D. Don* has highest antioxidant activity as compared to the other extracts such as 80% ethanol and ethanol extract of pseudobulb powder of *Crepidium acuminatum D. Don*. *In-vitro* antioxidant assay shows that *Crepidium acuminatum D.Don* pseudobulb powder has strong reducing power, high radical scavenging activities. Studies reveals that *Crepidium acuminatum D. Don* pseudobulb powder has poly-phenols, flavonoids etc. which are well known for their antioxidant activities.

Following relevant result are revealed for pseudobulbs:-
1. Reducing power
2. Ascorbic acid> Aqueous extract> 80% Ethanol> Ethanol Total antioxidant activity
3. Ascorbic acid> Aqueous extract> 80% Ethanol> Ethanol Ferric reducing antioxidant power

Ascorbic acid> Aqueous extract> 80% Ethanol> Ethanol
The results show that reducing power, total antioxidant activity and ferric reducing antioxidant power of the extracts showed similar pattern. Therefore, *Crepidium acuminatum D. Don* pseudobulb sample can protect cells through scavenging ROS in a dose-dependent manner. It is evident that the aqueous extract of pseudobulb powder of *Crepidium acuminatum D. Don* samples containing high levels of phenols and flavonoids has promising antioxidant activity than the 80% ethanol and ethanol extracts.

Discussion
The objective of the present study was to investigate the total phenolic content (TPC), total flavonoids content (TFC) and *in vitro* studies of *Crepidium acuminatum D. Don* (pseudobulb). Different extracts were used for this investigation. We used three types of extracts i.e. Alcoholic, hydroalcholic and aqueous. Extraction of phytoconstituents depends on various factors. Solvent is one of the important factor. It is observed that aqueous solvent give better yield than absolute solvents. Phenolic classes have capability of free radical scavengers therefore are evaluated as antioxidants. Therefore they are having lot of attraction in the field of food (21). Similarly Flavonoids are also plant secondary metabolites whose antioxidant potential depends on the presence of free OH groups. Generally Plant flavonoids have good antioxidant activity *in vitro* and also *in vivo*. Therefore in present study TFC was also calculated. It was found that aqueous extract of the plant have shown better *in vitro* activity. The *in vitro* antioxidant study was performed by different extracts (EE, 80% EE, AE) of *Crepidium acuminatum D. Don*. *In vitro* antioxidant activity was carried out by total antioxidant activity, reducing power assay and Ferric reducing ability of plasma (FRAP) assay. Ascorbic acid was used as a standard in total antioxidant activity and FRAP assay. Butylated hydroxyl toluene (BHT) was taken as a standard compound in reducing power assay. From antioxidant studies, it can be concluded that aqueous extract with high other extracts.
It is important to substitute synthetic antioxidants with naturally occurring safer antioxidants as the synthetics have been suspected of causing or provoking unfavourable side effects, while stronger restrictions are encountered on their application. Several reports have conclusively shown a correlation between the antioxidant activity and amount of total phenolics or total flavonoids. Plant extracts with high phenolic contents also possess high flavonoid content as reported for other plant species (22).Therefore estimation of TPC,TFC is to be evaluated to check the potential of the plant as antioxidant.

Conclusion
In this study the determination of TPC (Total phenolic content) and TFC (Total Flavonoid content) along with *in vitro* antioxidant studies by the 3 methods i.e. Reducing power Assay, TAC (Total antioxidant capacity) by phosphomolybdenum assay, Ferric Reducing Antioxidant Powder assay (FRAP) has been evaluated. These studies showed that this plant can be one of the potential sources of safer natural antioxidants. Further studies (*in vitro* and *in vivo)* are required for isolation and characterization of different moieties exhibiting budding anti-oxidation properties.

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