Int J Pharm Bio Sci 2012 July; 3(3): (P) 170 - 180

Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

PHYTOCHEMICAL EVALUATION AND IN VITRO STUDY OF ANTIOXIDANT POTENCY OF *AMORPHOPHALLUS CAMPANULATUS, ALOCASIA INDICA* AND *COLOCASIA ESCULENTA*: A COMPARATIVE ANALYSIS

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ABSTRACT

Amorphophallus campanulatus, Alocasia indica and *Colocasia esculenta* are very popular for their edible corms and leaves, especially in Assam and Bengal and are cultivated there as common food crops. Besides, they are also considered as medicinal plants in Sushruta Samhita and Ayurveda. So, the ethanolic extracts of these tubers were screened for the presence of in vitro antioxidant potential against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, ferric reducing power, hydroxyl radical and superoxide radical. Total phenolic and flavonoid contents of all the extracts were also evaluated. The results revealed that the ethanolic extracts of all the tubers possessed antioxidant property. Among the three, *Amorphophallus campanulatus* showed significantly higher ($P<0.01$) antioxidant potential in scavenging free radical than that of *Alocasia indica* and *Colocasia esculenta.* This higher ability to scavenge free radicals by *Amorphophallus campanulatus* may be attributed to the presence of higher amount of polyphenols and flavonoids in it, than the other two tubers as evidenced from our result. Thus the ethanolic extracts of these tubers can be utilized in future as therapeutic agent against free radical induced oxidative stress.

KEYWORDS; Oxidative stress; Antioxidant activity; DPPH; Free radical; Hydroxyl radical; Superoxide radical.

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INTRODUCTION

Free radicals are any atoms that have at least one unpaired electron in the outermost shell [1]. These uncoupled electrons are very reactive with adjacent molecules such as lipids, proteins, and carbohydrates and can cause cellular damage [2]. Excess free radical generation can result from tissue damage and hypoxia, overexposure to environmental factors (smoking, ultraviolet radiation, and pollutants), a lack of antioxidants, or destruction of free radical scavengers. The presence of free radicals in biological materials was discovered less than 50 years ago [3]. Today there is a large body of evidence indicating that uncontrolled production of free radical species (FRS) is responsible for several pathophysiological processes [4, 5]. The cause of a majority of disease conditions like atherosclerosis, hypertension, ischemic disease, Alzheimer's disease, Parkinsonism, cancer, diabetes mellitus and inflammatory conditions are being considered to be preliminarily due to imbalance between prooxidant and antioxidant homeostasis [6]. When the production of damaging free radicals exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs. Cells have a comprehensive array of antioxidant defense mechanisms to reduce free radical formation or limit their damaging effects[7]. These mechanisms are not sufficient when the balance shifts to the side of free radical generation [8], thus body requires antioxidant supplements to reduce oxidative damage. Nowadays, the use of synthetic antioxidants is limited because of inherent toxicity associated with them at optimum concentration. The use of natural antioxidants of plant origin is receiving great attention and is becoming one of the most acceptable modes of modern therapy [9].

Amorphophallus campanulatus, Alocasia indica and *Colocasia esculenta* are grown in East Asia, Malaysia and Pacific Coast. They are rhizomatous perennial herbs from the family Araceae. They are grown wild and cultivated all over India and are very popular for their edible corms and leaves. Especially in Assam and Bengal these are grown as food crop and the underground stems constitute a valuable and important vegetable of native diet. These are popularly known as elephant yam, giant taro and taro respectively in English while in Bengali these are referred to as 'ol kachu', 'mann kachu' and 'gathi kachu' respectively. In Ayurveda system of medicine specially the underground stem of these plants are used to cure various ailments like constipation, stomatitis, jaundice, diseases of abdomen, spleen, inflammation, hemorrhoids, hepato-splenopathies and general weakness. These are mainly starch based food item but also possesses several important antinutrient components like steroids, alkaloids, tannins, glycosides, phenols, flavonoids, saponins etc.

Therefore the objective of the present study was to perform a comparative evaluation of the antioxidant potential and free radical scavenging activity of ethanolic extracts of *Amorphophallus campanulatus, Alocasia indica* and *Colocasia esculenta.*

MATERIALS & METHODS

Procurement of plant materials

The tubers of *Amorphophallus campanulatus* (AC)*, Alocasia indica* (AI) and *Colocasia esculenta* (CE) were collected from local vegetable market of Kolkata district, West Bengal (India). Authentication was done by Dr. Krishnendu Sarkar, Associate Professor, Department of Botany, Rammohan College under University of Calcutta, West Bengal, (India).

Preparation of Ethanolic Extract

100 gm of dried powdered sample of each tuber were taken in the thimble of Soxhlet and extracted with 250 ml of ethanol (70%) in the round bottomed flask continuously for three days. The mixture was then filtered through muslin cloth, centrifuged and the collected filtrate was evaporated to dryness using rotary

evaporator (M/s B.C. Chatterjee & Co., Kolkata, West Bengal, India). The dried sample was collected and stored in air tight plastic vials for estimation of antioxidant property.

Reagents and Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), was obtained from Sigma, Aldrich. Gallic Acid, Quercetin, Rutin, Butylated hydroxytoluene (BHT), 2-deoxy-2-ribose, Potassium ferricyanide, Trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), Ethylenediaminetetraacetic acid (EDTA), Hydrogen peroxide (H_2O_2), Ascorbic acid, 2-thiobarbituric acid (TBA), Nitro blue tetrazolium (NBT), Riboflavin were procured from Hi- Media, Mumbai, India. Potassium chloride (KCl), Hydrochloric acid (HCl), Ethanol, Methanol, Aluminium chloride ($AlCl_3$), Sodium carbonate (Na_2CO_3), Sodium nitrite ($NaNO_2$), Sodium Hydroxide (NaOH), Di sodium hydrogen phosphate (Na_2HPO_4), Sodium dihydrogen phosphate (NaH_2PO_4) were procured from Merck India Ltd, Mumbai. All chemicals are of analytical grade.

Total phenols estimation

The total phenols of all extracts were measured at 765 nm by Folin Ciocalteu reagent[10]. The dilute ethanolic extracts of AC, AI and CE (0.5 ml of 1mg ml^{-1}) or Gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M). The mixture was allowed to stand for 30 min and the total phenols were determined by spectrophotometer (Systronics, India) at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/L solutions of Gallic acid in methanol: water (50:50, *v/v*). Total phenol values were expressed in terms of Gallic acid equivalent (mg/gm of dry mass), which is a common reference compound. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

Total flavonoid estimation

Total flavonoid contents were measured with the aluminum chloride colorimetric assay[11]. Ethanolic extracts of AC, AI and CE (1.0 ml of 1mg ml^{-1}) and different dilution of standard solution of Rutin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Total flavonoid content of the extracts were expressed in terms of Rutin equivalent (mg/gm of dry mass), which is a common reference compound. The standard curve was prepared using 20, 40, 60, 80, and 100 µg/ml rutin solution in methanol: water (50:50, *v/v*). All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

DPPH Radical Scavenging Activity

The stable DPPH radical was used for determination of free radical-scavenging activity of the extracts. 0.1 mM solution of DPPH in ethanol (22.2 mg in 1000 ml) was freshly prepared. 1 ml of different concentrations of ethanolic extracts of AC, AI and CE (100-1000 µg/ml) and standard ascorbic acid (10-100 µg/ml) were added to 2 ml of ethanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm[12]. Blank was performed in the same way with 1ml of ethanol instead of test substance. The percentage inhibition activity was calculated from:

Inhibition (%) = 1 – (sample OD/blank OD) × 100

All tests were performed in triplicate and the graph was plotted with the average of the three determinations. An IC_{50} was calculated as the concentration which brought about a 50% reduction in absorbance compared to blank.

Ferric Reducing Antioxidant Power (FRAP) Assay

Various concentrations of ethanolic extracts of AC, AI and CE (100-1000 µg/ml) and standard solutions of butylated hydroxytoluene (BHT) (100-1000 µg/ml) (1ml each), 2.5 ml of

phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were mixed separately and allowed to incubate at 50ºC for 30 min. After incubation period 2.5 ml of 10% TCA was added to the mixtures and centrifuged for 10 min at 3000 rpm. About 2.5 ml of the supernatant was diluted with 2.5 ml water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm[13]. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

Hydroxyl Radical scavenging Assay

Scavenging of hydroxyl free radical was measured by the method of Halliwell and Chirico[14], with minor changes. All solutions were prepared freshly. 200µL of 2.8mM 2-deoxy-2-ribose, 1ml of various concentrations of ethanolic extracts of AC, AI and CE (100-1000 µg/ml), 400 µL of 200 µM $FeCl_3$, 1.04mM EDTA (1:1 *v/v*), 200 µL of H_2O_2 (1.0 mM) and 200 µL ascorbic acid (1.0 mM) were mixed to form a reaction mixture. After an incubation period of one hour at 37ºC the extent of deoxyribose degradation was measured by the TBA reaction. 1.5 ml of 2.8% TCA and 1 ml of 0.336% TBA was added and boiled for 20 min on boiling water bath. After cooling the absorbance was read at 532 nm against a blank (containing all reagents except the extracts). The percentage inhibition activity was calculated from:

Inhibition (%) = 1 – (sample OD/blank OD) × 100

All tests were performed in triplicate and the graph was plotted with the average of the three determinations. An IC_{50} was calculated as the concentration which brought about a 50% reduction in absorbance compared to blank. Quercetin was used as standard.

Assay for superoxide radical scavenging activity

The assay was based on the capacity of the sample to inhibit blue formazon formation by scavenging the superoxide radicals generated in riboflavin-light-nitro blue tetrazolium (NBT) system. The reaction medium contained 2.5 mL of phosphate buffer (pH 7.6), 100 µL riboflavin (20 µg), 200 µL EDTA (12mM), 100µL NBT (0.1 mg) and 1ml of various concentrations of ethanolic extracts of AC, AI and CE (200-1200 µg/ml). The reaction was started by illuminating the reaction mixture for 5 minutes. The absorbance was measured at 590 nm. Blank was performed in the same way with 1ml of methanol instead of test substance[15]. The percentage inhibition activity was calculated from:

Inhibition (%) = 1 – (sample OD/blank OD) × 100

All tests were performed in triplicate and the graph was plotted with the average of the three determinations. An IC_{50} was calculated as the concentration which brought about a 50% reduction in absorbance compared to blank. Ascorbic acid was used as standard.

Statistical analysis

Results were subjected to statistical analysis using Student's t test. In all the cases, results are the mean ± SD of at least three individual experimental data, each in triplicate.

RESULTS

Total phenolic content and total flavonoid content:

The quantitative determination of the total phenolic content (TPC) is expressed as mg Gallic acid equivalents/gm dry weight of sample. TPC of ethanolic extract of *Amorphophallus campanulatus, Alocasia indica and Colocasia esculenta* is 190.42 ± 2.2 mg w*/w,* 87.54 ± 1.3 mg w*/w* and 66.25 ± 1.5 mg w*/w* respectively. Total flavonoid content of the extracts is expressed as mg of Rutin equivalent per gm dry weight of sample. Total flavonoid of ethanolic extract of *Amorphophallus campanulatus, Alocasia indica and Colocasia* esculenta is 6.23 ± 0.3 mg w/w, 3.5 ± 0.58 mg w/w and 1.48 ± 0.87 mg w/w respectively. Fig. 1a &1b shows the standard curve for total phenolic content with Gallic acid and total flavonoid content with rutin respectively.

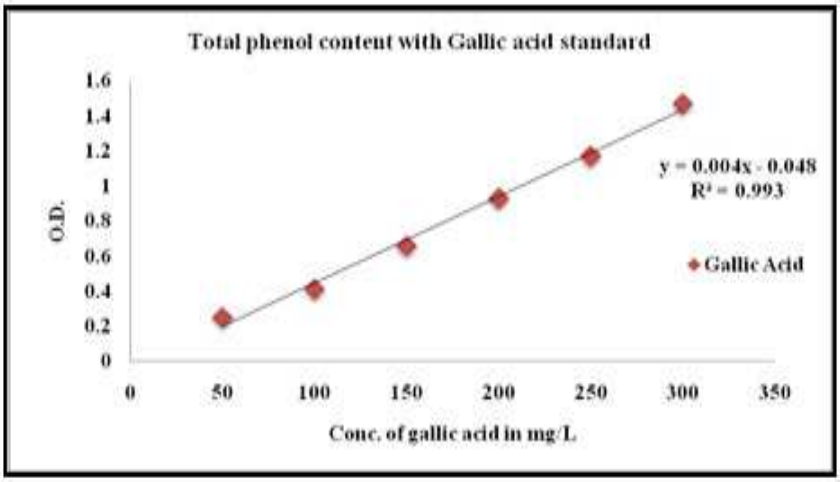

Figure 1a

Total phenol content with Gallic acid standard. The data are presented as means ± S.D of three independent experiments.

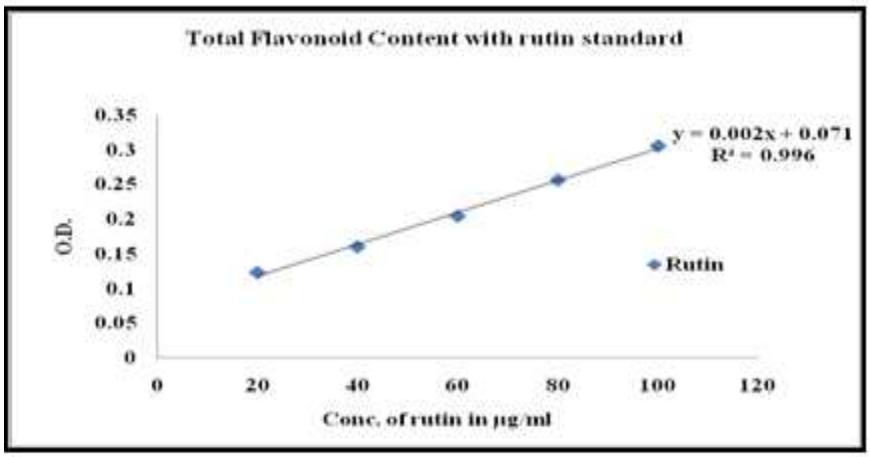

Figure 1b

Total Flavonoid Content with rutin standard. The data are presented as means ± S.D of three independent experiments.

DPPH Scavenging Assay

In the DPPH scavenging study, the ethanolic extracts of *Amorphophallus campanulatus, Alocasia indica and Colocasia esculenta* exhibited marked DPPH free radical scavenging activity in a concentration-dependent manner. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Fig. 2a & 2b shows that % inhibition decreases the concentration of DPPH radical due to scavenging ability of standard ascorbic acid, as a reference compound and that of the ethanolic extracts of the three tubers. The scavenging effect of AC, AI, CE and standard ascorbic acid on the DPPH radical decreased in the order: ascorbic acid > AC > AI > CE. The IC_{50} values for these compounds were found to be 67.79 µg/ml, 682.58 µg/ml, 1008.78 µg/ml and 1343.88 µg/ml respectively. The results also show that the ethanolic extract of AC possess significantly higher ($P<0.01$) DPPH scavenging activity than AI and CE and is most potent among the three extracts.

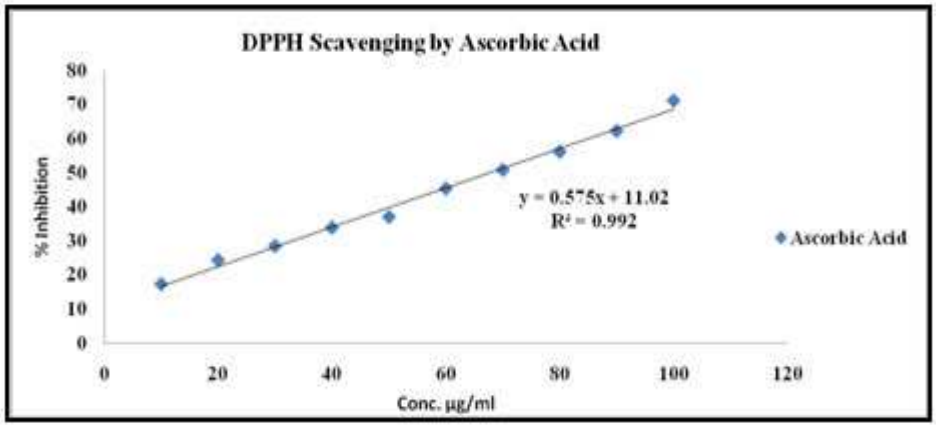

Figure 2a

DPPH scavenging by Ascorbic Acid. The data are presented as means ± S.D of three independent experiments.

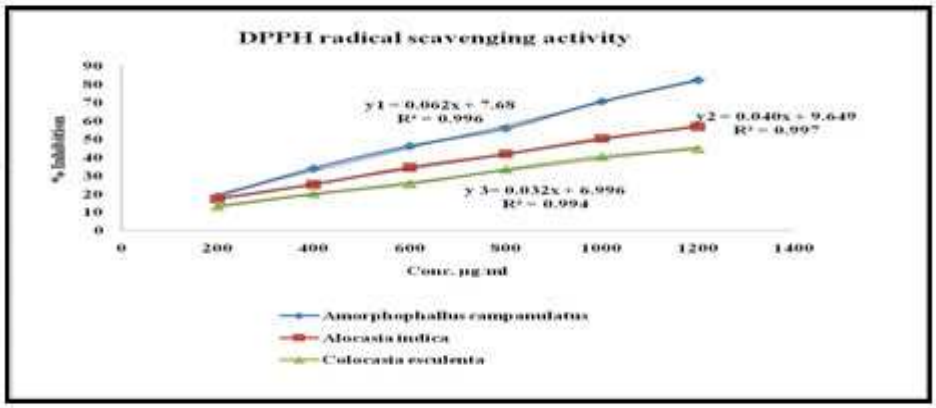

Figure 2b

DPPH scavenging by ethanolic extract of Amorphophallus campanulatus, Alocasia indica and Colocasia esculenta. The data are presented as means ± S.D of three independent experiments.

Ferric Reducing Antioxidant Power (FRAP) Assay:

Reducing power is a measure of reductive ability of antioxidants and is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of sample extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power of synthetic antioxidant BHT and the ethanolic extracts of AC, AI and CE are shown in Fig. 3. The data shows an increase in the reducing power for all the three extracts in a dose dependent manner. However, the ethanolic extract of AC presents better activity at all concentrations when compared to the other two extracts of AI and CE respectively.

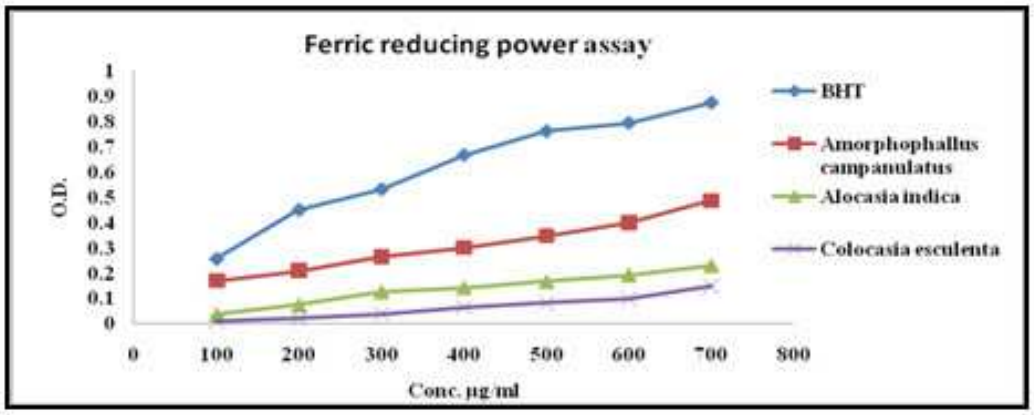

Figure 3

Reducing power (Fe^{3+} to Fe^{2+} conversion) shown by ethanolic extract of Amorphophallus campanulatus, Alocasia indica, Colocasia esculenta and known antioxidant BHT; Butylated hydroxytoluene. The data are presented as means ± S.D of three independent experiments.

Hydroxyl Radical scavenging Assay

Hydroxyl radicals are highly reactive and consequently short-lived. On generation in or exposure of biological systems to these radicals, they can cause damage to cells, including those in humans, where they react with DNA, lipids, and proteins. Here, in our study, it was found that the ethanolic extracts of all the three tubers: AC, AI and CE had the ability to scavenge hydroxyl radical generated in vitro by Fenton reaction. The ethanolic extracts exhibited antioxidant property by scavenging the hydroxyl radicals and thereby protecting the sugar from degradation. Percent inhibition of standard Quercetin and the ethanolic extracts of AC, AI and CE are illustrated in Fig. 4a & 4b. Besides quercetin, maximum % inhibition was shown by ethanolic extract of AC followed by AI and CE. The IC_{50} value of ethanolic extract of AC is 669.5 µg/ml which is significantly lower than ($P<0.01$) AI and CE which amounts to 988.37 µg/ml and 1059.36 µg/ml respectively.

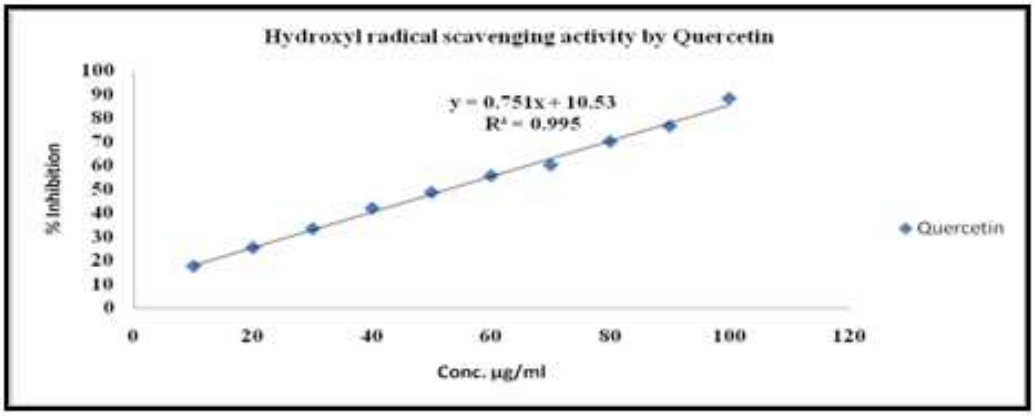

Figure 4a

Hydroxyl Radical Scavenging by Quercetin. The data are presented as means ± S.D of three independent experiments.

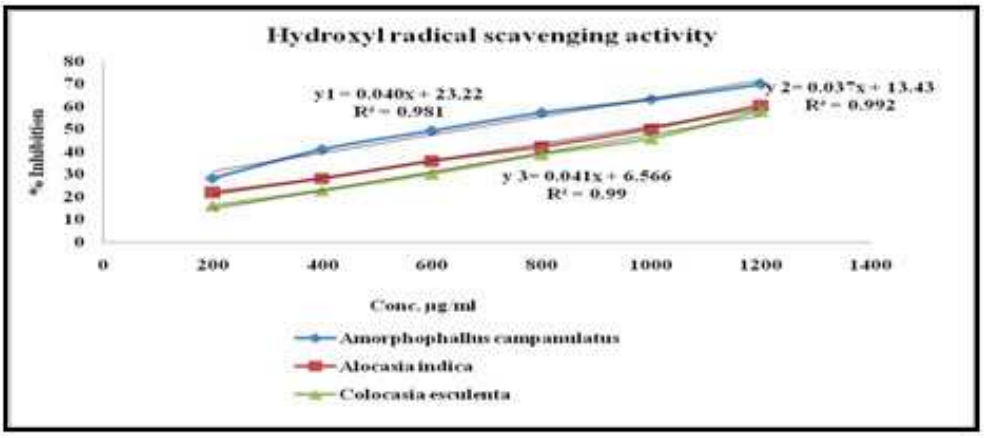

Figure 4b

Hydroxyl Radical Scavenging by ethanolic extract of Amorphophallus campanulatus, Alocasia indica and Colocasia esculenta. The data are presented as means ± S.D of three independent experiments.

Superoxide radical scavenging activity

Superoxide anions are a precursor to active free radicals that have potential for reacting with biological macromolecules, and thereby, inducing tissue damage. It has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical. Also, superoxide has been observed to directly initiate lipid peroxidation[16]. Superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of free radicals and oxidizing agents[17]. The ethanolic extracts of AC, AI and CE were found to possess concentration dependent scavenging activity on superoxide generated by photoreduction of riboflavin. Percent inhibition of standard ascorbic acid and that of the extracts is illustrated in Fig. 5a & 5 b. The ethanolic extracts show increased scavenging activity in dose dependent manner. Among the three, the ethanolic extract of AC shows maximum scavenging activity at all concentrations in comparison to AI and CE. It also possesses significantly lower ($P<0.01$) IC_{50} value of 703.33 µg/ml when compared to 1075.71 µg/ml and 1506.32 µg/ml for AI and CE respectively.

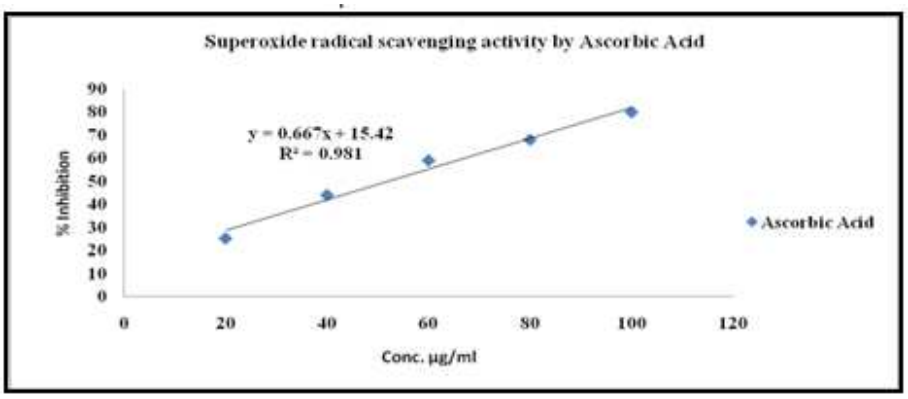

Figure 5a

Superoxide Radical Scavenging by Ascorbic Acid. The data are presented as means ± S.D of three independent experiments.

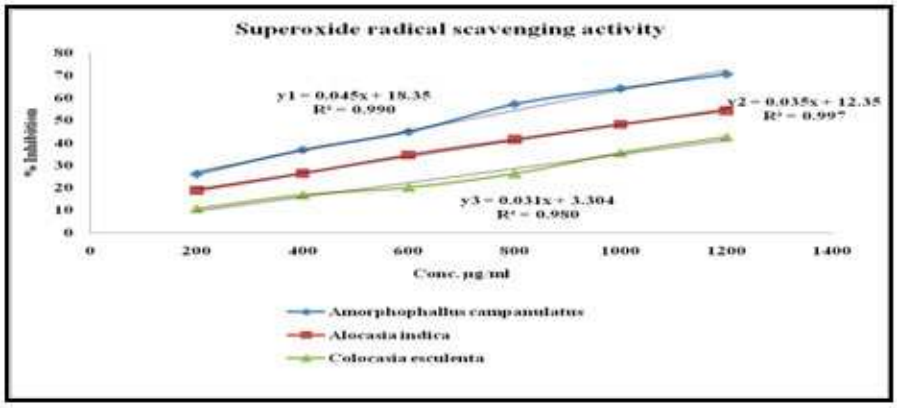

Figure 5b

Superoxide Radical Scavenging by ethanolic extract of Amorphophallus campanulatus, Alocasia indica and Colocasia esculenta. The data are presented as means ± S.D of three independent experiments.

Table 1 represents the equation, R^2 values and IC_{50} values for the ethanolic extracts of AC, AI, CE and the corresponding standards used to evaluate the DPPH, hydroxyl and superoxide radical scavenging activity.

Table 1

Results of all the % Inhibition studies

Results	Amorphophallus campanulatus (Ethanolic)	Alocasia indica (Ethanolic)	Colocasia esculenta (Ethanolic)	Standard
% Inhibition by DPPH				
Equation	y1 = 0.062x + 7.68	y2 = 0.040x + 9.649	y 3= 0.032x + 6.996	y = 0.575x + 11.02
R^2	0.996	0.997	0.994	0.992
IC_{50}	682.58 µg/ml**	1008.78 µg/ml*	1343.88 µg/ml	67.79 µg/ml
Hydroxyl Radical				
Equation	y1 = 0.040x + 23.22	y 2= 0.037x + 13.43	y 3= 0.041x + 6.566	y = 0.751x + 10.53
R^2	0.981	0.992	0.99	0.995
IC_{50}	669.5 µg/ml**	988.37 µg/ml	1059.36 µg/ml	52.55 µg/ml
Superoxide Radical				
Equation	y1 = 0.045x + 18.35	y2 = 0.035x + 12.35	y3 = 0.031x + 3.304	y = 0.667x + 15.42
R^2	0.99	0.997	0.98	0.981
IC_{50}	703.33 µg/ml**	1075.71 µg/ml*	1506.32 µg/ml	51.84 µg/ml
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IC_{50}	682.58 µg/ml**	1008.78 µg/ml*	1343.88 µg/ml	67.79 µg/ml
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Equation	y1 = 0.040x + 23.22	y 2= 0.037x + 13.43	y 3= 0.041x + 6.566	y = 0.751x + 10.53
R^2	0.981	0.992	0.99	0.995
IC_{50}	669.5 µg/ml**	988.37 µg/ml	1059.36 µg/ml	52.55 µg/ml
Superoxide Radical				
Equation	y1 = 0.045x + 18.35	y2 = 0.035x + 12.35	y3 = 0.031x + 3.304	y = 0.667x + 15.42
R^2	0.99	0.997	0.98	0.981
IC_{50}	703.33 µg/ml**	1075.71 µg/ml*	1506.32 µg/ml	51.84 µg/ml

Data set of n=3 and mean R^2 values obtained from the graphs.

***** Significantly lower (p<0.01) than ethanolic extract of Alocasia indica and Colocasia esculenta***

****Significantly lower (0.05) than ethanolic extract of Colocasia esculenta***

DISCUSSIONS

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals can bring about thousands of reactions and thus may cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals[18, 19], thus waving towards a number of oxidative stress-related disorders. Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing[20]. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals and there is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress. Protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants are also achieving importance in modern day lifestyle [21, 22].

Phenolic compounds and flavonoids are the major constituents in most plants reported to possess antioxidant and free radical scavenging activity. In our study, among the three ethanolic extracts, *Amorphophallus campanulatus* possesses highest phenolic and flavonoid content in terms of gallic acid and rutin equivalent respectively than that of *Alocasia indica* and *Colocasia esculenta*. Greater phenol and flavonoid content of *Amorphophallus campanulatus* is also reflected in its antioxidant potential when compared to *Alocasia indica* and *Colocasia esculenta.*

There are a number of assays designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to withstand free radical stress. Here, in this study, we have attempted to evaluate the antioxidant potential of the ethanolic extracts of *Amorphophallus campanulatus, Alocasia indica* and *Colocasia esculenta* by screening for their ability to scavenge DPPH radical and other reactive oxygen species like hydroxyl and superoxide radical. In all the tests performed it is found that ethanolic extract of *Amorphophallus campanulatus* show better free radical scavenging activity as well as greater ferric to ferrous reducing power, which is also an indirect measure of antioxidant potential of a compound, when compared to the other two extracts of *Alocasia indica* and *Colocasia esculenta.*

Thus it reveals that higher amount of polyphenols and flavonoids present in *Amorphophallus campanulatus* is responsible for making it the most potent antioxidant than the other two tubers, *Alocasia indica* and *Colocasia esculenta* belonging to the same family viz Araceae. The antioxidant potential of the three tuber extracts decrease in the following order: AC > AI > CE.

CONCLUSION

Amorphophallus campanulatus, Alocasia indica and *Colocasia esculenta* are popularly grown and available throughout the year all over West Bengal, India and are very common food item among the Bengali people. Besides, they are also affordable by people from all socio-economic strata because of their low cost, easy availability and wild growth. As we are gradually progressing towards an era of nutraceuticals, extracts from these tuberous vegetable especially that of *Amorphophallus campanulatus* can be explored further from pharmacognostic approach to formulate drug against lifestyle induced oxidative stress disorders, for which the results of this study will be of immense help.

ACKNOWLEDEMENT

The first author gratefully acknowledges the award of INSPIRE Fellowship from Department of Science and Technology, Government of India. The authors also wish to thank the authority of Rammohan College for providing the facilities to conduct the experiments.

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