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Comparative *in-vitro* Antioxidant Screening of Methanolic Extract of *Costus pictus* & Its Silver Nanoparticles

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ABSTRACT

To compare *in-vitro* antioxidant activity of Methanolic extract of *Costus pictus* (MECP) and its Silver nanoparticles (MECPAgNPs) by various methods. Preliminary phytochemical screening of MECP was done by standard procedure. Synthesis of silver nanoparticles from MECP was done. *In-vitro* anti-oxidant activity of the MECP & MECPAgNPs were studied by DPPH assay, H_2O_2 scavenging activity, Phosphomolybdenum Method, FRAP and reducing power assay. The total Phenolic content, Flavonoid content & Vitamin C were estimated by using Gallic acid, Quercetin and standard Ascorbic acid calibration curve respectively. Preliminary phytochemical screening showed the presence of carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, flavonoids, sterols and volatile oil. *In-vitro* antioxidant methods were resulted, the extract and the nanoparticles showed a dose dependent reducing ability. The nanoparticles at the same concentration offered much better activity than the extract alone. Phenolic content, Flavonoid content and Vitamin C amount of the MECPAgNPs was higher than MECP. These determination and quantification gives the information about the amount of secondary metabolites present in the MECPAgNPs was higher than the MECP which is responsible for the therapeutic or pharmacological activity of the plant. The MECPAgNPs showed very potent anti oxidant activity as compared to MECP.

Keywords: *Costus pictus*, *In-vitro* anti-oxidant, 1, 1-diphenyl-2-picryl hydrazyl (DPPH), Total Phenolic Content, Flavonoid content.

INTRODUCTION

Antioxidant protects the key cell components by neutralizing the damaging effects of free radicals, which are natural by-products of cell metabolism. [1-2] Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. [3-5] The plants contain a wide variety of free radical scavenging molecules such as flavonoids, phenols, terpenoids and vitamins. Natural antioxidants tend to be safer, therefore the evaluation of antioxidant activity of various plant extracts is considered as an important step in the identification of their ability to scavenge the free radicals. [6-7]

Screening of *In-vitro* antioxidant activity done by various methods such as, scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical, Nitric oxide scavenging activity assay, Total Antioxidant activity by phosphomolybdenum method and Ferric Reducing Antioxidant Power Assay (FRAP) etc. [8]

Costus pictus D. Don is commonly known as spiral ginger, belonging to the family Costaceae. It is a magical cure of diabetes. Its leaf helps to build up

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insulin the human body. So, it is commonly known as Insulin plant. [9-10] Leaf is traditionally used as anti diabetic [11-17], hypolipidemic [18], Antibacterial [19-20], Anti-cancer [21] and diuretic. [22-23]

The plant *Costus pictus* has been selected (specially the leaves) for the present investigation on the basis of the ethnomedical information and the review of literature as the plant is commonly available throughout India. Hence to exploit its potential use prompted the present study to investigate the leaves of this plant with clear scientific protocol. The leaf extract of *Costus pictus* contain bioactive compounds such as flavonoid, phenolic compound, tannin, triterpenes, sterols, alkaloids and vitamins. The extract may serve as a lead medicinal plant to synthesize various semi-synthetic drugs to treat various life threatening diseases such as diabetes, cancer etc. Antioxidant activity of *C. pictus* leaf extract was previously reported. [24-25] No studies available on antioxidant screening of its silver nanoparticles. This present study focused on comparative antioxidant activity of MECP & MECPAgNPs by various *in vitro* methods.

MATERIALS AND METHODS

Reagents: 0.1mM Diphenyl Picryl Hydrazyl Radical in Ethanol, 6% Hydrogen peroxide diluted with water in the ratio of 1:10, 0.1M Phosphate buffer (pH 7.4), 0.6M Sulphuric acid, 28mM Sodium phosphate, 4mM Ammonium molybdate, 1% Potassium Ferricyanide, 10% Trichloro acetic acid, Phosphate buffer (pH 6.6), 0.1% Ferric chloride.

FRAP Reagent contains,

a. Acetate buffer 30mM pH 3.6: Weigh 3.1 g Sodium acetate trihydrate and add 16 ml of Glacial acetic acid and make the volume to 1L with distilled water.
b. TPTZ (2, 4, 6-tripyridyl-s- triazine) (M.W. 312.34) 10mM in 40mM HCl
c. $FeCl_3 . 6H_2O$ (M.W. 270.30) 20mM

FRAP reagent was prepared freshly by mixing a, b & c in the ratio of 10:1:1 at the time of use.

Instruments: Shimadzu UV Visible spectrophotometer, Model 1800.

Collection and preparation of extract: The leaves of *Costus pictus* D. Don were collected and washed thoroughly and dried in shade. The shade dried leaves were powdered and sieved in a No. 60 sieve and used for the further studies. About 500g of the dried powdered leaf of *Costus pictus* D. Don was defatted with 1.5 L petroleum ether (60-80°C) by maceration. The solvent was removed by filtration and the marc was dried. To the dried marc 1.5 L of methanol was added and the extraction was performed by triple maceration (72 hours process). It was then filtered and the combined filtrate was evaporated to a cohesive mass using rota vapour.

Preliminary phytochemical screening [26-27]

The preliminary phytochemical screening helps us in identifying the type of secondary metabolites present in plants. Preliminary phytochemical screening of MECP was carried out by using standard procedure.

Synthesis of Methanolic leaf extracts of *Costus pictus* D. Don Silver nanoparticles (MECPAgNPs) [28]: 5ml of MECL was taken in the conical flask separately and placed on a magnetic stirrer with hot plate. To this 50 ml of 1mM $AgNO_3$ solution was added drop wise with constant stirring 120RPM at 50-60°C. The colour change of the solution was checked periodically.

***In vitro* ANTIOXIDANT ACTIVITY**

Free radical Scavenging activity using Diphenyl picryl hydrazyl (DPPH) free radical [29-31]: A stock solution of 0.5 mg/ml concentration of MECP and MECPAgNPs was prepared. To 1 ml of various concentrations of test samples, 4 ml of DPPH and the absorbance was measured at 517nm. Vitamin C was used as standard. The percentage scavenging & IC_{50} were calculated. A triplicate reading was taken and average was calculated.

Total antioxidant activity by Phosphomolybdenum Method [32-33]: An aliquot of 0.3 ml of different concentrations of sample solution (MECP & MECPAgNPs) was combined with 2.7 ml of the reagent solution (H_2SO_4, Sodium phosphate and Ammonium molybdate). In case of blank, 0.3 ml of Ethanol was used in place of sample. The tubes were incubated for 95°C for 90 min. The total antioxidant activity is expressed as the number of equivalents of Ascorbic acid (µg/g).

Ferric Reducing Antioxidant Power (FRAP) Assay [34-35]**:** MECP & MECPAgNPs was dissolved in Methanol to get a stock solution containing 1 mg/ml. Varying quantities of the stock solution were added to 3ml of FRAP reagent and absorbance was measured at 0min and 4min after vortexing at 593nm.

Reducing power assay [36-37]

Various concentrations of MECP & MECPAgNPs was mixed with 0.75 ml Phosphate buffer and 0.75 ml Potassium ferricyanide [$K_3Fe(CN_6)$], then the mixture was incubated at 50°C for 20 min. 0.75 ml of Trichloro acetic acid was added to the mixture, which was then centrifuged at 3000rpm for 10 min. Finally 1.5 ml of the supernatant solution was mixed with 1.5 ml of distilled water and 0.1 ml of Ferric chloride ($FeCl_3$) and absorbance was measured at 700nm.

Determination of scavenging activity against Hydrogen peroxide [38]

Varying quantities of the stock solution were added to 3.8 ml of 0.1M Phosphate buffer solution (pH 7.4) and then 0.2 ml of Hydrogen peroxide solution was added and the absorbance was measured at 230nm after 10 min. Ascorbic acid was used as standard. The percentage inhibition and IC_{50} of extract were determined.

Quantitative estimation of phytoconstituents

Estimation of total Phenolic content [39-41]

Phenolic substances are water soluble and they have been reported to have multiple biological effects, including antioxidant activity.

The total Phenolic content of the MECP & MECPAgNPs was determined by Folin-Ciocalteau reagent and it was expressed as milligram of Gallic acid equivalent (GAE) per g of extract.

Total Flavonoid content estimation [42-44]

Flavonoids have ability to complex with metal ions and act as an antioxidants and bind to proteins such as structural proteins and enzymes. The Aluminum chloride colorimetric technique was used for estimation of total Flavonoid content. The total Flavonoid content in MECP & MECPAgNPs was expressed as mg of Quercetin equivalents per g of extract.

Estimation of Vitamin C [45-46]

Vitamin C is also an important physiological antioxidant and has been shown to regenerate other antioxidants within the body, including α-Tocopherol (Vitamin E). Ascorbic acid was weighed and dissolved in water to get stock solution of 1mg/ml. Further dilutions were made to get the concentrations ranging from 40-200µg/ml. To 1 ml of sample 0.5 ml of Dinitro phenyl hydrazine solution was added and incubated for 3 h at 37ºC. After 3 h, 2.5 ml of 85% Sulphuric acid was added and the absorbance was measured after 30 min at 520nm. The amount of Vitamin C can be determined by linear regression analysis and it was expressed as mg/g of extract.

RESULTS

Preliminary phytochemical screening: Preliminary phytochemical screening showed the presence of carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, flavonoids, sterols and volatile oil.

Synthesis of Methanolic Leaf extracts of *Costus pictus* D. Don silver nanoparticles (MECPAgNPs): There was a visible color change after the substrate was added to the plant extract. Initially the plant extract was colourless. Upon adding the silver salt, it turned brown. After 5 h, no significant colour change was observed. Increased concentrations of silver nitrate resulted in a brown solution of nanosilver indicating the completion of reaction.

Table 1: Percentage inhibition of MECP, MECPAgNPs & standard Ascorbic acid against DPPH at 517nm

S. No	Conc. in µg/ml	Percentage inhibition by Ascorbic acid	Percentage inhibition by MECP	Percentage inhibition by MECPAgNPs
1	10	48.91 ± 0.60	35.31±0.115	43.3±0.085
2	20	48.91 ± 0.60	44.62±0.115	48.71±0.066
3	40	67.86 ± 0.27	48.64±0.394	60.82±0.085
4	60	79.49 ± 0.30	60.95±0.167	66.14±0.085
5	80	85.36 ± 0.29	64.61±0.227	70.23±0.182
IC_{50}		27.29µg/ml	46.66µg/ml	37.64µg/ml

*mean of three readings ± SEM

In-vitro ANTIOXIDANT ACTIVITY

Free radical Scavenging activity using 2, 2-Diphenyl-1-Picryl hydrazyl (DPPH)

The results for DPPH assay & graphical representation were presented (Table 1 & Fig. 1). From the Table 1, it can see that the MECP, MECPAgNPs and Ascorbic acid were showed percentage inhibition of 64.61±0.227, 70.23±0.182 and 85.31±0.29 at a concentration of 80µg/ml. The IC_{50} value was found to be 46.66, 37.64 and 27.29µg/ml for MECP, MECPAgNPs and Ascorbic acid respectively (Fig. 1). The extract possessed a good radical scavenging capacity.

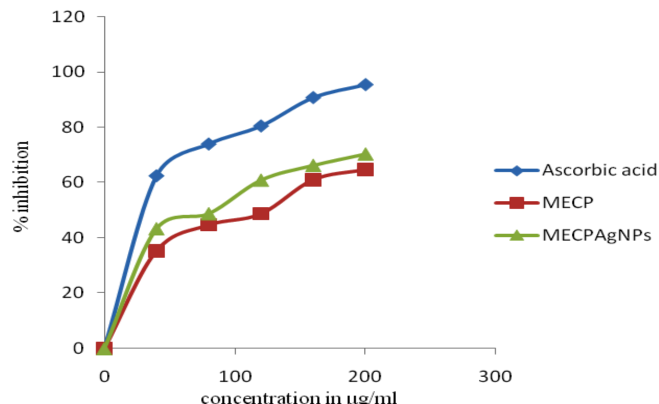

Fig. 1: Free radical scavenging of MECP, MECPAgNPs and Ascorbic acid against DPPH at 517nm

Antioxidant activity by Phosphomolybdenum method

The results obtained for the Phosphomolybdenum method and the graphical representation were presented (Table 2 & Fig. 2). From the Table 2, it can be seen that the MECP, MECPAgNPs and Ascorbic acid were showed an absorbance of 0.332±0.005, 0.355±0.007 & 0.371 ± 0.005 at a concentration of 100µg/ml. The extract showed a dose dependent reducing ability.

Table 2: Absorbance of MECP, MECPAgNPs and standard Ascorbic acid by Phosphomolybdenum method

S. No.	Conc. in µg/ml	Absorbance of Ascorbic acid	Absorbance of MECP	Absorbance of MECPAgNPs
1	16.66	0.085 ± 0.005	0.057±0.003	0.065±0.008
2	33.33	0.165 ± 0.004	0.126±0.005	0.138±0.003
3	50.00	0.206 ± 0.008	0.185±0.006	0.199±0.005
4	66.66	0.323 ± 0.004	0.28±0.008	0.305±0.005
5	83.33	0.371 ± 0.005	0.332±0.005	0.355±0.007

*mean of three readings ± SEM

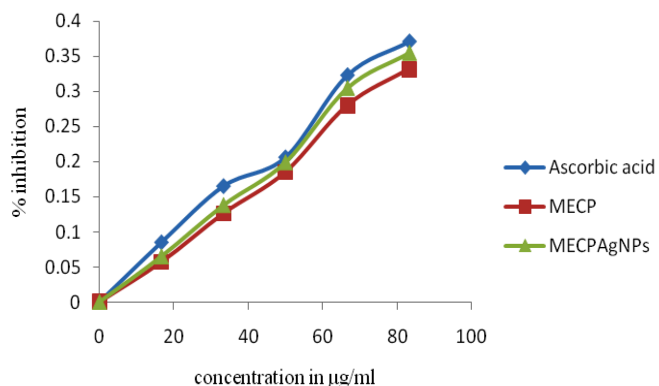

Fig. 2: Antioxidant activity by Phosphomolybdenum method

Ferric Reducing Antioxidant Power (FRAP) Assay

The results obtained for the Ferric Reducing Antioxidant Power assay and the graphical representation were presented (Table 3 & Fig. 3). From

the Table 3, it can be seen that the MECP, MECPAgNPs and Ascorbic acid were showed an absorbance of 0.7 ± 0.0011, 0.87 ± 0.002 and 0.936 ± 0.002 at a concentration of 100µg/ml.

Table 3: Ferric reducing anti-oxidant power assay of MECP, MECPAgNPs & standard Ascorbic acid

S. No	Conc. in µg/ml	Absorbance of Ascorbic acid	Absorbance of MECP	Absorbance of MECPAgNPs
1	12.5	0.457 ± 0.001	0.36±0.0006	0.38±0.0014
2	25	0.576 ± 0.004	0.44±0.0017	0.47±0.0060
3	50	0.667 ± 0.003	0.57±0.0017	0.59±0.0043
4	75	0.821 ± 0.001	0.72±0.0014	0.76±0.0072
5	100	0.936 ± 0.002	0.79±0.0011	0.87±0.0020

*mean of three readings ± SEM

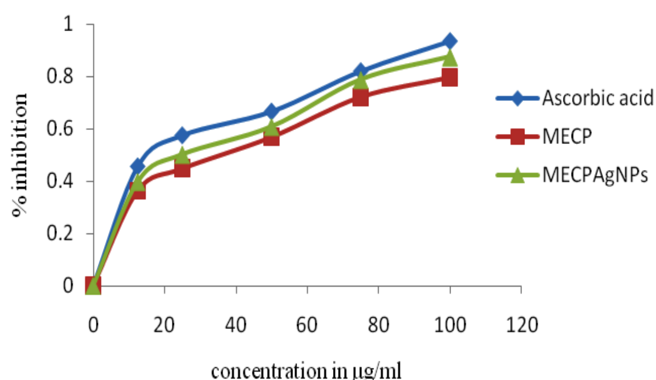

Fig. 3: Ferric reducing anti-oxidant assay of MECP &MECPAgNPs

Reducing Power Assay

The results obtained for the Reducing Power assay and the graphical representation were presented (Table 4 & Fig. 4). From the Table 4, it can be seen that the MECP, MECPAgNPs and Ascorbic acid were showed the absorbance of 0.780±0.007, 0.842±0.007 & 1.052±0.007 at a concentration of 100µg/ml.

Table 4: Reducing Power Assay of MECP, MECPAgNPs and standard Ascorbic acid

S. No.	Conc. in µg/ml	Reducing power of Ascorbic acid	Reducing power of MECP	Reducing power of MECPAgNPs
1	20	0.745 ± 0.012	0.340±0.002	0.451±0.005
2	40	0.820 ± 0.003	0.414±0.004	0.543±0.004
3	60	0.930 ± 0.002	0.641±0.002	0.702±0.003
4	80	0.958 ± 0.059	0.689±0.003	0.738±0.005
5	100	1.052 ± 0.007	0.780±0.007	0.842±0.007

*mean of three readings ± SEM

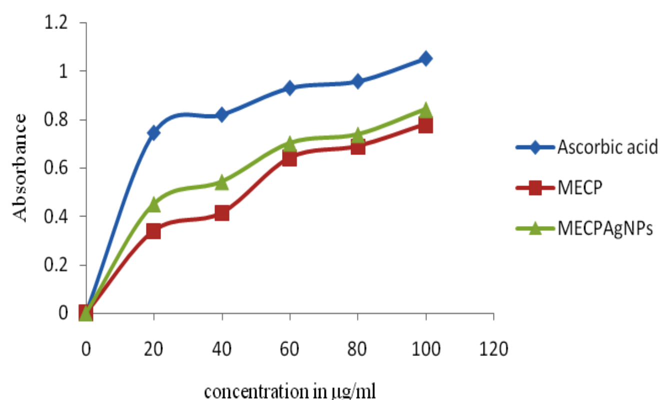

Fig. 4: Antioxidant activity by reducing power assay

Determination of scavenging activity against Hydrogen peroxide

The results obtained for Hydrogen peroxide assay and the graphical representation was presented (Table 5 & Fig. 5). From the Table 5, it can seen that the MECP, MECPAgNPs and Ascorbic acid were showed percentage inhibition of 74.07 ± 0.29 & 74.56 ± 0.28 and 79.99 ± 0.51 at a concentration of 400µg/ml. The IC_{50} value calculated using the linear regression analysis was found to be 287.14, 251.82 and 221.21µg/ml for MECP, MECPAgNPs and Ascorbic acid respectively.

Table 5: Percentage inhibition of Hydrogen peroxide by MECP, MECPAgNPs & standard Ascorbic acid

S. No	Conc. in µg/ml	Percentage inhibition by Ascorbic acid	Percentage inhibition by MECP	Percentage inhibition by MECPAgNPs
1	80	32.44 ± 0.81	23.61±0.35	27.16±0.28
2	160	35.47 ± 0.91	32.9±0.46	32.17±0.83
3	240	60.73 ± 0.51	43.45±0.28	45.01±0.43
4	320	67.32 ± 0.53	64.36±0.33	66.49±0.21
5	400	79.99 ± 0.51	74.07±0.29	74.56±0.28
IC_{50}		221.21 µg/ml	287.14 µg/ml	251.82 µg/ml

*mean of three readings ± SEM

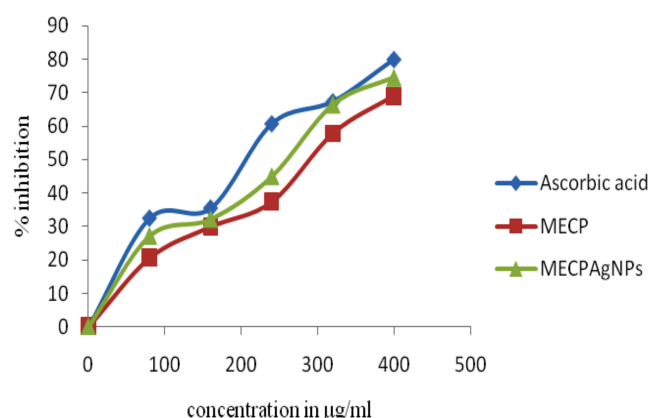

Fig. 5: Scavenging activity by Hydrogen peroxide method

Quantitative estimation of phytoconstituents

Estimation of total phenolic content: The results for the total phenolic content of MECP & MECPAgNPs and Gallic acid calibration curve were presented (Tab. 6 & Fig 6).

Table 6: Total phenolic content of MECP & MECPAgNPs by Folin-ciocalteu method

Conc. of Gallic acid (µg/ml)	Absorbance at 760nm	Conc. of Methanolic extract (µg/ml)	Absorbance at 760nm		Amount of total Phenolic content in terms of mg GAE/g of extract*	
			MECP	MECPAgNPs	MECP	MECPAgNPs
2	0.229± 0.010	50	0.530± 0.001	0.542± 0.002	92.06± 1.2	94.13±0.3
4	0.452± 0.006	100	0.980± 0.008	0.991± 0.001	84.82± 0.5	85.77±0.8
6	0.695± 0.005					
8	0.918± 0.031		**Average**		**88.44± 0.85**	**90±0.55**
10	1.162± 0.028					

*mean of three readings ± SEM

The linear regression equation was found to be y = 0.116x-0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the MECP & MECPAgNPs in terms mg GAE/g of extract was found to be 88.44±0.85 & 90±0.55 by using the above linear regression equation.

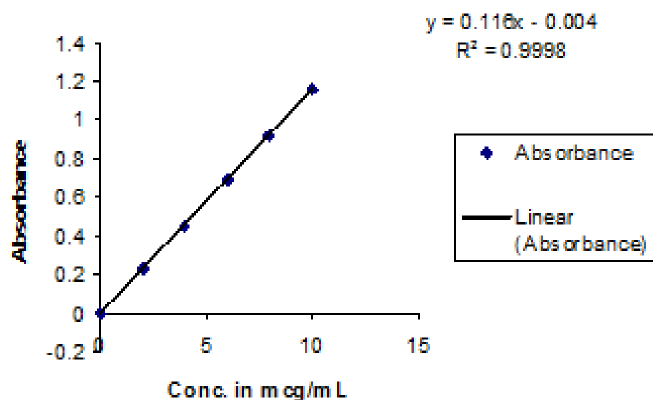

Fig. 6: Calibration curve of Gallic acid for estimation of total Phenolic content

Estimation of total Flavonoid content

The results for total Flavonoid content of MECP & MECPAgNPs and Quercetin calibration curve were presented (Table 7 & Fig. 7).

Table 7: Total Flavonoid content in MECP & MECPAgNPs by Aluminum chloride method

Conc. of Quercetin in µg/ml	Absorbance at 415nm	Conc. of extract in µg/ml	Absorbance at 415nm*		Amount of total flavonoids content in terms mg QE/g of extract*	
			MECP	MECP AgNPs	MECP	MECP AgNPs
20	0.589 ± 0.01	100	0.337± 0.011	0.35±0.011	115.96 ±0.26	117.45 ±0.37
40	1.151 ± 0.04	200	0.669± 0.017	0.65±0.017	123.76 ±3.69	128.07 ±0.37
60	1.710 ± 0.09					
80	2.390 ± 0.03		Average		**119.86 ±1.97**	**122.76 ±0.37**
100	3.112 ± 0.03					

*mean of three readings ± SEM

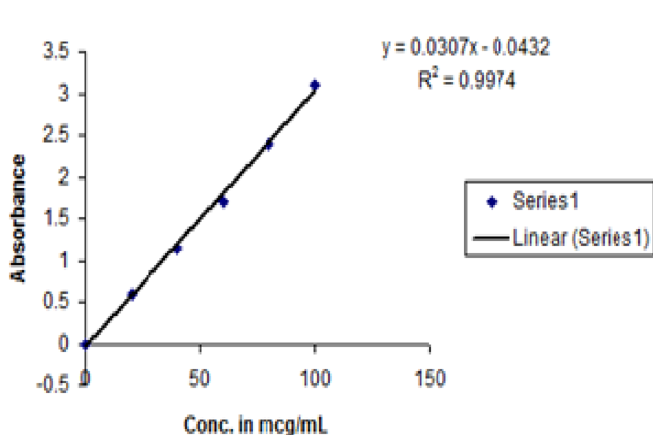

Fig. 7: Calibration curve of Quercetin for estimation of Flavonoid content

The linear regression equation was found to be y = 0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of Flavonoid content present in the MECP & MECPAgNPs in terms mg Quercetin equivalent/g of extract was found to be 119.86±1.97 & 122.76±0.37mg/g of extract by using the above linear regression equation.

Estimation of Vitamin C Content

The results for Vitamin C content of MECP & MECPAgNPs are presented (Table 8 & Fig. 8).

Table 8: Vitamin C content of MECP & MECPAgNPs

Conc. of Ascorbic acid (µg/ml)	Absorbance at 520nm	Conc. of extract (µg/ml)	Absorbance at 520nm*		Amount of Vitamin C present/g of extract*	
			MECP	MECP AgNPs	MECP	MECPAgNPs
40	0.135 ± 0.000					
80	0.265 ± 0.015					
120	0.346± 0.010	50	0.0866 ±0.012	0.094±0.066	746.66 ±12.01	830.04±10.03
160	0.468 ± 0.011					
200	0.525 ± 0.010					

*mean of three readings ± SEM

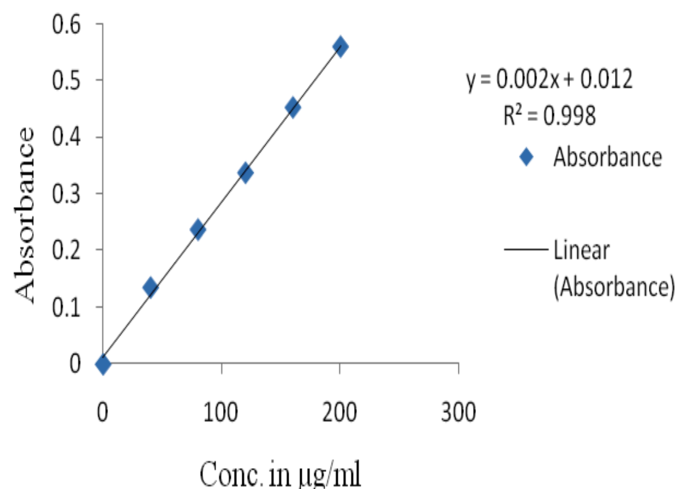

Fig. 8: Calibration curve of Ascorbic acid

DISCUSSION

The colour change of the medium from colourless to brown after 5 h was indicated the formation of silver nanoparticles. It showed that aqueous silver ions could be reduced by the Methanolic extract of *Costus pictus* (MECP) D. Don to generate extremely stable silver nanoparticles. All *In-vitro* antioxidant methods were resulted, the extract and the nanoparticles showed a dose dependent reducing ability. The nanoparticles at the same concentration offered much better activity than the extract alone. Polyphenols are naturally occurring compounds largely found in the herbals and medicinal plants. Phenolic compound may protect cell constituents against oxidative damage and, therefore,

limit the risk of various degenerative diseases associated with oxidative stress. [47] Consumption of plant polyphenols offer protection against development of diabetes, cancers, cardiovascular diseases and neurodegenerative diseases. [48-49] Phenolic content of MECPAgNPs was higher than MECP.

More than 4,000 varieties of flavonoids have been identified, many of which are responsible for the therapeutic activity in humans. Quercetin, Myricetin, Catechin etc., are some most common flavonoids. [50] Quercetin is known to possess strong anti diabetic activity. Recently reported Quercetin has ability to protect the alterations in diabetic patients during oxidative stress. Quercetin significantly protected the lipid peroxidation and offer antioxidant effect in Diabetes. [51] Flavonoid content of MECPAgNPs was higher than MECP.

Free radicals are capable of damaging essential biomolecules such as Proteins, DNA and Lipids which implicated in the etiology of several degenerative diseases such as Stroke, Coronary artery diseases, Rheumatoid arthritis, Diabetes and Cancer. Vitamin C offer antioxidant effect which protect from several degenerative diseases. [52] Vitamin C content of MECPAgNPs was higher than MECP.

The amount of secondary metabolites present in the MECPAgNPs was higher than the MECP which is responsible for the therapeutic or pharmacological activity of the plant. The MECPAgNPs showed very potent anti oxidant activity as compared to MECP.

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