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A Study on phytochemical properties and Anti-oxidant activity of different solvent extract of siddha drug Paruthi Chooranam in *In -Vitro* model.

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Abstract

Siddha system of medicine has holistic approach in treating diseases pertains to mankind. According to the principles of siddha human body is composed of distinct fluid called humors. Change in the proportion of vata, pitta and kapha leads to several physiological changes which may render dreadful diseases. Further it was also evident that deficiency in anti-oxidant defense system contributes stress related disorders. A role of oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory condition, certain cancers, and the process of aging. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases. Modern research outcomes even abide with the concept of therapeutic anti-oxidants. Siddha formulations comprises of herbs with bioactive phytocomponents that may acts as powerful antioxidants. Research interest in the field of anti-oxidant therapy is emerging in recent times. There is a profound risk of using synthetic antioxidants which are known to have side effects and have been proved carcinogenic. The main aim of the present study is to evaluate the anti-oxidant potential of the siddha formulation *Paruthi Chooranam (PC)*which consists of *Gossypium herbaceum* as a major ingredient using *In-vitro* antioxidant and metal chelating assays. The results of phytochemical analysis revealed the presence of active phytocomponents such as alkaloid, flavonoid, saponin, triterpenoids and polyphenols. Total phenolic and flavonoid content of *PC*was found to be 61.54 and 54.23 µg equivalent. Siddha formulation *PC* has shown significant percentage reduction of 81.23% on ABTS radicals scavenging activity. Followed by this 72.43% of metal chelating activity and 77.48 % of activity observed in LPO assay. The results of superoxide radical scavenging assay has shown that the percentage inhibition of 73.49 %. Similarly the NO radical scavenging activity of the *PC* ranges from 22.15 to 79.23%. From the result analysis of the present investigation it was concluded that the formulation *PC* possess significant radical scavenging activity in all the tested in-vitro assays which may be due to the presence of core phytochemicals present in the formulation this unique blend of novel phytocomponents makes this formulation to be drug of choice in management of stress related disorders.

Keywords: Siddha system, *ParuthiChooranam* ,*Gossypiumherbaceum*, Phytocomponents, DPPH assay

1. Introduction

Oxidative stress caused by generation of free radicals and their derivatives leads to disturbances in redox homeostasis [1]. Reactive oxygen species (ROS) are endogenously produced during intracellular metabolic processes but can also be generated by exogenous stimuli such as UV radiation, pollutants, smoke, and drugs [2-3]. When intracellular oxidative status increases, the cell triggers its defense systems or undergoes apoptosis. These responses to oxidative stress influence numerous cellular processes including core signaling pathways, which are associated with development of systematic and/or chronic disorders including aging and cancer [4]. Therefore, it is critical to remove cellular oxidants and restore redox balance.
Plants are rich in antioxidants; so much attention has been directed towards the development of ethnomedicines as they contain phenols, flavonoids, alkaloids, tannins, vitamins, terpenoids, and many more phytochemicals responsible for different pharmacological activities [5]. Current research has proofed that ingestion of natural antioxidants has been associated with reduced risk of cancer and many chronic diseases [6].

Siddha system of traditional medicine has unique tendency of rejuvenating the body and soul. Several combinations of formulations included metals, herbs, herbominerals having class of ingredients and phytocomponents that possess the tendency to completely alleviate the generation of free radicals and also quenches the free unstable radicals. *Paruthi Chooranam (PC)*is one such novel siddha formulation which consists of *Gossypium herbaceum* as a major ingredient.

Gossypium spp was one of the earliest plants that have been cultivated by mankind and it has been used for over 4,000 historic years. It was evident through several research that Gossypium possess several significant pharmacological activities such as neurotonic for memory and learning [7], Anti-epileptic [8], Anti-oxidant [9], Anti-diabetic [10], Anti-hyperlipidemic [11],wound healing [12],Anti-Microbial [13], Diuretic [14], Ulcer healing [15]. Medicinally, cotton seeds were used as pain reliever, as a nervine tonic in treating of headache and migraine; the decoctions of the seed were given in intermittent fever. The seeds and flowers in the form of poultice were applied to burns. Seeds were also used in epilepsy and as an antidote to snake poison. The juice of the leaves and the decoctions of the seed were used in dysentery [16-18]

The generation and subsequent involvement of free radicals in a large number of diseases prompted us to study the antioxidant potential of siddha drug *PC*. In order to consider any substrate or plant extract as an effective antioxidant, it should act as antioxidant under both *in vivo* and *in vitro* conditions by decreasing the level of pro oxidant like radicals. The main aim of the present study is to evaluate the anti-oxidant potential of the siddha formulation PC using In-vitro antioxidant and metal chelating assays.

2. Materials and Methods

2.1.Extraction of *Paruthi chooranam:*

Paruthi chooranam (Gossypium herbaceum) dry leaves were finely powdered and triturated in house hold mixer grinder without adding water. Then the powdered leaves were made aqueous decoction into sterile distilled water in water both 100 ^{0}C for 20 minutes. The extracts were filtered and evaporated to dryness and kept for further studies.

2.2. Preliminary phytochemical Evaluation [19]:

Test drug *PC* was subjected to class of preliminary phytochemical screening of the following components

Test for Alkaloid- Mayer's reagent

To the test drug about 2ml of Mayer's reagent was added and was observed for the presence of alkaloids. Appearance of dull white precipitate indicates the presence of alkaloids.

Test for flavonoid

To 0.1ml of the test sample about 5 ml of dilute ammonia solution were been added followed by addition of few drops of conc. Sulfuric acid. Appearance of yellow color indicates the presence of Flavonoids.

Test for Glycosides -Borntrager's Test

Test drug is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests. To 2 ml of filtered hydrolysate, 3 ml of choloroform is added and shaken, choloroform layer is separated and

10% ammomia solution is added to it. Pink colour indicates presence of glycosides.

Test for Triterepnoids

To the test solution 2ml chloroform was added with few drops of conc. Sulphuric acid (3ml) at the side of the test tube. An interface with a reddish brown coloration is formed if terpenoids constituent is present.

Test – Phenol- Lead acetate test

The test sample is dissolved in of distilled water and to this 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds.

Test for tannins

About 0.5ml of test sample is boiled in 20 mL of distilled water in a test tube and then filtered. The filtration method used here is the normal method, which includes a conical flask and filter paper. The 0.1% FeCl3 is added to the filtered samples and observed for brownish green or a blue black coloration, which shows the presence of tannins

Test for Saponins

The test drugs were shaken with water vigorously for 10 mins , copious lather formation indicates the presence of saponins.

Test for Anthocyanin

About 0.2 ml of the extract was weighed in separate test tube, 1ml of 2N Sodium hydroxide was added, and heated for 5 minutes at 100 ± 2°C. Observed for the formation of bluish green color which indicates the presence of anthocyanin.

2.3. Determination of total Phenol content of *PC:*

The total phenol content was determined using Folin–Ciocalteu reagents with analytical grade Gallic acid as the standard. 1 ml of sample was added to deionized water (10 ml) and Folin–Ciocalteu phenol reagents (1ml). After 5 minutes, 20% sodium carbonate (2 ml) was added to the mixture. After being kept in total darkness for 1 hr, the absorbance was measured at 750 nm using a spectrophotometer. Amounts of total Phenol was calculated using Gallic acid calibration curve. The results were expressed as catechin equivalents mg/g of the extract [20].

2.4. Determination of Total Flavonoid of *PC:*

Total flavonoid content in the drug was determined using aluminum chloride method. In this method Quercetin was used as standard and flavonoid contents were measured as quercetin equivalent. For this purpose, the calibration curve of quercetin was drawn. 1ml of standard or extract solution was taken into 10ml volumetric flask, containing 4ml of distill water. 0.3ml of 5%NaNO2 added to the flask. After 5min, 0.3ml 10%AlCl3 was added to the mixture. At the 6th min add 2ml of 1M NaOH was added and volume made up to 10ml with distills water. The absorbance was noted at 510nm using UV-Visible spectrophotometer [21].

2.5. Reducing power ability of the *PC:*

The reducing power of the *PC* extract was investigated by the Fe^{3+}-Fe^{2+}transformation in the presence of the PC. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. One ml of the test drug with varying concentration, 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000 *g*. About 2.5 ml of the supernatant was diluted with 2.5 ml of distilled water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Butylated hydroxytoluene (BHT) and ascorbic acid were used as the standards. All tests were performed in triplicate and the graph was plotted with the average of the three determinations [22-23].

2.6. ABTS radical scavenging activity of *PC:*

This assay carried out for the purpose of evaluating the anti-oxidant potential of test drug *PC*against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS radicals. The ABTS radical cation method was modified to evaluate the free radical-scavenging effect of one hundred pure chemical compounds. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1 : 44, v/v). To determine the scavenging activity, 100 µL ABTS reagent was mixed with 100 µL of test sample (10-100µg/ml) and was

incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm. 100% methanol was used as a control. Gallic acid with same concentrations of test drug PC was measured following the same procedures described above and was used as positive controls. The antioxidant activity of the test sample SC was calculated using the following equation: The ABTS scavenging effect was measured using the following formula

$$\text{Radical scavenging (\%)} = \left[\frac{(A)_{control} - (A)_{sample}}{(A)_{control}}\right] \times 100.$$

2.7. Metal Chelating activity of *PC*[25]:

Metal chelating activity was measured as described previously, by adding 0.1 mM $FeSO_4$ (0.2 mL) and 0.25 mM ferrozine (0.4 mL) subsequently into 0.2 mL of the *PC* . After incubating at room temperature for 10 min, absorbance of the mixture was recorded at 562 nm. Chelating activity was calculated using the following formula:

Metal chelating activity =

$$(A_{control} - A_{sample})/A_{control} \times 100$$

Where $A_{control}$ is the absorbance of control reaction (without plant extract), and A_{sample} is the absorbance in the presence of a plant extract.

2.8. Lipid peroxidation Inhibition activity of *PC*:

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of Polyphenol rich fraction of *Paruthi chooranam* (*Gossypium herbaceum*) in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample [26].

2.9. Superoxide radical scavenging activity of *PC:*

This assay was based on the capacity of the Polyphenol rich fraction of both country and hybride variety of *paruthi chooranam* (*Gossypium herbaceum*) to inhibit the photochemical reduction of Nitrobluetetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer [27]. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:

% Super oxide radical scavenging capacity=

$$[(A_0-A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of both plant extracts fraction.

2.10. Nitric oxide radical scavenging activity of *PC:*

The concentrations of test sample PC are made into serial dilution from 10−100 µg/mL and the standard gallic acid. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediaminedihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the test drug (10−100 µg/mL) and incubated at 25°C for 180 mins [28]. The test drug

PC was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the test drug but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The absorbance was measured at 546 nm using a Spectra Max Plus UV-Vis microplate reader (Molecular Devices, GA, USA). Gallic acid was used as the positive control. The percentage inhibition of the test drug SC and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the test drug SC and gallic acid were calculated using the following formula:

percentage nitrite radical scavenging activity:

$$\text{nitric oxide scavenged } (\%) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100,$$

where A_{control} = absorbance of control sample and A_{test} = absorbance in the presence of the samples extracts or standards.

3. Results

3.1. Result Analysis of Qualitative and Quantitative Phytochemical Investigation of *PC:*

The results of preliminary qualitative phytochemical analysis of the sample PC have shown the presence of certain biologically active phytocomponents such as alkaloid, flavonoid, saponin, triterpenoids and polyphenols (Table 1). Results of quantitative phytochemical investigation of *PC* reveals that total phenolic and flavonoid content of *PC* was found to be 61.54 and 54.23 µg equivalent. As listed in Table 2.

Table No 1: Preliminary qualitative phytochemical analysis of the sample *PC :*

Sl. No.	Phytochemical Constituents	Observation	*Paruthi chooranam* (*Gossypium herbaceum*)
1	**Alkaloids** -Dragendorff's Test -Mayers test	Orange / red precipitate Yellow or white precipitate	+ +
2.	**Flavonoids** -Alkalai Reagent -Lead acetate test	Intense yellow colour Precipitate formed	+ +
3.	**Glycosides** Keller-Killiani test	Reddish brown colour ring formed	-
4.	**Tannin** -$FeCl_3$ test	Blue black coloration	+
5.	**Saponins** -Frothing test	Foam	+
6.	**Terpenoids** *-Salkowski test*	Dark reddish brown color in interface	+
7.	**Polyphenols** -Ferrozine test	Raddish blue	+
8.	**Anthocyanin test Ammonia**	Ammonia layer yellow in color	-

+ Indicate positive result; -- Indicate negative result

Table No 2: Quantitative phytochemical analysis of the sample *PC:*

Sample	Yield of extract (g/100 g of defatted Content)	Total phenolic content (µg catechin equivalents per gram Aqueous sprouts extract)	Total flavonoid content (µg catechin equivalents per gram Aqueous sprouts extract)
Fresh sprouts aqueous extract of *Gosssypium herbaceum*	85.49 ±1.49 [a]	61.54±1.49[b]	54.23±1.69

[a]Data are expressed as mean ± standard deviation (n = 3) on a fresh weight basis.
[b]Means in each column sharing the same letter are not significantly (P = 0.05) different from other.

3.2.Anti-Oxidant Activity:

Result Analysis of Percentage reduction activity of *PC:*

The reducing power of different concentration of aqueous extract of *Paruthi Chooranam* aqueous decoction were performed. Fresh aqueous decoction of *PC* exhibits good reducing power activity than positive control. The reducing ability of aqueous decoction *of PC* generally depends on the presence of reductants which have been exhibiting anti-oxidative potential by breaking the free radical chain and donating a hydrogen atom. The respective absorbance of different concentration of *PC* was illustrated in figure 1.

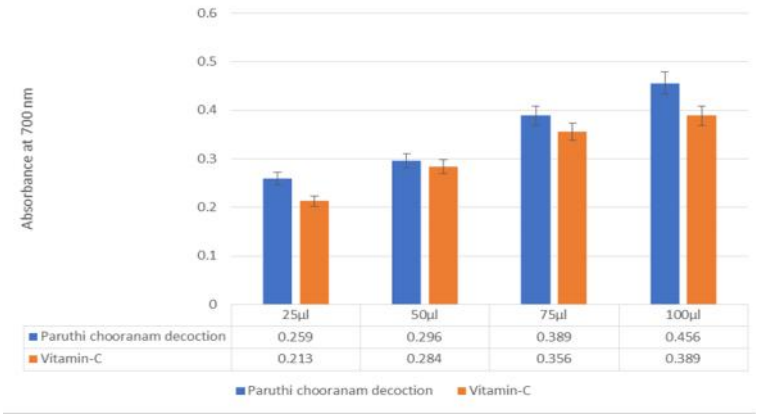

Figure 1: Percentage reducing property of *PC* in potassium ferricyanide assay

3.3. Result Analysis of ABTS radical scavenging activity of *PC:*

The results of ABTS radical scavenging activity of the sample *PC* shown that the test drug has significant ABTS radical scavenging activity dose dependently. Sample *PC* has exerted potential ABTS scavenging property whose values radical ranges from 32.16 to 81.23 % when compared with standard ascorbic acid with percentage inhibition ranges from 27.46 to 76.13 %. The values of ABTS free radical scavenging activity of the PC was illustrated in figure 2.

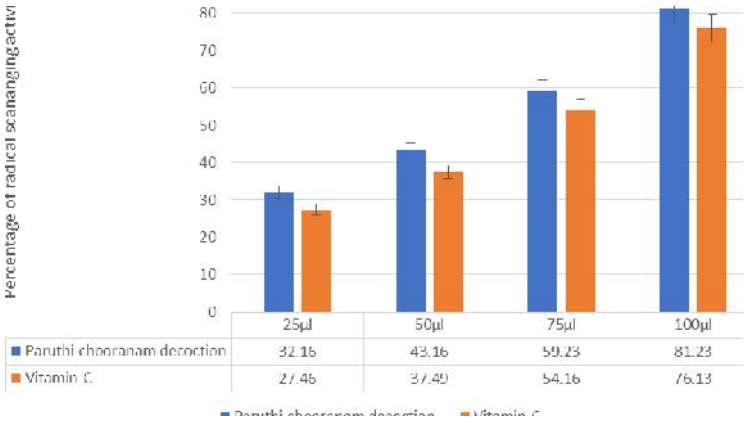

Figure 2: ABTS radical Inhibition potential of *PC* and Ascorbic acid

3.4. Result Analysis of Percentage reduction activity of *PC:*

Sample *PC* exhibits excellent metal chelating activity in the tested medium with the percentage inhibition of 74.23 % when compare to that of the standard ascorbic acid with percentage inhibition of 71.46 %.The respective percentage inhibition of different concentration of *PC* and ascorbic acid was illustrated in figure 3.

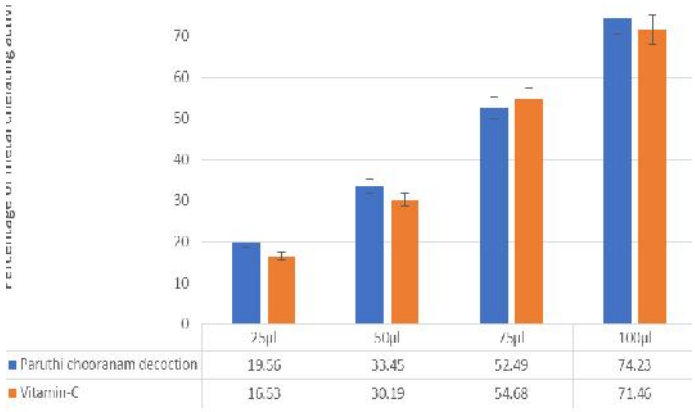

Figure 3: Metal chelating potential of *PC* and Ascorbic acid

3.5. Result Analysis of Lipid peroxidation activity of *PC :*

The results of LPO radical inhibition activity of the sample *PC* shown significant radical inhibition activity dose dependently. Sample *PC* exerted percentage inhibition of LPO ranges from 23.46 to 77.48 % when compared with standard ascorbic acid with percentage inhibition ranges from 19.26 to 71.49%. The values of LPO inhibition activity of the *PC* was illustrated in figure 4.

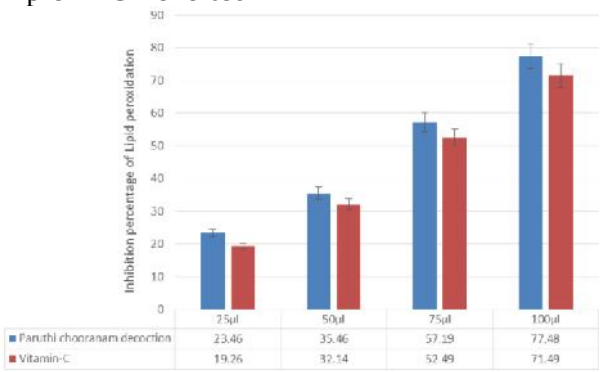

Figure 4: Lipid peroxidation Inhibition potential of *PC* and Ascorbic acid

3.6. Result Analysis of Superoxide radical scavenging activity of *PC:*

The results of SO radical scavenging activity of the sample *PC* shown that the test drug has significant SO radical scavenging activity dose dependently. Sample *PC* has exerted potential SO radical scavenging property whose values ranges from 22.6 to 73.49 % when compared with standard ascorbic acid with percentage inhibition ranges from 18.98 to 68.49%. The values of SO free radicals cavenging activity of the *PC* was illustrated in figure 5.

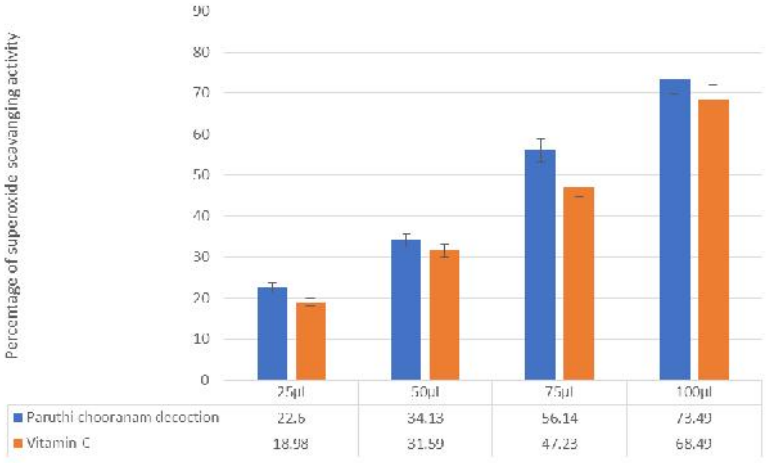

Figure 5: SO radical Inhibition potential of *PC* and Ascorbic acid

3.7. Result Analysis of Nitric oxide radical scavenging activity of *PC:*

The results of NO radical scavenging activity of the sample PC reveals significant NO radical scavenging activity dose dependently. Sample *PC* has exerted potential NO radical scavenging property whose values ranges from 22.15 to 79.23 % when compared with standard ascorbic acid with percentage inhibition ranges from 19.68 to 76.21 %. The values of NO free radical scavenging activity of the *PC* was illustrated in figure 6.

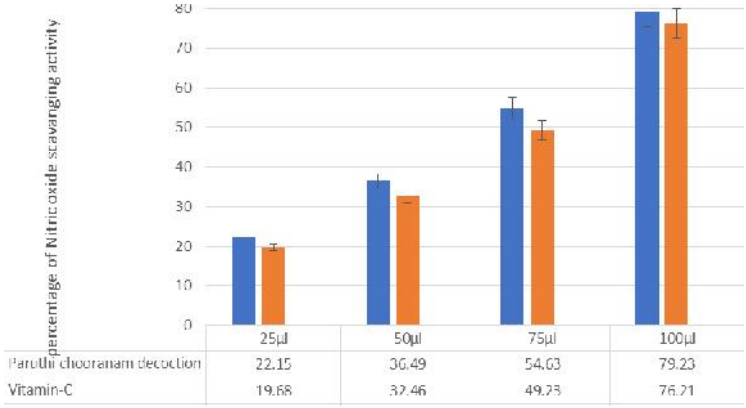

Figure 6: NO radical Inhibition potential of *PC* and Ascorbic acid

4. Discussion

Due to the high level of complexity in the nature of phytochemicals, a single method to evaluate the antioxidants activity cannot be screened. In this context, different standard methods were used to validate nature of plant extract in terms of antioxidants. The phytochemical analysis carried out in *PC* extract revealed the presence of alkaloid, flavonoid, saponin, triterpenoids and polyphenols. The results indicate that *PC* extract contains significant amounts of flavonoids and phenolic compounds. These identified bioactive compounds have good antioxidant potential and their effects on human nutrition and health are considerable. The mechanism of action of flavonoids is through scavenging or chelation [29]. Flavonoids due to actions by its anion radicals serve as health promoting compound [30]. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability.

The scavenging activity of $ABTS^{+}$ radical of the *PC* extract was found to be substantially reported. Sample *PC* has exerted potential ABTS scavenging property whose values radical ranges from 32.16 to 81.23 % when compared with standard ascorbic acid with percentage inhibition ranges from 27.46 to 76.13 %.

Reducing power of the *PC* extract was compared with standard ascorbic acid and found to be superior indicating its potential antioxidant behavior. Test drug *PC* exhibits excellent metal chelating activity in the tested medium with the percentage inhibition of 74.23 % when compare to that of the standard ascorbic acid with percentage inhibition of 71.46 %.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals [32] and also possess significant degeneration to the cell molecular components [33]. Sample *PC* has exerted potential SO radical scavenging property whose values ranges from 22.6 to 73.49 % when compared with standard ascorbic acid with percentage inhibition ranges from 18.98 to 68.49%.

Lipid peroxidation inhibition assay of *PC* extract inhibits the $FeSO_4$ induced lipid peroxidation in egg yolk, which is the net result of iron-mediated hydroxyl radicals. Sample *PC* exerted percentage inhibition of LPO ranges from 23.46 to 77.48 % when compared with standard ascorbic acid with percentage inhibition ranges from 19.26 to 71.49%.

Nitric oxide (NO) is a reactive free radical produced by phagocytes and endothelial cells and plays an important role in inflammatory process. Sample *PC* has exerted potential NO radical scavenging property whose values ranges from 22.15 to 79.23 % when compared with standard ascorbic acid with percentage inhibition ranges from 19.68 to 76.21 %.

5. Conclusion

Generation of free radical is a continuous and spontaneous process majorly because of the metabolism and change in the pathology is concern. Whereas failure in mechanism which counteracts this generated radicals surely causes degeneration and abnormalities in health organs and damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases and extensive lysis. From the data's obtained from the present study it was evidently proven that the test drug *Paruthi Chooranam* possess significant anti-oxidant property in all the tested module and Furthermore, the *in vivo* antioxidant activity of *PC* extract needs to be assessed prior to clinical use.

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