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ANTIOXIDANT ACTIVITY OF ADATHODAI CHOORANAM BY IN-VITRO APPROACH

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ABSTRACT The Siddha poly herbal drug Adathodai Chooranam is indicated for respiratory disorders like Bronchial Asthma, bronchitis, COPD etc. Bronchial asthma is a clinical syndrome with possible correlation to oxidative stress; therefore the effectiveness of some antioxidant drugs has been studied in management of chronic bronchial asthma. Antioxidants can compensate the oxidative stress that correlates with asthma, can reduce the symptoms of asthma, and improve pulmonary functions.[1] This study was also intended to explore the anti-oxidant activity of the Adathodai Choorananam by in-vitro methods. The antioxidant capacity of the drug was evaluated with the estimation of total Flavonoids, estimation of total phenols, estimation of Vitamin C, DPPH spectrometric assay, Metal chelating assay, Assay of catalase, Lipid peroxidation assay, and assay of glutathione peroxidase. In DPPH method, 100 µg/ml of the drug and ascorbic acid exhibit 98.9% and77.7% inhibition respectively and the IC 50 value of the drug is

KEYWORDS : Siddha poly herbal drug, Adathodai Chooranam, anti-oxidant activity, Bronchial asthma, in-vitro.

Introduction

Oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others[2]. Antioxidants are substances that may protect our body cells against the effects of free radicals. Free radicals are molecules produced when our body breaks down food. They can also be produced by environmental exposures like tobacco smoke and radiation. Free radicals can damage cells, and may play a role in heart disease, cancer and other diseases. Antioxidants cause protective effect by neutralizing free radicals which are toxic byproducts of natural cell metabolism. Antioxidants improve the quality of life by preventing or postponing the onset of degenerative diseases.[3] This study proved the anti-oxidant activity of the Adathodai Choorananam by in-vitro methods.

Materials and methods

Estimation of total flavonoids

The amount of total Flavonoids in the Adathodai chooranam was estimated by the method Proposed by Cameron et al. (1943).

Principle:

The method is based on the formation of the flavonoids - aluminium complex which has an absorptivity maximum at 415nm.

Reagents

Ethanol

Standard (Quercetin)	: 1mg/ml Ethanol (Stock)
Working Concentration	: 50µg/ml
Aluminium Chloride	: 10% in 10% Methanol
Potassium Acetate	: 0.282mg in 10ml of distilled Water

Estimation of total phenols

The amount of total phenols in the Adhatodai chooranam was estimated by the method Proposed by Mallick and Singh (1980).

Principle

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent to produce a blue-coloured complex in alkaline medium, which can be estimated spectro photometrically at 725nm.

Reagents

Folin-Ciocalteau reagent
Saturated Sodium carbonate
Gallic acid (standard) - 1mg/mL
Working Concentration: 0.1mg/mL

Procedure

Test tubes were labeled as S1-S5 and different concentration of working standard solution were added as 20, 40, 60, 80,100µL. The drug extract was added as the test solution in tube T1 as 20µL. Folin's reagent 0.5mL was added to each tubes and the test tubes were incubated at room temperature for 10 minutes. Then 2.5mL of saturated sodium carbonate was added to all the tubes and it was incubated at dark for 40 minutes. Blue color was developed which can be estimated spectrophotometrically at 725nm.

ESTIMATION OF VITAMIN C

The amount of vitamin C in the Aadhatodai chooranam was estimated by the method Proposed by Roe and keuther (1943).

PRINCIPLE

Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2, 4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 530nm.

REAGENTS

Ascorbic acid (standard)	:1mg/mL
Working standard	: 0.2mg/mL
Thiourea	: 4%
Sulphuric acid	: 85%
DNPH	: 10mM
TCA	: 5%

PROCEDURE

Test tubes were labeled as S1-S5 and different concentration of working standard solution were added as 20, 40, 60, 80,100µL. The drug extract was added as the test solution in tube T1 as 20µL. 150µL of 5% TCA was added to all the tubes and centrifuged for 5 minutes at 3500g.Then 0.5mL of 10mM DNPH and 0.5mL of 4% thiourea was

added to all the tubes and the test tubes were incubated at room temperature for 3 hours. Then post incubation by adding 2.5mL of 85% sulphuric acid was added to all the tubes then which can be estimated spectrophotometrically at 530nm.

DPPH SPECTROPHOTOMETRIC ASSAY

The scavenging ability of the natural antioxidants of the drug extract adhatodai chooranam towards the stable free radical DPPH was measured by the method of Mensor et al. (2001).

PRINCIPLE

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenylpicryl hydrazine. This can be identified by the conversion of purple to light yellow color.

REAGENTS

Standard (Ascorbic Acid)	: 1mg/ml in Dis. Water
DPPH	: 0.006% in 10% Methanol(2, 2-diphenyl-1-picrylhydrazine) 95% Methanol

PROCEDURE

Test tubes were labeled as S1-S5 and different concentration of standard solution were added as 20, 40, 60, 80,100µL. The drug extract was added as the test solution in tubes T1-T5 as 20, 40, 60, 80,100µL. 1.0mL of DPPH was added to all the tubes. All the tubes were made up to 3.0mL of 95% methanol and it was incubated at dark for 30 minutes. Pale yellow color was developed which can be estimated spectrophotometrically at 515nm. The radical scavenging activity was calculated as follows:

% of DPPH radical scavenging activity:

$$\frac{\text{Absorbance of control - Sample}}{\text{Absorbance of Control}} \times 100$$

METAL CHELATING ASSAY

The method proposed by Stookey (Stookey, 1970) was used to estimate the metal chelating activity in the drug extract Adhatodai chooranam.

PRINCIPLE

The ferrozine is used for the measurement of iron- chelating capacity, because it can specifically react with ferrous ions to form a stable, magenta colored solution. The complex (Ferrozine-Ferrous ion chromophore) has an absorptive peak at 562nm so that it can measured spectrophotometrically.

REAGENTS

Standard (EDTA)	: 1mg/ml in Dis.Water (Stock)
Working Concentration	: 0.1mg/ml
Ferrozine	: 5mM
Ferric Chloride	: 2mM

PROCEDURE

Test tubes were labeled as S1-S5 and different concentration of working standard solution were added as 100,200,300,400,500µL.

The drug extract was added as the test solution in tubes T1-T5 as 100,200,300,400,500µL. 100µL of Ferric chloride was added and then 200µL of Ferrozine was added to all the tubes. All the tubes were made up to 2.0mL of distilled water and it was incubated at dark for 10 minutes. Magenta colour was developed which can be estimated spectrophotometrically at 515nm.

ASSAY OF CATALASE

Catalase activity was assayed by the method of Sinha (1972).This method is based on the fact dichromate in acetic acid is reduced to chromic acetate when treated in the presence of H_2O_2 with the formation of perchloric acid as an unstable intermediate.

REAGENTS

Phosphate buffer (0.01M, pH 7.0) 0.2M Hydrogen peroxide

PROCEDURE

To 0.1ml of cell supernatant, 1.0ml of buffer and 0.5ml of H_2O_2 were added and the time was noted. The tubes heated in a boiling water bath for 10 minute. The green colour developed was read at 610nm.

Catalase activity is expresses as Nano moles of H_2O_2 consumed/min/mg protein.

LIPID PEROXIDATION ASSAY

PRINCIPLE

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for the determination of the extent of peroxidation reaction. MDA, a product of lipid peroxidation reacts with TBA (thiobarbituric acid) to give a pink coloured product having absorption maxima at 535nm.

REAGENTS

TBA (0.375%)
TCA (15%)
Hydrochloric acid (0.25N)
Malondialdehyde (working standard 2µg/ml)

PROCEDURE

Test tubes were labeled as S1-S5 and different concentration of working standard solution were added as 0.1µg,0.3 µg,0.6 µg,0.8 µg,1,2µg/mL. The HepG2 cell supernatant was added as the test solution in tube T1 as 1Ml.Distilled water 1mL was added to each tubes. Then 1mL of TBA-TCA-HCL reagent was added into all the tubes. The contents were incubated in a boiling water bath for 15 minutes and the pink colour developed.

ASSAY OF GLUTATHIONE PEROXIDASE

Reduced glutathione was determined by the method of Moron et al. (1979).

PRINCIPLE

Reduced glutathione on reaction with DTNB (5, 5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm.

REAGENTS

TCA (10%)
Tris-HCL (400mM)
DTNB (2.5mM)

PROCEDURE

The supernatant (0.1mL) was made upto 1mL with 10% TCA. The sample was centrifuged at 4000rpm for 10 minutes at 10°C.Pellet was discarded and supernatant was used for assay. The tubes were cooled and centrifuged. Absorbance was measured at 535nm by using spectrophotometer

% Enzyme activity:

$$\frac{\text{Absorbance of sample X total vol. mixture X Dilution factor}}{\text{Ext. co. eff. X Vol. of sample tested}}$$

Add 700µL of 400mM Tris-HCL (pH 8.9)
Incubation for 10 minutes at room temperature
Then add 100µL of 2.5mM DTNB dissolved in Tris-HCL buffer (pH 8.9)
Incubation for 10 minutes at room temperature
The absorbance was read at 412 nm

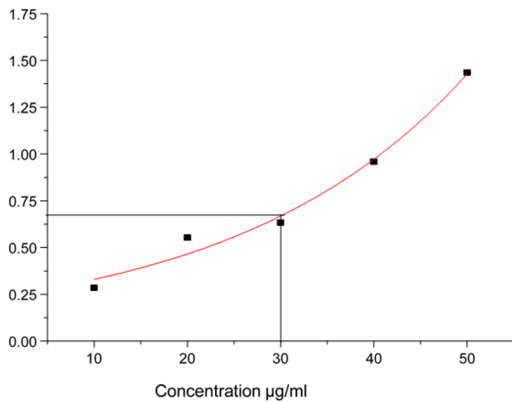
Results:

The results were shown in the Fig (1 – 8) and the table (1- 5). The present study supports the antioxidant activity of Adhatodai chooranam. The assays Flavanoid estimation, phenols estimation, Glutathions peroxidase, lipid peroxidase, Vitamin C, Metal chelating activity and catalase activity, DPPH scavenging activity evidenced the antioxidant activity of the Adathodai chooranam.

Conclusion:

Adhathodai chooranama is a potential antioxidant and it acts as an antiasthmatic agent.

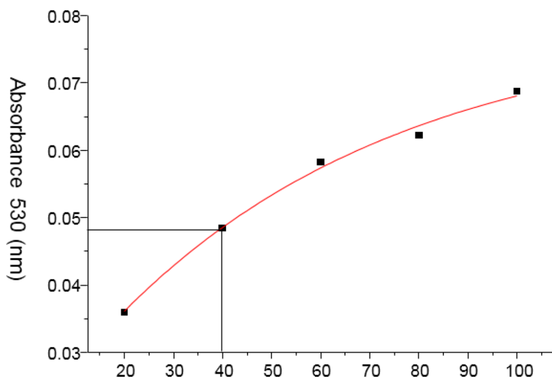
Total flavonoid estimation from Adathodai chooranam



Standard – Quercetin 50µg/ml
50µl sample contains 30µg/ml of flavonoids.

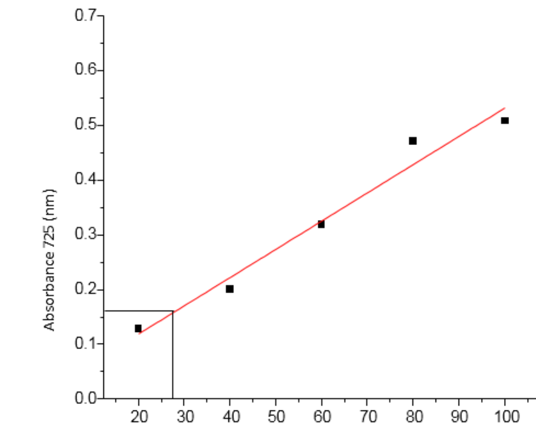
Fig.1. Estimation of Total Flavanoids

Estimation of Vitamin C



Standard – Ascorbic acid 0.2mg/ml
25µl of sample contains 40µg of Vitamin c

Fig.2. Estimation of Vitamin C
Estimation of total phenol

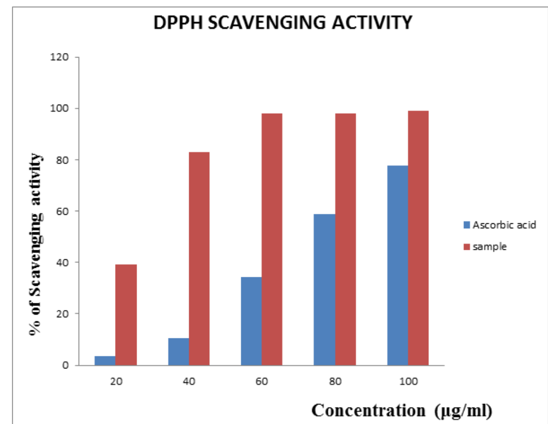


Standard –Gallic Acid 0.1mg/ml
25µl of sample contains 28µg of phenol

Fig.3. Estimation of total phenol

Table. 1. DPPH SPECTROPHOTOMETRIC ASSAY

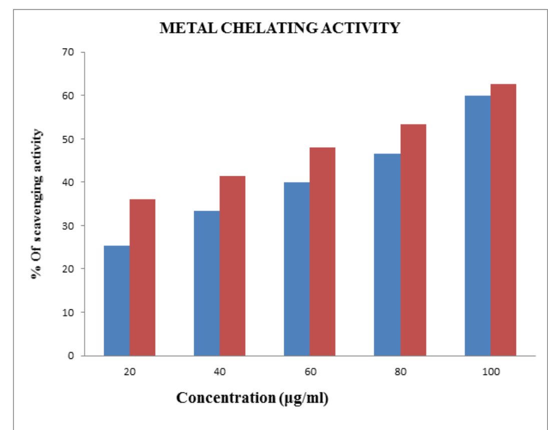
Concentration (µg/ml)	% of scavenging activity	
	Ascorbic acid	sample
Control	0.00	0.00
20	3.47	39.27
40	10.62	82.94
60	34.16	98.01
80	58.82	98.1
100	77.7	98.9



Standard – Ascorbic acid 0.1mg/ml

Table.2. METAL CHELATING ASSAY

Concentration (µg/ml)	% of scavenging activity	
	EDTA	sample
Control	0.00	0.00
10	25.3	36
20	33.3	41.3
30	40	48
40	46.6	53.3
50	60	62

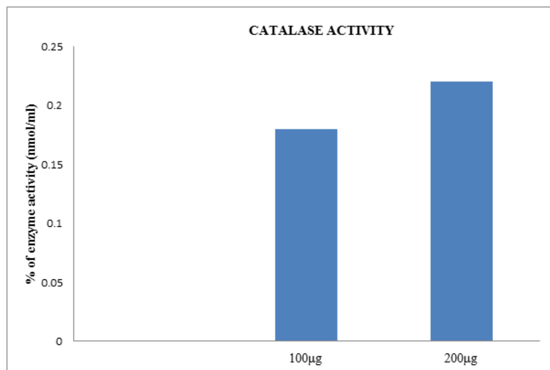


Standard –EDTA 0.1mg/ml

Table.3. Catalase activity of Adathodai chooranam

Content	% of Enzyme activity (nanomoles/mL)
HepG2 cell	0.16
100µg AC treated HepG2 cell	0.18
200µg AC treated HepG2 cell	0.22

Extinction coefficient of enzyme 39.4mM



Concentration of Adatodai chooranam

Table .4. LIPID PEROXIDATION ASSAY

Content	Concentration (µg/mL)
HepG2 cell	0.6
100µg AC treated HepG2 cell	0.26
200µg AC treated HepG2 cell	0.23

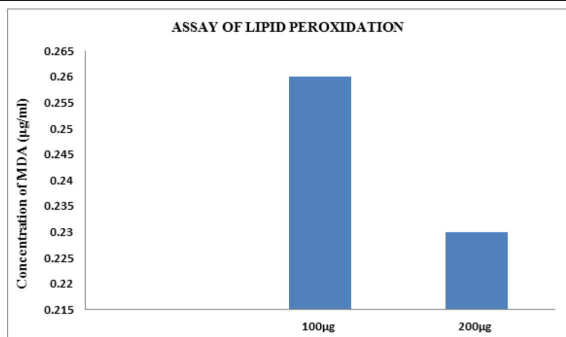


Table. 5. Glutathione peroxidise activity of Adathodai chooranam

Content	% of Enzyme activity (Unit/mL)
HepG2 cell	0.1
100µg AC treated HepG2 cell	0.11
200µg AC treated HepG2 cell	0.13

Extinction Coefficient = 6.22

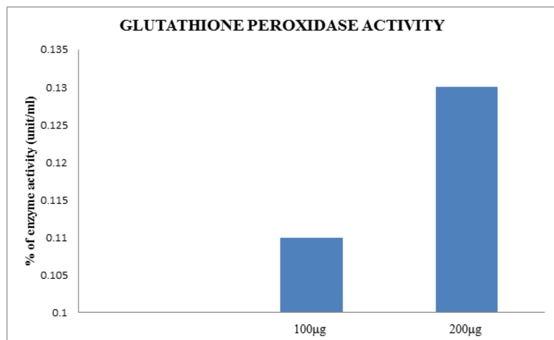


Fig.8. Glutathione Peroxidase activity of Adathodai chooranam

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