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Preliminary Phytochemical Analysis, Antioxidant Activity, Phenolic and Flavonoid Contents of *Annona reticulata* Leaf Extract

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Abstract : *Annona reticulata* (custard apple) has been utilized widely in India as traditional medicine for cure of many ailments. Plants of the *reticulata* species are an important resource of many curative agents like antioxidant, anti bacterial and antimicrobial. Present study reports the wide variety of phytochemicals present in the leaf extract viz. Alkaloids, Terpenoids, Flavonoids, Carbohydrates, Glycosides, Tannins and Phenolic compounds, Amino acids and Proteins. The study also evaluated total phenolic and flavonoid contents for their in vitro antioxidant potential. The DPPH free radical scavenging and hydrogen peroxide assays were employed for assessment of antioxidant property of the leaf extract. The total phenol content of ethanol extract of *A. reticulata* measured by Folin-Ciocalteau reagent in terms of gallic acid equivalent achieved 11.57±2.057 µg/100 µg Gallic acid. However, the flavonoid content of the plant extract as rutin equivalent achieved 23±3 µg/100 µg. The *in vitro* antioxidant effect of *A. reticulata* was estimated by DPPH assay with IC50 value of 0.67 mg/ml.

Key Words: *Annona reticulata*, custard apple, phytochemicals, antioxidant, total phenol, flavonoid.

Introduction

The plants and plant based chemicals have been the most important sources of medicines since ages. Phytochemicals and different yields obtained from plant are used as medication, pharmaceuticals, cosmetics and food supplements. Plants are gifted with a range of phytochemical bioactive molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant activity (Pitchersky and Gang, 2000; Sheikh and Tembhre, 2016). Studies have shown that the consumption of natural antioxidants has been associated with decreasing ageing, risks of cancer, cardiovascular and renal disease and diabetes hence people have attracted worldwide to explore the natural phytochemicals (Raskin *et al.,* 2002; Huang *et al.,* 2009; Akram and Tembhre, 2016).

Reactive oxygen species (ROS) are oxygen free radicals generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion. Higher concentrations of free radicals induce oxidative stress in the cell, which causes disruption in the cell function. Overproduction of ROS or oxidants induces oxidative stress in the cell due to imbalance owing to excess ROS over the ability of the cell to mount an effective antioxidant response. Multiple recent studies have shown that deleterious free radicals cause various degenerative diseases, such as diabetes, cancer, and neurodegeneration, which can be ameliorated with the use of natural antioxidants (Ola *et al.,* 2020). Antioxidants are substances, present at low concentration relative to the oxidizable substrate, which significantly delay or prevent oxidation of substrate (Islam *et al.,* 2012). Evidence suggests that the human body does not synthesize sufficient quantity of antioxidants to balance with the damaging effects of ROS. It has been reported that there are over 400,000 species of plants on earth which have a huge reservoir of bioactive compounds including antioxidants, but only a small percentage of these have been examined in the research studies (Shoemaker *et al.,* 2005; Sheikh and Tembhre, 2016). Therefore, the attention of scientists has been redirected towards ethno-medicines due to the reinforcement of knowledge in regular health practices all over the world. In recent years the demand for herbal medicines and several natural products from a variety of plant species is constantly rising. Hence a need of new potential natural sources of antioxidants is of great significance.

Plant *Annona reticulata* of genus *Annona* and family Annoanaceae (Pinto *et al.*, 2005) also known as custard apple or bullock's heart (Nirmal *et al.*, 2010) grows in a tropical climate. It is a versatile tree with a spreading crown and thick trunk (Pinto *et al.*, 2005). Several folkloric medicinal uses of this plant have been reported. Parts of *A. reticulata* are used as source of medicine and industrial products as well. It's leaves, bark, seed and root are reported to have many therapeutic activities as anticancer, CNS depressant, analgesic, anti hyperglycemic, anti-inflammatory, antiproliferative, wound healing, antiulcer activity

and cytotoxic effects (rahman *et al.*, 2011) Jamkhande and Wattamwar, 2015). The paste of leaves of the plant is used as a poultice on boils, abscesses and ulcers is commonly known (Jansen *et al.*, 1991). Tender shoots and the leaves are used in tanning and dyeing black leather and cloth (Hanelt *et al.*, 2001). Recently a phytochemical study on custard apple has revealed that it contains numerous phenol-based compounds, e.g., proanthocyanidins, with 18 different phenolic compounds, mainly alkaloids or flavonoids (Mannino *et al.,* 2020).

The present study was carried out to screen preliminary phytochemical compounds and the antioxidant potential of ethanol extract obtained from extraction of *Annona reticulata* leaves *in-vitro*. Attempt also has been made to estimate the flavonoid and phenolic content of the extract.

Materials and Methods

The leaves of *Annona reticulata* were collected from local area of Bhopal, India, and were thankfully identified and authenticated by Dr. Zia Ul Hassan (H.O.D. Botany, Safia Science College, Bhopal, Madhya Pradesh, India). The voucher specimens bearing numbers 508/Bot/Safia/14 were submitted in the Botany department for future reference.

Preparation of plant extract

About 300 gms of the powder of shade dried leaves of *Annona reticulata* was extracted with 1.2 L of 90% ethanol using Soxhlet apparatus for 72 hrs at 40-50°C.

Phytochemical evaluation of leaf extract of *Annona reticulata*

The biological potential of major phytoconstituents of ethanolic extracts of *Annona reticulata* was screened by using the standard procedures (Kokate *et al.,* 2006).

***In vitro* antioxidant assay**

The antioxidant potential of leaf extract of *Annona reticulata* was determined by estimating DPPH radical scavenging activity **(**Gulcin *et al*., 2006; Jain and Jain, 2011**)** and reducing power assay (Jain and Jain, 2011).

DPPH radical scavenging activity

Different concentrations of test sample were prepared using 0.1 mM DPPH in methanol. The DPPH solution was added to test samples followed by 10 minutes incubation at room temperature. The absorbance was taken at 515nm against blank (methanol). Percentage Inhibition was calculated by using following formula:-

% inhibition = Absorbance of control - Absorbance of sample × 100/ Absorbance of control

A curve for % inhibition and concentration was plotted and IC50 was estimated by using line of regression.

Reducing power assay

For assessment of reducing power, phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (0.5 ml, 1%W/V) were added to different concentrations of the test sample. The reaction mixture was incubated at 50°C for 20 min. After cooling, trichloroacetic acid solution (10% W/V) was added to terminate the reaction followed by addition of ferric chloride (0.1% W/V) and then absorbance was measured at 700 nm. The curve between absorbance and concentrations was plotted. Increased absorbance of the reaction mixture indicated increase in reducing power.

Estimation of total phenolic content

The total phenolic content of the ethanolic extract of*, A. reticulata was* determined by using the Folin-Ciocalteu reagent (Ainsworth and Gillespie, 2007). Varying concentrations of Gallic acid (10 to 100 µg/ml) and the test sample were prepared in methanol (100 µg/ml). To 0.5 ml of each concentration of gallic acid/ test sample was added with 2 ml Folin-Ciocalteu reagent (1:10 in de-ionized water), then 4 ml of sodium carbonate solution was added. The testing mixture was incubated at room temperature for 30 minutes with intermittent shaking. The absorbance of reaction mixture was spectrophotometrically measured at 765 nm (due to blue colour formation) using methanol as blank. A standard curve of different concentrations of gallic acid was prepared and line of regression was found. The absorbance of test sample was placed in line of regression of standard curve of gallic acid. Total phenolic content was calculated and expressed as µg/mg galic acid equivalent.

Estimation of Total Flavonoid Content

The total flavonoid content (TFC) of *A. reticulata* extracts was determined according to Alhakmani *et al*. (2013). Different concentrations of rutin (10 to 100 µg/ml) and the test sample were prepared in methanol (100 µg/ml). 0.5 ml aliquots were mixed with distilled water (2 ml), followed by addition of 5% $NaNO_2$ solution (0.15 ml). After 6 minutes, 0.15 ml of a 10% $AlCl_3$ solution was added to the mixture, which was allowed to incubate for 6 minutes. Then 4% NaOH solutions (2 ml) was added and after that distill water was added to bring the final volume to 5 ml. After 15 minute incubation, the absorbance was determined at 510 nm using water as the blank. The standard curve for different concentration of rutin was prepared and line of regression was drawn. Total flavonoid content was calculated and expressed µg/mg rutin equivalent.

Statistical Analysis: The data were subjected to statistical analysis. All the values are expresses as mean ± SD.

Results

Phytochemical Analysis of the *A. reticulata* Leaves Extract

The experiments conducted in ethanolic extract of leaves of *Annona reticulata* for analysis of phytochemical constituents. The different qualitative tests were performed for its chemical composition. The ethanol extract of *Annona reticulata* leaves was analyzed for the presence of various phytoconstituents by following standard phytochemical tests (Table-1) showed presence of fair amount of preliminary phytochemical active constituents such as alkaloids, flavonoids, carbohydrate, glycosides, tennin, phenol, amino acids and protein while saponin was not present.

Table-1. Phytochemiclas present in ethanolic extract of *A. reticulata* Leaves

Phytochemical	Tests	*Positive/Negative A. reticulata*
Alkaloids	Mayer's Test	+
	Wagner's Test	-
	Hager's Test	+
	Dragendroff's Test	-
Terpenoids	Salkowski Test	+
	Libermann Burchards Test	-
Flavonoids	Lead Acetate Test	++
	Alkaline Reagent Test	++
	Shinoda Test	-
Carbohydrates	Molish's Test	+
	Fehling's Test:	+
	Benedict's Test:	-
	Barfoed's Test	+
Glycosides	Killer Killians Test	+
	Borntrager's Test	-
	Legal's Test	+
Tannins and Phenolic compounds	$FeCl_3$ Test	-
	Dilute Iodine Solution Test	+++
	Lead Acetate Test	-
	Gelatin Test	++
Saponins	Froth Test	-
Amino acids Proteins	Biuret's Test	+
	Millon's Test	++
	Ninhydrin Test	-

(+) Low levels; (++) Moderate levels; (+++) High levels.

Analysis of *in-vitro* antioxidant activity of *Annona reticulata* leaf extract

DPPH Assay:

The *in vitro* antioxidant effect of *Annona reticulate* ethanolic leaf extracts was evaluated by using DPPH assay. The DPPH radicals react with suitable reducing agents losing colour stoichometrically with the number of electrons consumed, which is measured spectrophotometrically at 517 nm. The scavenging activity of the extracts was observed to be 0.67 mg/ml IC (Table-2). The scavenging effect was compared to that of the standard ascorbic acid (Fig.1 and 2). The results, thus obtained suggest that ethanolic extracts of leaf the selected medicinal plant have the proton donating ability and can serve as free radical inhibitors or scavenger or exhibit significant DPPH radical inhibition.

Table: 2 - Showing the % Inhibition of DPPH by ethanolic extract of *Annona reticulata.*

S. No.	Conc. (mg/ml) of extract	% Inhibition	IC_{50}
1	0.4	31.09	**0.67 mg/ml**
2	0.6	36.05	
3	0.8	43.94	
4	1	56.93	
5	1.2	65.69	
6	1.4	74.89	
7	1.6	79.12	

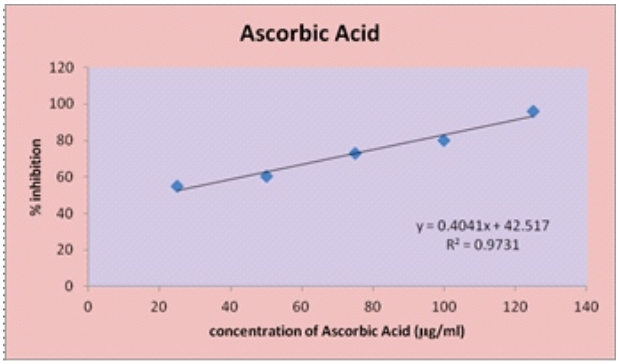

Fig. 1 - Represents the regression curve of ascorbic acid by DPPH assay method

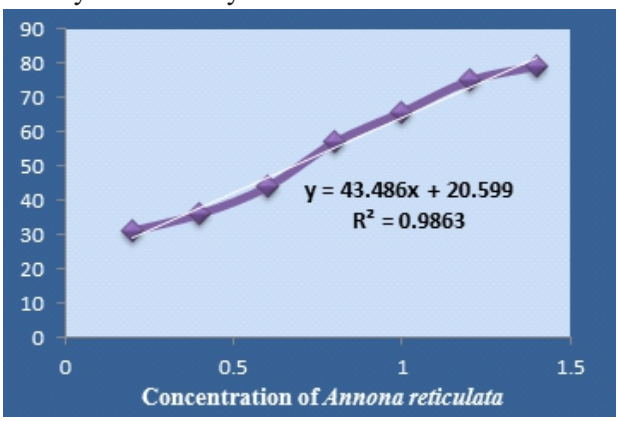

Fig. 2 - Represents percentage inhibition of DPPH by ethanolic leaf extract of *Annona reticulata.*

Reducing Power Assay:

Another method used for determining the antioxidant activity of *Annona reticulata* was reducing power assay. In this assay the higher the absorbance of the reaction mixture, the higher would be the reducing power. Table-3 clearly shows an increase in absorbance of leaf extract of *Annona reticulata* with its increasing concentrations.

Table: 3- Showing the reducing power of ethanolic extract of *Annona reticulata.*

S. No.	Concentration µg/ml	Absorbance
1	100	0.366
2	200	0.455
3	300	0.523
4	400	0.685
5	500	0.801

Total Phenolic Assay (TPC):

The total phenolic content was calculated and it was found that the ethanolic extract of leaves of *Annona reticulata* had the TPC of 11.57±2.057 µg/100 µg gallic acid equivalent (Fig. 3 & Table-4).

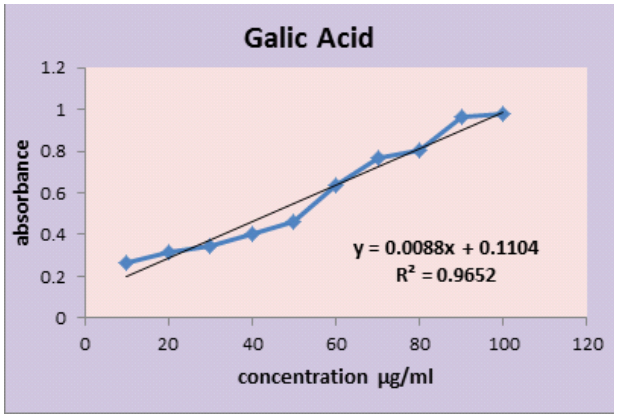

Fig.3 - Represents the standard curve of Gallic Acid.

Table: 4 - Showing total phenolic content in ethanolic extract of *Annona reticulata* leaves.

S. No.	Concentration	Absorbance	Total Phenolic content in µg/100 µg Gallic acid equivalent
1	100 µg/ml	0.187	9.62
2	100 µg/ml	0.201	11.37
3	100 µg/ml	0.227	13.72
Mean	-	0.205	**11.57**
S. D.	-	0.020298	**2.057**

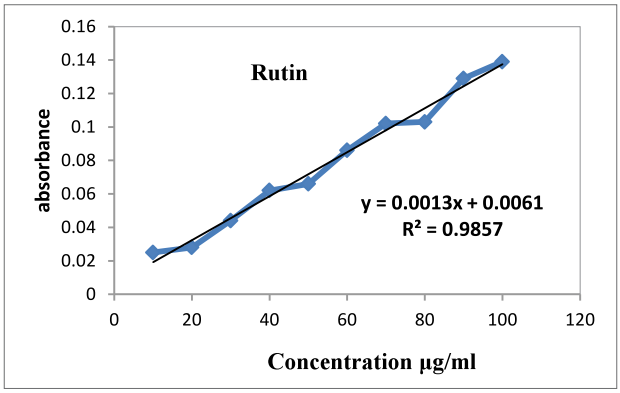

Fig. 4 - Represents the Standard curve of Rutin.

Table:5 -Showing total flavonoid content in ethanolic extract of *Annona reticulata* leaves.

S. No.	Concentration	Absorbance	Total Flavonoid content in µg/100 µg rutin equivalent
1	100 µg/ml	0.029	23
2	100 µg/ml	0.032	26
3	100 µg/ml	0.026	20
MEAN±SD			**23 ± 3**

Total Flavonoid Assay:

The total flavonoid content was also estimated in the selected extracts and the extract of *Annona reticulata* contained 23±3 µg/100 µg rutin equivalent, (Fig. 4 & Table- 5).

Discussion

Annona reticulata is one of the traditionally important plants used for the treatment of various ailments. The present study on preliminary phytochemical active constituent in ethanolic leaf extract of *Annona reticulata* clearly shows the presence of alkaloids, flavonoids, carbohydrate, glycosides, tennin, phenol, amino acids and protein while, saponin was not present. Earlier, ethanolic *Annona reticulata* leaf extract was found to be more prominent than the aqueous extract (Gowdhami *et al.,* 2014). The screening of the plant extract also showed the presence of alkaloids, carbohydrates, coumarins, flavonoid, glycosides, phenolic compounds, phytosterols, proteins, quinones, saponins, steroid and terpenoids (Florence *et al.,* 2013). Phytochemical screening of the *Annona squamosa* leaf extract showed the presence of flavonoids, terpenoids, saponins, tannins and reducing sugars (Suresh *et al.,* 2006).

The results of the DPPH assay have been presented in many ways by the investigators. The majority of studies express the results as the IC_{50} value, defined as the amount of antioxidants necessary to decrease the

initial DPPH concentration by 50%. This value is calculated by plotting inhibition percentage against extract concentration (Sokmen *et al.,* 2004; Tepe *et al.,* 2005; Ani *et al.,* 2006; Sheikh *et al.,* 2015). In present study IC_{50} of the *Annona reticulata* leaves extract was found 0.67 mg/ml showing antioxidant potential with the selected concentration range for antioxidant activity in present study was 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mg/ml extract. Similarly, Subba and Aryal (2016) showed the Antioxidant activity of the leaf extract of *Annona reticulata* using scavenging activity of DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) radical method and found the IC50 value of 41 µg/ml. The results of this study are in line with the published result of other group reporting the IC50 value of methanolic extract of *A reticulate* leaf was 52.08µg/ml (Jamkhande *et al.,* 2016).

Further, the *in vitro* antioxidant potential of the extracts was authenticated by using reducing power assay (Oktay *et al.,* 2003; Akram *et al.,* 2016; Ahirwar *et al.,* 2016). Compounds with reducing power indicate that they are electron donors and associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay *et al*., 2003). The higher the absorbance of the reaction mixture, the higher would be the reducing power. The ethanolic extract of *Annona reticulata* leaves showed higher absorbance and hence shows reducing power.

In the present study the total phenolic content of *Annona reticulata* leaf extract was also assessed and it was 11.57±2.057 µg/100 µg Gallic acid equivalent. Measurement of total phenolic compounds by Folin-Ciocalteu's phenol reagent indicated that 1mg of the methanol extract of the leaves *of Annona reticulata* contained 146.20µg146.20µg equivalent of pyrocatechol (Mondal *et al.,* 2008).

In our study the total flavonoid content was also estimated in *Annona reticulata*, which was estimated to be 23±3 µg/100 µg rutin equivalent. Flavonoids are the phenolic compounds, which are synthesized by plants due to adaptation in response to biotic and abiotic stresses (infection, water stress, cold stress, and high visible light) (Pitchersky and Gang, 2000). Flavonoids inhibit the oxidation reaction through radical scavenging mechanisms by donating an electron to the unpaired electrons in free radicals.

This study revealed that ethanolic leaf extract of *A. reticulata* comprises effective potential source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Further the study on quantitative estimation of the extract would reveal better insight on phytochemical properties of this plant.

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