



Pharmacological Study

In vitro antioxidant activity of hydro alcoholic extract from the fruit pulp of *Cassia fistula* Linn.

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Abstract

The present study is aimed to investigate antioxidant activity of the extracts of *Cassia fistula* Linn. (*Leguminosae*) fruit pulp. *Cassia fistula* Linn., a Indian Laburnum, is widely cultivated in various countries and different continents including Asia, Mauritius, South Africa, Mexico, China, West Indies, East Africa and Brazil as an ornamental tree for its beautiful bunches of yellow flowers and also used in traditional medicine for several indications. The primary phytochemical study and *in vitro* antioxidant study was performed on hydro alcoholic extract of fruit pulp. Phytochemical screening of the plant has shown the presence of phenolic compounds, fatty acids, flavonoids, tannins and glycosides. Phenolic content was measured using Folin-Ciocalteu reagent and was calculated as gallic acid equivalents. Antiradical activity of hydro alcoholic extract was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and was compared to ascorbic acid. Ferric reducing power of the extract was also evaluated by Oyaizu method. In the present study, three methods were used for evaluation of antioxidant activity. First two methods were for direct measurement of radical scavenging activity and third method to evaluate the reducing power. Results indicate that hydro alcoholic fruit pulp extracts have marked amount of total phenols which could be responsible for the antioxidant activity. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, *Cassia fistula* fruit pulp extract shows lower activity in DPPH and total phenol content as compared with standard which might be helpful in preventing the progress of various oxidative stresses.

Keywords: Antioxidants, *Aragwadha*, *Cassia fistula*, free radical, phenols

Introduction

Natural antioxidants present in the plants scavenge harmful free radicals from our body. Free radicals are any species capable of independent existence that contains one or more unpaired electrons which react with other molecule by taking or giving electrons, and involved in many pathological conditions,^[1] and they play very important role in human health and beneficial in combating against several diseases like cardiovascular disorders, lung damage, inflammation etc. These free radicals are highly unstable and when the amount of these free radicals exceed in the body, it can damage the cells and tissues and may involve in several diseases. Thus, there is a need of antioxidants of natural origin because they can protect the human body from the diseases caused by free radicals.^[2,3]

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. The most common reactive oxygen species (ROS) include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxy (ROO^-) radicals, and reactive hydroxyl (OH^-) radicals. The nitrogen-derived free radicals are nitric oxide (NO) and peroxynitrite anion ($ONOO^-$).^[4] Under normal state of affairs, the ROS generated are detoxified by the antioxidants nearby in the body and there is symmetry between the ROS generated and the antioxidant present. However, due to ROS over production and/or derisory antioxidant argument, this equilibrium is hindered favoring the ROS gain that culminates in oxidative hassle. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA.^[5] This oxidative damage is decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing course.^[6] Based on growing interest in free radical biology and the lack of effective therapies for most chronic diseases, the expediency of antioxidants in protection against these diseases is defensible. Epidemiological studies have shown that the intake of antioxidants such as vitamin C reduces the risk of

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coronary heart diseases and cancer.^[7] It is possible to reduce the risk of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants.^[8]

Currently available synthetic antioxidants like BHT, butylated hydroxyl anisole (BHA) and tertiary butylated hydroquinones have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants.^[9] Several studies revealed that phenols, mainly the type of flavonoids, from some medicinal plants are safe and bioactive, and have antioxidant properties and exert anticarcinogenic, antimutagenic, antitumoral, antibacterial, antiviral and anti-inflammatory effects.^[10] Therefore in current years, substantial attention has been directed towards credentials of plants with antioxidant ability that may be used for human expenditure.

Due to their redox properties, they act as reducing agents, hydrogen donors, singlet oxygen quenchers and chelating metals.^[11-13] The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability.^[14] One of the areas, which attracted a great treaty of attention in recent years is antioxidant in the control of degenerative disease in which oxidative dent has been implicated. Several plant extracts have shown antioxidant activity.^[15-17]

Materials and Methods

Collection of plant materials

The ripe pods of *Cassia fistula* were collected from the local gardening area located in Jamnagar, Gujarat, India in the month of June-Aug 2009. The plant was authenticated in the Pharmacognosy laboratory, I.P.G.T. and R.A. Jamnagar, Gujarat, India. Each specimen was labeled, numbered, annotated with date and place of collection.

Preparation of plant extract

Extraction of *Cassia fistula* fruit pulp was carried out using known standard procedures.^[18] The fresh pulp of *C. fistula* pods (25.0 g) was extracted with 900 ml of diluted methanol in a Soxhlet apparatus. The extract were then combined, filtered and evaporated to dryness on a hot water bath to yield a Soxhlet crude extract (9.7 g).

The extract were kept in sterile bottles, under refrigerated conditions, until further use. They were used directly for DPPH assay, total phenol and ferrus-reducing power content and also for assessment of antioxidant capacity.

Preliminary phytochemical testing

The extract were subjected to preliminary phytochemical testing to detect for the presence of different chemical groups of compounds. Air-dried and powdered plant materials were screened for the presence of saponins, tannins, alkaloids, flavonoids, triterpenoids, steroids, glycosides, anthraquinones, coumarin, saponins, gum, mucilage, carbohydrates, reducing sugars, starch, protein and amino acids as described in literatures.^[19-21]

Chemicals and Instruments

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH, Lancaster-UK), gallic acid (Loba-India) were purchased from Krishna scientific traders, Rajkot, Gujarat, India. Folin Ciocalteu's reagent, sodium carbonate, ascorbic acid, hydrogen peroxide, potassium ferricyanide, trichloroacetic acid, ferric chloride, and all other reagents of analytical grade were obtained from the pharmaceutical chemistry laboratory, I.P.G.T and R.A., Jamnagar, Gujarat, India.

Instruments

UV spectrophotometer (Systronic double beam-UV-2201).
Centrifuge machine (Remi instruments-C24).

Determination of total antioxidant activity

In-vitro antioxidant activity

Free radical scavenging activity (DPPH assay)

The anti-oxidant potential of any compound can be determined on the basis of its scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by Sadhu *et al.*^[23] DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis.

The absorption maximum of a stable DPPH radical in methanol was at 517 nm. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation.

Preparation of standard solution

Required quantity of ascorbic acid was dissolved in methanol to give the concentration of 5, 10, 15, 25, 50 and 60 µg/ml.

Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of dried hydro alcoholic extract in 10 ml of methanol to give a concentration of 1 mg/ml. Then prepared sample concentrations of 10, 15, 25, 50 and 60 µg/ml.

Preparation of DPPH solution

3.9 mg of DPPH was dissolved in 3.0 ml methanol; it was protected from light by covering the test tubes with aluminum foil.

Protocol for estimation of DPPH scavenging activity

Antiradical activity was measured by a decrease in absorbance at 517 nm of a solution of colored DPPH in methanol brought about by the sample.^[23-26] A stock solution of DPPH (1.3 mg/ml in methanol) was prepared such that 75 µl of it in 3 ml methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of sample extract and standard at different concentrations was noted after 30 min. EC₅₀ was calculated from % inhibition. A blank reading was taken using methanol instead of sample extract. Absorbance at 517 nm is determined after 30 min using UV spectrometer (Systronic double beam- UV-2201). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The EC₅₀ value was calculated graphically.

The capability to scavenge the DPPH radical was calculated using the following equation.

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

Where, A_{control} is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts.

Reducing power assay

For the measurement of the reductive ability, we investigated the Fe^{3+} Fe^{2+} transformations in the presence of *Cassia fistula* hydro alcoholic extract following the standard method.^[27] The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Like the antioxidant activity, the reducing power of *Cassia fistula* hydro alcoholic extract increases with increasing concentration.

Preparation of standard solution

3 mg of ascorbic acid was dissolved in 3 ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentrations of 10, 25, 50, 75 and 100 $\mu\text{g/ml}$.

Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of dried hydro alcoholic extract in 10 ml of methanol to give a concentration of 1mg/ml. Then sample concentrations of 10, 25, 50, 75 and 100 $\mu\text{g/ml}$ were prepared.

Protocol for reducing power

According to this method, the aliquots of various concentrations of the standard and test sample extracts (10 to 100 $\mu\text{g/ml}$) in 1.0 ml of deionized water were mixed with 2.5 ml of (pH 6.6) phosphate buffer and 2.5 ml of (1%) potassium ferricyanide. The mixture was incubated at 50°C in water bath for 20 min after cooling. Aliquots of 2.5 ml of (10%) trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution 2.5 ml was mixed with 2.5 ml distilled water and a freshly prepared 0.5 ml of (0.1%) ferric chloride solution. The absorbance was measured at 700 nm in UV spectrometer (Systronic double beam-UV-2201).^[28] A blank was prepared without adding extract. Ascorbic acid at various concentrations (10 to 100 $\mu\text{g/ml}$) was used as standard. As illustrated in figures, Fe^{3+} was transformed to Fe^{2+} in the presence of *Cassia fistula* extracts. This result indicates that increase in absorbance of the reaction mixture indicates increase in reducing power.

Total phenolic content

Preparation of standard solution

3 mg of gallic acid was dissolved in 3 ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentrations of 25, 50, 75, 100, 200 and 250 $\mu\text{g/ml}$.

Preparation of test sample

Stock solutions of samples were prepared by dissolving 10 mg of dried hydro alcoholic extract in 10 ml of methanol to give a concentration of 1 mg/ml. Then sample concentrations of 25, 50, 75, 100, 200 and 250 $\mu\text{g/ml}$ were prepared.

Protocol for total phenolic content

Total phenolic content was determined using Folin-Ciocalteu was established by following standard methods.^[29] The powdered extracts of plant were dissolved in methanol to obtain a concentration of 10 mg/10 ml. From this solution, 1 ml was taken and gave dilution up to 10 ml (100 $\mu\text{g/ml}$ of stock solution) with the same solvent. It was considered as the stock solution. From this stock solution, different concentrations 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 75 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$, were made following the same procedures for standard. Gallic acid was used as the standard. 1.0 ml of Folin-Ciocalteu reagent was added in these concentrations samples and the content of the flask was mixed thoroughly. The mixture was then kept for 5 min and to it 4 ml of 20% w/v sodium carbonate solution was added, the volume was made with double distilled water. The mixture was kept for 30 min until blue color develops. The absorbance of blue color developed which was recorded at 765 nm in UV spectrophotometer. The % of total phenolic was calculated from the calibration curve of gallic acid plotted by using the similar procedure.^[30,31]

Results and Discussion

Preliminary phytochemical screening

It was found that hydro alcoholic extract of *Cassia fistula* contained saponins, steroids, triterpenoids, and higher amount of phenolic compounds, flavonoids, tannins, glycosides, anthraquinones, gums, mucilage and amino acids.

DPPH free radical scavenging activity

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm, which is induced by different antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progress, results in the scavenging of the radical by hydrogen donation. *Cassia fistula* exhibited a comparable antioxidant activity with that of standard ascorbic acid at varying concentrations tested (5, 10, 15, 25, 50, 60 $\mu\text{g/ml}$). There was a dose dependant increase in the percentage antioxidant activity for all concentrations tested [Tables 1 and 2]. The extract at a concentration of 10 $\mu\text{g/ml}$ showed a percentage inhibition of 9.67 ± 0.28 and for 60 $\mu\text{g/ml}$ it was 17.03 ± 0.27 . Ascorbic acid was used as

Table 1: Percentage inhibition of standard at various concentrations ($\mu\text{g/ml}$) in hydrogen peroxide scavenging model

Concentration ($\mu\text{g/ml}$)	% inhibition	EC ₅₀
5	44.50±0.59	
10	52.74±0.22	
15	62.63±0.34	6.1
25	78.57±0.32	
50	98.79±0.28	
60		

Values are mean±SD of three parallel measurements. STD: Ascorbic acid

the standard drug for the determination of the antioxidant activity by DPPH method. The concentration of ascorbic acid varied from 1 to 50 µg/ml. Ascorbic acid at a concentration of 5 µg/ml exhibited a percentage inhibition of 44.50 ± 0.59 and for 50 µg/ml 98.79 ± 0.28 [Tables 1 and 2]. A graded increase in percentage of inhibition was observed for the increase in the concentration of ascorbic acid. The EC₅₀ value of ascorbic acid was found to be 6.1 µg/ml. EC₅₀ value of sample extracts could not be calculated because of lower values of inhibition than 50%. All determinations were done in triplicates and the mean values were determined [Figures 1 and 2].

Reducing power assay

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe³⁺) to form potassium ferrocyanide (Fe²⁺), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. The reducing power of the hydro alcoholic extracts and standard increases with the increase in amount of sample and standard concentrations [Tables 3 and 4]. The reducing power shows good linear relation in both standard (R₂ = 0.981) and sample extract (R₂ = 0.970) [Figures 3 and 4].

Total phenolic content

The total phenolic content of hydro alcoholic extract of *Cassia fistula* calculated as gallic acid equivalent of phenols was detected [Tables 5 and 6].

Free radicals are produced under certain environmental conditions and during normal cellular function in the body; these molecules miss an electron, giving them an electric charge. To neutralize this, charge, free radicals try to withdraw an electron from, or donate an electron to a neighboring molecule. The newly created free radical, in turn, looks out for other molecules and withdraws or donates an electron, setting off a chain reaction that can damage hundred of molecules. The total phenol content shows good linear relation in both standard and sample extracts [Figures 5 and 6]. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability.

Conclusion

From the results obtained in the present study, it is concluded that, the hydro alcoholic extract of *Cassia fistula* fruit pulp shows antioxidant activity by inhibiting DPPH and hydroxyl radical, total phenol content and reducing power activities. The preliminary phytochemical investigation indicates that the *Cassia fistula* contains saponins, steroids, triterpenoids, and higher amount of phenolic compounds, flavonoids, tannins, glycosides, anthraquinones, gums, mucilage and amino acids. The presence of phenols and flavonoids in the plant plays a major role in controlling antioxidants and are also major constituents of anthraquinones. It contains phenolic groups which are responsible for higher antioxidant activity. The results of this study show that the hydro alcoholic extract of *Cassia fistula* can be used as a easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. However, the activity of *Cassia fistula* fruit pulp extract shows lower activity in DPPH and total phenol content as compared

Table 2: Percentage inhibition of hydro alcoholic extracts of fruit pulp at various concentrations (µg/ml) in hydrogen peroxide scavenging model

Concentration (µg/ml)	% inhibition	EC ₅₀
10	09.67±0.28	
15	10.98±0.31	
25	12.63±0.26	
50	14.06±0.39	
60	17.03±0.27	

Values are mean±SD of three parallel measurements

Table 3: Absorbance of standard at various concentrations (µg/ml) in ferric reducing power determination model

Concentration (µg/ml)	Absorbance
10	0.090±0.002
25	0.122±0.003
50	0.240±0.002
75	0.352±0.004
100	0.471±0.001

Values are mean±SD of three parallel measurements

Table 4: Absorbance of hydro alcoholic extract of fruit pulp at various concentrations (µg/ml) in ferric reducing power determination model

Concentration (µg/ml)	Absorbance
10	0.030±0.002
25	0.052±0.004
50	0.075±0.001
75	0.125±0.003
100	0.165±0.004

Values are mean±SD of three parallel measurements

Table 5: Absorbance of standard gallic acid at various concentrations (µg/ml) in total phenolic content determination model

Concentration (µg/ml)	Absorbance
25	0.194±0.012
50	0.395±0.010
75	0.652±0.009
100	0.790±0.015
200	1.192±0.010

Values are mean±SD of three parallel measurements

Table 6: Absorbance of hydro alcoholic extract of fruit pulp at various concentrations (µg/ml) in total phenolic content determination model

Concentration (µg/ml)	Absorbance
25	0.010±0.001
50	0.025±0.001
75	0.058±0.003
100	0.069±0.005
200	0.102±0.004
250	0.121±0.008

Values are mean±SD of three parallel measurements

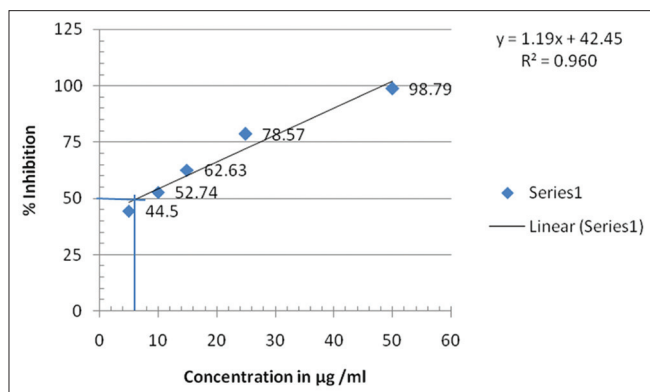


Figure 1: DPPH free radical scavenging activity of standard ascorbic acid

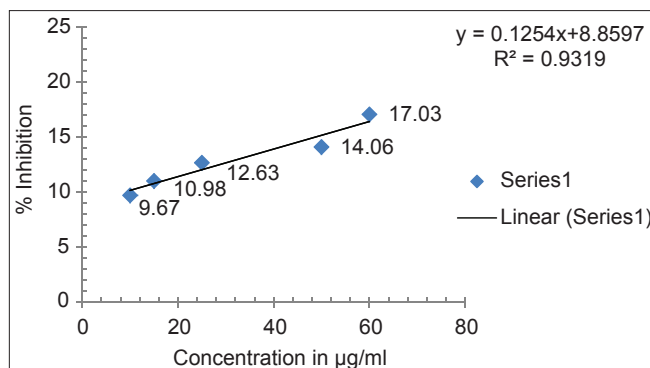


Figure 2: DPPH free radical scavenging activity of hydro alcoholic extract of fruit pulp

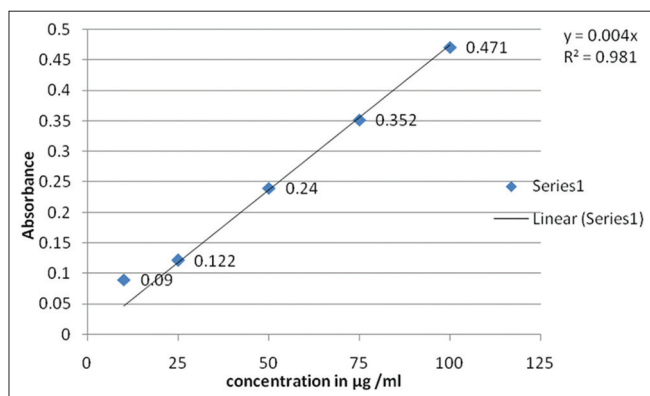


Figure 3: Ferric reducing power determination of standard ascorbic acid

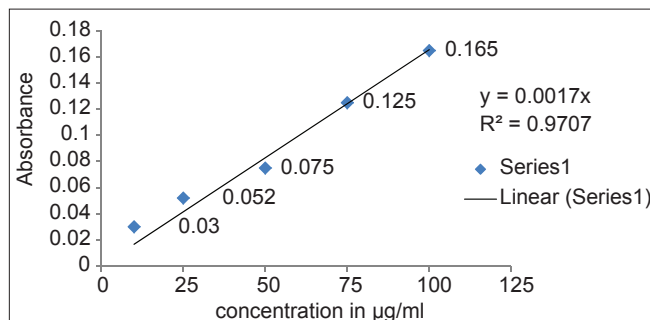


Figure 4: Ferric reducing power determination of hydro alcoholic extract of fruit pulp

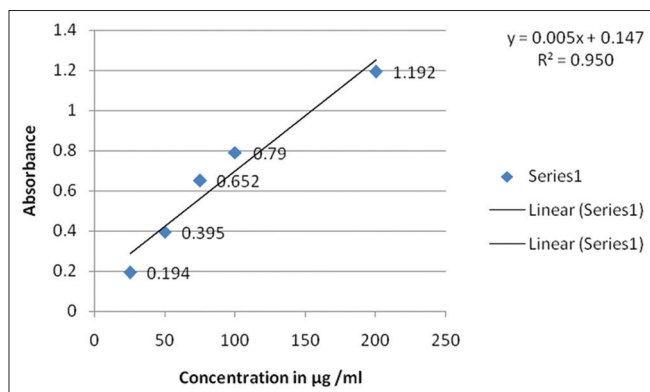


Figure 5: Total phenolic content of standard gallic acid

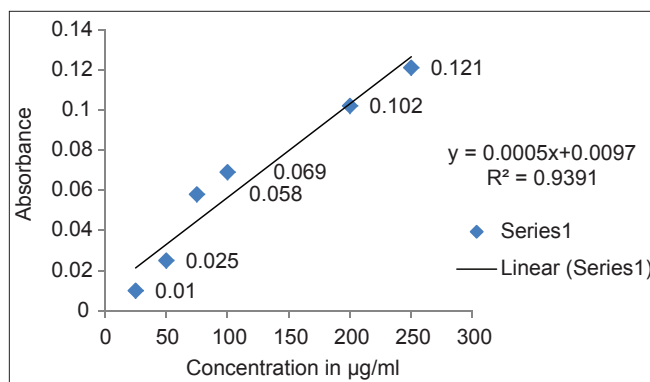


Figure 6: Total phenolic content of hydro alcoholic extract of fruit pulp

with standard. Further works may be taken-up the other extracts of various parts of *Cassia fistula*.

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हिन्दी सारांश

आरग्वध (कॅशिया फिस्टुला) फलमज्जा के हायड्रो-अल्कोहोलिक कार्यकारी तत्वों की इन व्हिट्रो अँन्टिऑक्सिडेन्ट कार्यकारीता

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प्रस्तुत अध्ययन आरग्वधफलमज्जा के हायड्रोअल्कोहोलिक कार्यकारी तत्वों की अँन्टिऑक्सिडेन्ट कार्यकारीता परीक्षण हेतु किया गया है। एशिया, मॉरिशस, दक्षिण आफ्रिका, मेक्सिको, चीन, वेस्ट इंडिज, पूर्व आफ्रिका और ब्राजील आदि देशों में इसके मनोहर पीले फूलों के कारण सुशोभिकरण हेतु इसे लगाया/उगाया जाता है और कई व्याधियों में उपयोग करते हैं। आरग्वध फलमज्जा के हायड्रो-अल्कोहोलिक कार्यकारी तत्वों की प्राथमिक रासायनिक परिक्षण द्वारा इसमें फिनॉलिक कम्पाउण्ड, वसीय अम्ल, फ्लेवोनोंईड, टॅनिन और ग्लायकोसाईड पाये गये। अँन्टिऑक्सिडेन्ट कार्यकारीता का परीक्षण तीन प्रकार के प्रयोगों/विधियाँ द्वारा किया गया। प्रस्तुत अध्ययन में देखा गया की आरग्वध फलमज्जा के हायड्रोअल्कोहोलिक कार्यकारी तत्वों में अँन्टिऑक्सिडेन्ट कार्यकारीता है और यह प्रायः ऑक्सिडेटिव्ह स्ट्रेस/भार बढ़ानेवाली व्याधियों में उपयोगी हो सकती है।