International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2019): 7.583

Phytochemical Analysis, Antioxidant and Antihemolytic Activity of Hibiscus and Amla

Deepak T[1], Shashank A S[2], K Yeshwanth Kumar[3], Vinanthi Rajalakshmi K S[4]

[1, 2, 3, 4]Department of Studies and Research in Zoology, Tumkur University, Karnataka, India

Abstract: ***Now a day our traditional medicine practice was replaced by western medicines due to the lack of Ayurvedic knowledge. It leads to the development of synthetic drugs. These drugs are very adverse to the human life. So that we can replace this through our folk medicinal plants like Hibiscus, Amla and Neem etc. These plants are widely used for diabetics, cancer and osteoporosis treatment because of their good medicinal properties. In our research we selected Hibiscus and Amla to know its medicinal value. Hibiscus flower and leaves is used for the treatment of constipation, ulcer and also it promotes growth and color of hair. Amla fruit is rich in vitamin C and this fruit is also used to control vaginal discharge and it has anti diabetic agent. The plants samples were extracted using Methanol. This methanolic sample is used to the phytochemical analysis. It was confirmed that samples contain many biological active compound like Flavonoids, Polyphenols, Tannins, Alkaloids and Terpinoids etc. It leads to the structures for the development of poly herbal formulation. In the DPPH assays the samples quenches the free radical of DPPH and prevents oxidation so it shows that samples have high anti-oxidant property. After this we subjected the samples to hemolytic assay to know the hemolytic activity. The negligible amount of hemolysis is happened compared with SDS solution so it is confirmed that the samples have anti-hemolytic activity. Thus these plants have good therapeutic potential.***

Keywords: Hibiscus, Amla, antioxidant, antihemolytic, biological active molecule.

1. Introduction

Ayurveda is one of the oldest medical systems in the world. The treatment in ayurveda is based on diet, exercises and medicinal plants. These medicinal plants have an ability to cure piles, fever, cough, some infection and many other common diseases. In ayurveda the Amla and Hibiscus are widely used because they have many medicinal benefits. These plants contain biologically active molecules like Flavonoids, Alkaloids, Tannins, Terpinoids, Steroids and many others. These biological active molecules are responsible for biological activities like anti cancer, anti microbial, anti inflammatory, anti diabetic, anti biotic and anti fungal activities (prachi javale et,. Al 2010). Two assays are carried out for the study of hemolysis and anti oxidant properties of methanolic extraction of Amla and Hibiscus. Breakdown or destruction of red blood cells is called as hemolysis. Interaction between the blood and other bio-materials may leads to the hemolysis. The hemolysis assay is a test based on erythrocyte lyses. Oxidative damage in biological system which is the basis of number of many physiological phenomena (Halliwell et,. Al 1986). Erythrocytes have been used as a convenient model for these studies. This method is suited to evaluate the hemo compatibility of biomaterials and medical devices according to the international standard ISO10993-4 2002. Assessments of antioxidants properties of natural compounds are very important because of their uses in medicine, food and cosmetics (Halliwell 1997, Live 2003, Sanchez-Moreno 2002). Free radicals are commonly introduced by many environmental like smoke, radiation, drug, pollution and sunlight (Honzel et al 2008). Radioactive oxygen species (ROS) are very reactive on molecules which are metabolism of oxygen. These ROS regulates the oxidative stress. Which involve number of chronic diseases Diabetes Cancer ,Cardiovascular disease, Cell aging, DNA damaging(Valko et al 2007).The exogenous antioxidants is necessary to maintain the proper physiological function for offsetting this oxidative stress(Shumin liv et al 2014).The global trend is to use natural substances present in the medicinal plants and dietary plants as therapeutic antioxidant after appropriate evolution(Lobo et al 2016).The DPPH assay is widely used to evaluate the antioxidant property of the plant extract. The DPPH is violet in aqueous solution. When it follows reduction process it turns to yellow color. It means the quenching of free scavenging molecules shows the antioxidant property. The aim of the work is to evaluate the antioxidant and hemolytic activities of the methanolic extraction of Amla and Hibiscus

2. Methods and Materials

2.1. Plant Materials

The flowers of Hibiscus (*Rosa sinensis)* and fruits of Amla (*Phyllanthus emblica*) were used for the study of hemolysis and Antioxidant activity.

2.2. Preparation of Extract

Weighed 20g of dried Sample powder and dissolved in 100ml of Methanol in 500ml beaker with aluminum foil covered on it. Then the beaker was kept on hot water bath at 50º C for 4 hours. After incubation period the extract was filtered with Whatmann filter paper and the filtrate was collected in 50ml beaker. Residue present over the filter paper was discarded and filtrate was taken for further use. Then the filtrate was kept at 50ºC for few hours until the extract got completely dried and turned into semisolid form. This semi solid sample was weighed and the yield was noted.

2.3. Phytochemical Analysis

Test for Alkaloids

Dragendoff's test: 0.2ml of sample was taken and 0.2ml of HCl was added, 2 to 3 drops of Dragendoff's reagent was

added. The appearance of orange red precipitate and turbid solution indicates the presence of alkaloids.

Test for Carbohydrates:
Molisch's test: 0.2 ml of sample was mixed with few drops of Molisch's reagent (α- napthol dissolved in alcohol).0.2 ml of sulphuric acid was added along the sides of the test tube and observed for the appearance of a purple color ring for positive test.

Test for Tannins:
Braymer's test: 0.2 ml of plant extract was mixed with 2 ml water and heated on water bath for 10 minutes. The mixture was filtered and ferric chloride was added to the filtrate and observed for dark green solution which indicates the presence of tannin.

Test for Terpinoids:
Salkowki's test: 0.2 ml of plant extract was taken in a test tube with 0.2 ml of chloroform. To this, concentrated sulphuric acid was added carefully to form a layer. Presence of reddish brown color at the interface would show the presence of Terpinoids.

Test for Glycosides:
0.2 ml of sample was mixed with 0.2 ml of chloroform. 0.2ml of acetic acid was added to this solution and the mixture was cooled on ice. Sulphuric acid was added carefully and the color change from violet to blue to green indicates the presence of steroidal nucleus (A glycogen portion of glycoside).

Test for Steroids:
Lieberman Burchard tests: 0.2 ml of sample was mixed with 0.2 ml of chloroform. To this 0.2ml of concentrated sulphuric acid was added. The appearance of red color in the lower layer of chloroform indicates the presence of steroids.

Test for Saponins:
Test for Saponins (Foam test): 0.2ml of extract was added 0.6ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of Saponins.

Test for Flavonoids:
Alkaline reagent test: 0.2 ml of plant extract was taken in a test tube and mixed with dilute sodium hydroxide solution. To this diluted hydrochloric acid was added. Observation of yellow solution that turn colorless later would indicate the presence of flavonoids.

Mucilage test (Glycoprotein):
0.2ml of extract was taken in a test tube and 0.2ml of absolute alcohol was added and allowed to dry. If the precipitation occurs then mucilage is present.

Volatile oil:
0.2ml of extract was treated with few drops of dilute hydrochloric acid. The appearance of white precipitate indicates the presence of volatile oils.

Test for phenols:
0.2 ml of extract was added to 0.4 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution. Formation of blue or green color indicated the presence of phenols.

2.4. Isolation of Erythrocytes

Five ml of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and centrifuged at 1000 rpm for 10 min at 4^0C. Plasma was removed carefully and the white buffy layer was completely removed by aspiration with a pipette with care. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4. Washed erythrocytes were stored at 4^oC and used within 6 h for the hemolysis assay.

2.5. HEMOLYSIS ASSAY

Take 50 µl of 10 dilution (100µl Erythrocytes suspension: 900 µl1 X PBS) of erythrocytes suspension into 2 ml Eppendorfs tube and add 100 µl of test samples (plant extracts, compounds, etc.), 100 µl of 1XPBS as negative control, 100 µl of 1% Triton X-100 or 100 µl of 1% SDS as positive controls. Reaction mixture is incubated at 37^0C for 60 min. adjust the volume of reaction mixture to 1 ml by adding 850µl of 1 X PBS. Finally centrifuge at 300 rpm for 3 min and the resulting hemoglobin in supernatant was measured at 540 nm by spectrophotometer to determine the concentration of hemoglobin.

2.6. DPPH ASSAY

DPPH assay is carried out as per the method of Rajakumar *et al*. 1994. In brief, 80µl of DPPH solution; various concentration of test solution and quantity sufficient to 240 µl with HPLC grade methanol. The different concentrations tested for reference standard are 0.3125, 0.625, 1.25, 2.5, 5, 10 µg/ml. The different concentrations tested for test samples are 3.125, 6.25, 12.5, 25, 50, 100 µg/ml. The reaction mixture is mixed and incubated at 25°C for 15 minutes. The absorbance is measured at 510nm using semi auto analyzer. A control reaction is carried out without the test sample.

2.7. Statistical Evoluation

Half maximal Inhibitory concentration (IC50) is the concentration of the substance required to inhibit a biological process such as an enzyme, cell, cell receptor or microorganism by half. IC 50 value is calculated using Graph Prism software version 5.0 by nonlinear regression analysis of % inhibition recorded for different concentrations of test substances/standard. For compounds showing <50% inhibition, IC50 value is not calculated. The relative activity of the sample can be determined by comparing the IC50 value of sample with standard. Higher the IC50 value, lower will be the relative activity in comparison to standard & vice versa.

3. Results and Discussion

3.1. Phytochemcal Analysis

The extract was subjected to different qualitative test. Many phytocomponents was observed during the test such as alkaloids, Terpinoids, tannins, steroids and flavonoids were present in the extract. The result was shown in the table 1. These phytocomponents have major important role in biological activities.

Table 1: Preliminary phytochemical analysis of MeOH of extraction Hibiscus and Amla

Types Of Tests	Amla	Hibiscus
Alkaloid	+	+
Carbohydrate	-	-
Tannin	-	+
Terpenoid	+	-
Glycoside	-	-
Steroid	-	+
Saponin	-	-
Flavanoid	+	+
Proteins (Myllon's Test)	-	-
Glycoprotein Test	-	-
Volatile Oil	-	-

3.2. HEMOLYSIS ASSAY

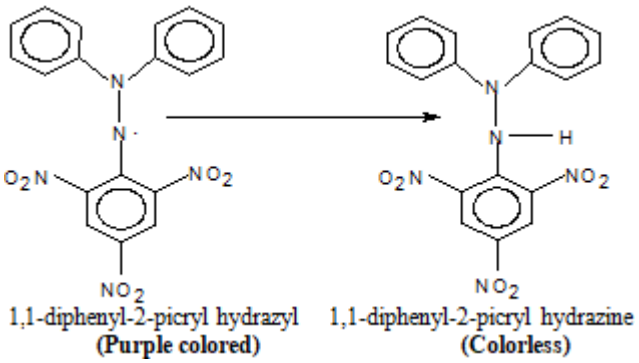
An erythrocyte suspension is incubated for 1 hour with test material during rotation at 37^0C. After incubation, samples are collected and centrifuged to obtain supernatant, containing free hemoglobin. The hemoglobin concentration is measured by means of a spectrophotometer (540 nm).Test samples are compared to reference materials (1% TritonX-100 and 1% SDS). The different concentration of plant sample is added to the erythrocyte suspensions into 2ml Eppendorfs tube. The negligible hemolysis was observed but the erythrocytes in the SDS solution show the major hemolysis. The detailed result was showed in table 2. Sample Hibiscus and Amla did not show any significant hemolytic activity. The obtained data has negligible hemolysis and samples screened can be considered for pharmaceutical studies.

Table 2: Hemolysis activity of plant samples of different concentration observed at 540nm. Here positive control is SDS and Control is PBS. Maximum hemolysis is noticed in positive control, no hemolysis in control and negligible hemolysis in plant extract

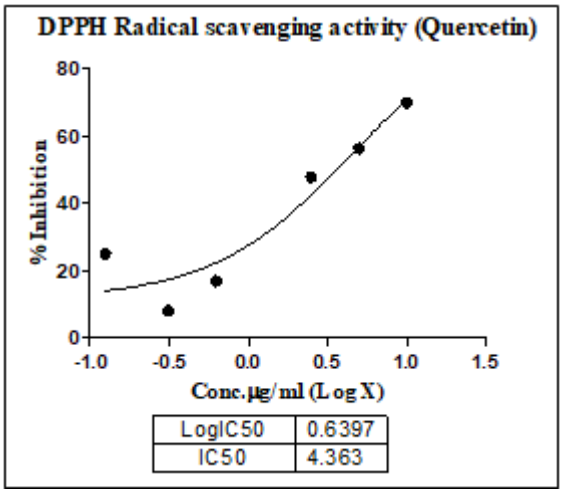
Sample	Treatment	Absorbance	% Hemolysis
Control	PBS	0.6794	0.00
Positive control	1% SDS	0.08	88.22
Hibiscus	10	0.6332	6.80
	20	0.612	9.92
	40	0.583	14.19
	80	0.552	18.75
	160	0.522	23.17
	320	0.499	26.55
Amla	10	0.641	5.65
	20	0.603	11.25
	40	0.574	15.51
	80	0.543	20.08
	160	0.521	23.31
	320	0.504	25.82

3.3. DPPH ASSAY

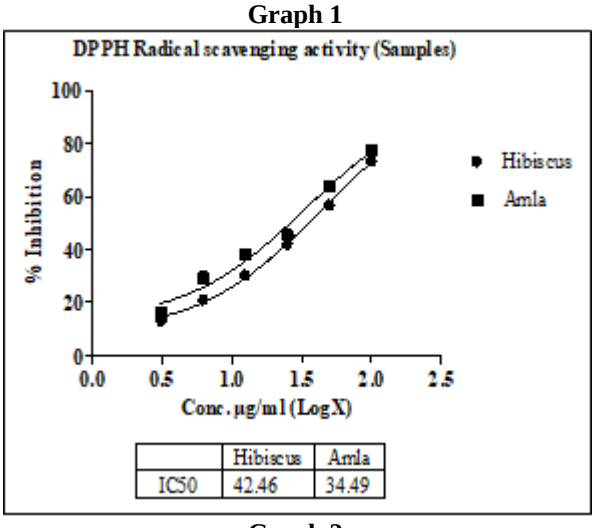
DPPH [1, 1-diphenyl-2-picryl hydrazyl] is a stable free radical with purple color. Antioxidant reduces DPPH to 1, 1-diphenyl-2-picryl hydrazine, colorless compound which is measured at an absorbance of 590nm.



In the DPPH test the ability of compound to act as donor for hydrogen atoms or electrons was measured spectrophotometrically. Both the Amla and Hibiscus were able to reduce the stable radical DPPH to the yellow colored Diphenyl picryl hydrazine. The strongest effect was measured for the Amla and Hibiscus with an IC_{50} of quercetin 4.363µg/ml, Amla 34.49µg/ml and Hibiscus 42.46µg/ml. The results are shown in Graph 1, Graph 2 and table 3.



Graph 1



Graph 2

Table 3: The sample tested above, Hibiscus and Amla have shown activity having an IC50 value of 42.46 and 34.49 µg/ml. Standard Quercetin has shown IC50 value of 4.363µg/ml

Sample	Conc. (µg/ml)	OD @ 510nm	% Inhibition	IC50 (µg/ml)
C	0	0.654	0.00	
Quercetin	0.3125	0.600	8.20	
	0.625	0.543	17.00	
	0.125	0.490	25.08	4.363
	2.5	0.341	47.88	
	5	0.285	56.43	
	10	0.196	70.02	
Hibiscus	0	0.655	0.00	
	3.125	0.568	13.24	
	6.25	0.517	21.12	
	12.5	0.456	30.33	42.46
	25	0.380	42.06	
	50	0.283	56.78	
	100	0.174	73.48	
Amla	3.125	0.548	16.33	
	6.25	0.463	29.38	
	12.5	0.404	38.40	
	25	0.358	45.44	34.49
	50	0.238	63.71	
	100	0.146	77.67	

4. Conclusion

The insufficient diet leads to the oxidative stress in the human these oxidative stress causes cancer, diabetes, cell damage, DNA damage etc. Naturally antioxidants are rich in leaves, flowers and fruits. Dietary supplement having antioxidants is best and effective to boost up the immunity. Present world facing many diseases and disorders due to the lack of immunity. From our study, we can develop a poly herbal formulation with a combination of Amla and Hibiscus for hemolytic and oxidative stress. This has pharmaceutical potential.

5. Acknowledgment

We thank to Skanda Life Science Pvt. Ltd, R and D center Bangalore for providing laboratory for this study and also for Tumkur University for the great support for this work.

References

[1] Arjun HB, Yasuhiro T, Ketut AI, Kiyoshi M, Katsumichi M, Dejair M, Alfredo AGH,Shigetoshi K. (2000) *J.* Ethnopharmacol. 72: 239-246.
[2] Burits M, Bucar F. (2000) Phytotherapy Res. 14: 323-328.
[3] Eklund, P. C., Langvik, O. K., Warna, J. P., Salmi, T. O., Willfor, S. M., & Sjoholm, R. E. (2005). Chemical studies on antioxidant mechanisms and free radical scavenging properties of lignans. Organic and Bimolecular Chemistry, 21, 3336–3347.
[4] Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S. I., & Chun, O. K. (2011). Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. Journal of Food Composition and Analysis, 24(7), 1043–1048. doi:10.1016/j.jfca.2011.01.008
[5] Halliwell B, Gutteridge JMC. (1989) Free Radicals in Biology and Medicine. Clarendon Press, Oxford, 2nd edn., 416-419.
[6] Harboe. A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry, Scand. Clin. Lab. Invest.1959; 11:66-70.
[7] Henkelman S, Rakhorst G, Blanton J, van Oeveren W. Standardization of incubation conditions for hemolysis testing of biomaterials. Materials Science & Engineering C – Biomimetic and Supramolecular Systems.2009; 29:1650-1654.
[8] Honzel, D., Carter, S. G., Redman, K. A., Schauss, A. G., Endres,J. R., & Jensen, G. S. (2008). Comparison of chemical and cell based antioxidant methods for evaluation of foods and natural products: Generating multifaceted data by parallel testing using erythrocytes and polymorph nuclear cells. Journal of Agricultural and Food Chemistry, 56, 8319–8325.
[9] Hopps, E., Noto, D., Caimi, G., & Averna, M. R. (2010). A novel component of the metabolic syndrome: The oxidative stress. Nutrition, Metabolism, and Cardiovascular Diseases, 20, 72–77.
[10] http://www.bentham.org/cmciema1-1/vaya/vaya-ms.htm
[11] http://ecso2.hcc.ru/DP_TOP3/dp130/dp130.htm
[12] I. H. Ibrahim, S. M. Sallam, H. Omar, M. Rizk, Oxidative Hemolysis of Erythrocytes Induced by Various Vitamins. International journal of biomedical science. 2006, 295- 298.
[13] Liu, S., & Huang, H. (2015). Assessments of antioxidant effect of black tea extract and its rational by erythrocyte haemolysis assay, plasma oxidation assay and cellular antioxidant activity (CAA) assay. Journal of Functional Foods, 18, 1095–1105. doi:10.1016/j.jff.2014.08.023
[14] Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews, 4, 118–126.
[15] Mishra, K., Ojha, H., & Chaudhury, N. K. (2012). Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. Food Chemistry, 130(4), 1036–1043. doi:10.1016/j.foodchem.2011.07.127
[16] Mebrahtom Gebrelibanos. In vitro Erythrocyte Hemolysis Inhibition Properties of Sennasingueana Extracts. Momona Ethiopian Journal of Science (MEJS), V4 (2):16-28,2012.
[17] MehboobHoque, Sandeep Dave, Pawan Gupta, Mohammed Saleemuddin. Oleic Acid May Be the Key Contributor in the BAMLET Induced Erythrocyte Hemolysis and Tumoricidal Action.Plosone, 1 September 2013, Volume 8, Issue 9, e68390.
[18] Mishra, K., Ojha, H., & Chaudhury, N. K. (2012). Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. Food Chemistry, 130(4), 1036–1043. doi:10.1016/j.foodchem.2011.07.127
[19] Rajakumar DV. (1994) Biochemical & Pharmacological studies on the antioxidant properties of

Dehydrozingerone and its analogs, University of Mangalore (unpublished Ph.Dthesis).

[20] Shumin Liu, Huihua Huang. Assessments of antioxidant effect of black tea extract and its rationals by erythrocyte haemolysis assay, plasma oxidation assay and cellular antioxidant activity (CAA) assay 2014.

[21] Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. International Journal of Biochemistry and Cell Biology, 39, 44–84.