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In-vitro ANTIOXIDANT AND MICROSCOPICAL EVALUATION OF *Hippeastrum puniceum* BULB (Lam) Voss.

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Abstract

Biologically active substances derived from natural sources are of interest at the mom ent since numerous new therapeutic targets for various diseases have been identified. Humans have been exploring the natural world's bounty for many ages in an effort to find cur es for every ailment that affects people.The monocotyledonous plants of the Amaryllidaceae family, which includes the genus Hippeastrum, are very useful to the global economy and the pharmaceutical sector.The Amaryllidaceae family, which has over 1100 species in 75 genera, is widely distributed throughout the tropical, subtropical, and warm regions of the world.For centuries, members of the Amaryllidaceae family of plants have been farmed for their decorat ive value as well as for use in numerous nations and areas as a source of traditional herbal re medies. The plants of Amaryllidaceae family have been grown for ages as ornamental plants and also for folk herbal treatments in many different countries and regions to treat variety of ailments. *Hippeastrum puniceum,* a parent of many ornamental hybrid plants, is generally a bulbous plant which is harvested from the wild for local use as a medicine. It produces a

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clump of 4 - 6 grass-like leaves up to 60cm tall from a globose bulb about 5cm in diameter. It is commonly grown as an ornamental in tropical and subtropical gardens, and also as a pot plant in cooler zones. The objective of this study is to investigate medicinal, and *in vitro* antioxidant activities of bulb of *Hippeastrum puniceum* and is collected from Kottayam, Kerala. The different pharmacognostical parameters are evaluated as per standard protocols with required modifications. The *in vitro* antioxidant activity of petroleum, ether, chloroform, ethyl acetate and ethanolic extract of the bulb is assessed against DPPH free radical scavenging assay, and reducing power assay method using standard protocols which proves that the activities of all bulb extracts against DPPH and reducing power assay method are concentration dependent.

Key words: *Hippeastrum puniceum*, microscopy, antioxidant, DPPH, Reducing power assay

INTRODUCTION

Medicinal plants are the major sources available in nature for the treatment and management of diseases. Herbal medications and phytopharmaceuticals use plant extracts and isolated pure components as a base[1]. A healthcare product must undergo scientific validation to determine its degree of purity, potency, efficacy, and safety for wide recognition[2,3]. World Health Organisation has established standard criteria, such as physicochemical and phytochemical evaluation of crude pharmaceuticals to assess the quality, safety, and effectiveness of herbal plants[4]. Setting these pharmacognostic standards is actually consists of several processes to create a monograph for a basic medication. The quality of a medicinal plant is evaluated for it is to be accepted into the traditional medical system. The established standards promote uniformity of quality which is formal numerical attributes by which the quality of herbs may be judged.

Metabolic process and external factors generate free radicals that interact with the biological system, such as the environment, food, and smoking products. Molecules that are extremely reactive because of their electronic instability are called as reactive species. Reactive oxygen species (ROS) is the main catalyst for starting the complete oxidation process. This oxidative stress plays as a significant health risk factor as per the etiology of many chronic diseases. Free radicals and other reactive oxygen species are known to have an important role in the pathophysiology of diseases like atherosclerosis, Parkinson's and Alzheimer's disease,

diabetes, Parkinson's disease, and asthma. It is professed that reactive oxygen species is the cause for human ageing[5-13].

Any agent that prevents or slows down oxidative damage from a target molecule is termed as an antioxidant. An antioxidant's main quality is its capacity to capture free radicals. By scavenging free radicals like peroxide, hydroperoxide, and lipid peroxyl, antioxidant substances including phenolic acids, polyphenols, and flavonoids repress the oxidative pathways that cause degenerative illnesses. Since ancient times, herbal plants have been considered as good antioxidants[14]

The evaluation standards and quality control of medicinal plants depend heavily on pharmacognostic study[15]. For authenticating herbal plants, a systematic strategy and carefully developed standardised methodologies are needed[16]. The misuse of herbal remedies starts with improper identification of basic plants, which is a common mistake if two or more different plant species under use have the same local name[17]. Another significant issue with the usage of herbal medicine is adulteration, which happens when the original plant material is replaced with another plant material or when foreign ingredients are purposefully added to increase weight, potency, or lower cost. By formulating proper pharmacognostic guidelines for the research of medicinal plants, all of these problems can be resolved.

Monocotyledonous plants in the Amaryllidaceae family are very much useful in medicinal industry. Their vast pharmacological effects have also been recorded. This family's species have historically been used for treatments of circulatory, neurological, and inflammatory diseases. Tyrosine-derived alkaloids, which are only produced by the Amaryllidaceae family, is considered to be the source of the medicinal value of the Amaryllidaceae species. These alkaloids have a wide range of physiological effects, including acetylcholinesterase (AchE) inhibitory, anti-inflammatory, and antibacterial activity, and also have cytotoxic and antiviral properties. One of the 20 most important plant families that contain alkaloids is the Amaryllidaceae family.

EXPERIMENTAL

Plant collection and identification

The *Hippeastrum puniceum* bulbs that have been utilised in this investigation are gathered in Kottayam, Kerala, India when the plant is in its blooming stage, which usually takes place

between March and April. Following collection, the plant material is taxonomically identified and authenticated at the Southern Regional Centre of the Botanical Survey of India (B.S.I.). For future reference and verification, a voucher specimen is provided with the number BSI/SRC/5/23/2020/Tech/67.

Morphological and microscopical studies

The freshly collected plant material be used for evaluating the morphology and anatomy. The anatomical studies are performed as per standard method[19,20]. In order to obtain thin sections, freehand sectioning is done so that cellular details are clearly visible. Sections are stained with safranin. The stained sections are mounted with glycerin on the glass slide and then observed under a light microscope. Leica DM 1000 LED. Trinocular 'Leica' microscope attached with Leica DFC 295 digital camera connected to the computer and Leica Application Software LAS Version 3.6.1.

Fluorescence Analysis

The coarsely powdered samples of the bulb are treated with different reagents such as acids and alkaline solutions along with other solvents. The treated powder is observed under visible light and UV light for their characteristic colour and all the observations are recorded[21,22].

Extraction and phytochemical analysis

The H. puniceum bulbs that are gathered for the investigation are first dried and then powdered into a coarse powder. Following a procedure known as a continuous percolation extraction, this powder is put through a series of extractions using several solvents with variable polarity. Petroleum ether, chloroform, ethyl acetate, ethanol, and water are the solvents employed in the present study, going from least polar to most polar. The objective of this extraction method is to separate the various chemical elements that are present in the plant material according to their solubility in various solvents. Each solvent extract is concentrated after extraction is finished in order to get rid of the solvent and leave only the extracted chemicals. The ethanolic extract is picked out of all the extracts for additional purification and analysis. An ethanolic extract's preliminary phytochemical analysis is examined[23].

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Determination of In Vitro Antioxidant Activities of different extracts

Ferric thiocyanate method (FTC)

The antioxidant activity of the different extracts obtained is tested using ferric thiocyanate (FTC) method. The FTC method is used to calculate the quantity of peroxide present at the start of the lipid peroxidation, when peroxide combines with ferrous chloride to produce ferric ion. After that, the ferric ion and ammonium thiocyanate unite to form ferric thiocyanate. The material is coloured red and the absorbance increases with increasing colour thickness.

The standard method as described by Kikuzaki and Nakatani, is used here for the determination of the antioxidant activity[24,25.] A vial with a screw cap is put into an oven at 40 ° C in the dark with a mixture of 4.0 mg of various plant extracts in 4 ml absolute ethanol, 4.1 ml of 2.5% linolenic acid in absolute ethanol, 8.0 ml of 0.05M phosphate buffer (pH 7.0), and 3.9 ml of water. 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate are added to 0.1 ml of this solution. The absorbance of red colour was calculated at 500 nm every 24 hours until the day after the absorbance of the control attained its maximum, precisely 3 min after adding 0.1 ml of 0.02M ferrous chloride in 3.5% HCl to the reaction mixture.

Determination of 1,1, dipheny-2-picrylhydrazyl (DPPH) Radical Scavenging Activities.

Using 1,1 diphenyl-2-picrylhydrazyl (DPPH), the DPPH radical scavenging assay is estimated using the method reported by Brand-Williams et al. with a few modifications. Briefly, four diverse concentrations of the studied plant extracts (0.2, 0.4, 0.8, and 1 mg/ml) are prepared in methanol (analytical grade). The same concentrations of L-ascorbic acid are used as the standard. DPPH 0.5 ml of 0.3 mM in methanol is added to a clean test tube containing 1 ml of each extract under study. After shaking, the mixture was allowed to place at room temperature in the dark for 15 minutes. The examined extract solutions (2. 5 ml) and 1 ml of methanol are used to create blank solutions as a starting point. The negative control included 2.5 ml of DPPH solution and 1 ml of methanol, whereas the positive control constituted L-ascorbic acid at the same concentrations as the examined extracts. The absorbance readings are calculated at 517 nm using a spectrophotometer after incubation in the dark. The experiments are carried out in triplicate. The DPPH radical scavenging activity is vagued using the equation depicted by Brand-Williams et al[26].

% Radical scavenging activity = Ac − As /Ac × 100,

where as is the absorbance of the sample, and Ac is the absorbance of the control. A plot of the percentage of DPPH free radical inhibition vs extract concentration is used to calculate the half maximum inhibitory concentration (IC_{50}) of the extracts.

Results:

Morphological evaluation

Hippeastrum puniceum bulb is of spherical shape. It has large and white coloured with a thin scale of leaves on it. The diameter of the bulb is almost 6-10 cm. The bulb has no specific odour and has a bitter taste (Figure 1).

Figure 1: A) *Hippeastrumpuniceum* bulbs B) A single bulb with leaf scales and roots

Microscopical evaluation:

Transverse section:

The study of *Hippeastrum puniceum* bulb is done anatomically to understand the cellular details. As per the study, transverse section of the bulb shows presence of epidermis and cortex. The epidermis is single layered. The upper cortical region is comprised of polygonal cells with abundant starch grains. The lower cortical region holds polygonal cells with scattered starch grains along with vascular bundles. Acicular calcium oxalate crystals are present. The powder microscopy of the bulb is also studied.(Figure 2).

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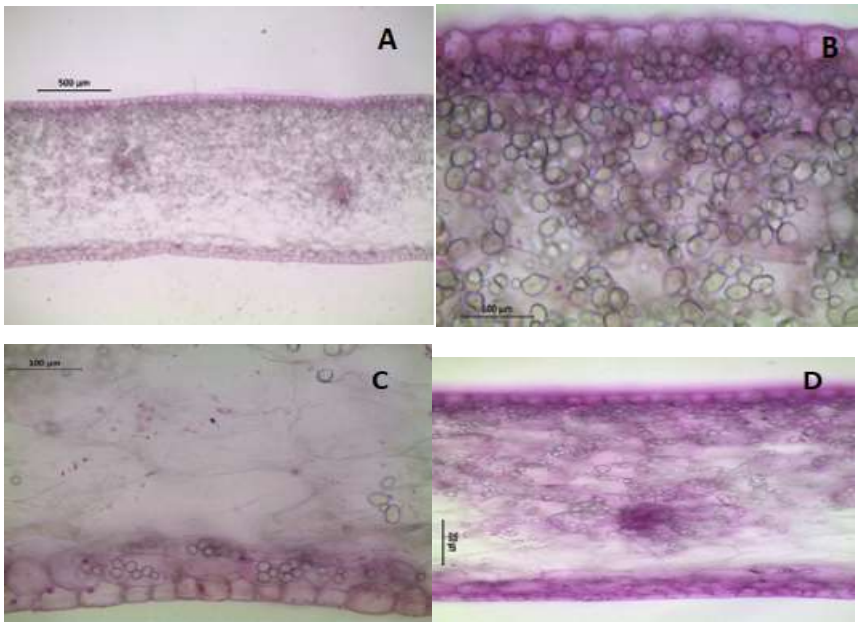

Figure 2: A) transverse section of the bulb with upper and lower epidermis B) Outer cortical region with abundant starch grains C) Lower cortical region with scattered starch grains D) Vascular bundles in cortex

Powder microscopy:

The powder is light brown in colour with bitter taste and no particular odour. The presence of epidermal cells, paranchyma cells with starch grains, parenchyma cells containing vascular strands, fragments of pitted vessel, fragments of spiral vessel, and presence of acicular calcium oxalate crystals are shown in the Figure 3.

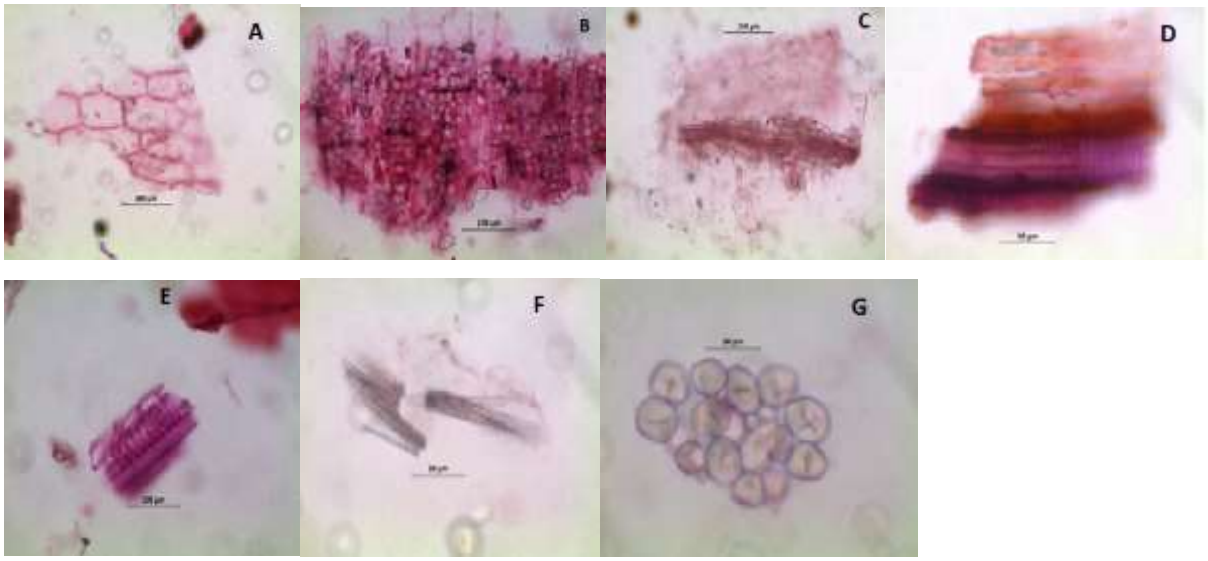

Figure 3: A) Epidermal cells B) Parenchyma cells with starch grains
C) Parenchyma cells containing vascular strands D) Fragments of pitted vessel
E) Fragments of spiral vessel F) Acicular calcium oxalate crystals G) Starch grains

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Fluorescence Analysis

The dried coarsely powdered bulb is dealt with various reagents and its colouration under visible light and UV light of short and longer wavelength is analysed. This is an important criterion to decide the authenticity of the powder of the traditional medicinal plants available in the market. The fluorescence characters of the bulb are noted in Table 1.

Table 1: Fluorescence properties of *Hippeastrum puniceum* bulb under visible, long and short wavelength

S. No	Experiment	Long wavelength (365nm)	Short wavelength (255nm)	Visible
1	Powder +Benzene	Colorless	Colourless	Colourless
2	Powder + Ethylacetate	Blue	Colourless	Colourless
3	Powder + Aceticacid	Fluorescent green	Light green	Pale yellow
4	Powder + $FeCl_3$	Darkbrown	Yellowish green	Pale brown
5	Powder + Ethanol	Whitish	Colourless	Colourless
6	Powder + Picricacid	Yellow	Bright yellow	Yellow
7	Powder + Petether	Colorless	Colourless	Colourless
8	Powder + Conc.HNO_3	Darkbrown	Palebrown	Brown
9	Powder + Conc.HCl	Darkbrown	Light brown	Light brown
10	Powder + Alcoholic KOH	Fluorescent green	Light green	Brown
11	Powder + $CHCl_3$	Fluorescent green	Yellowish green	Brown
12	Powder + Aqueous KOH	Fluorescent green	Yellowish green	Brown

Determination of antioxidant potential by FTC method

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Table 2. % Inhibition and IC_{50} values of total antioxidant activity of *Hippeastrum puniceum* bulb extracts

S.No	Concentration (µg/ml)	Standard (Ascorbic acid)	Petroleum Ether Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% inhibition	% inhibition	% inhibition	% inhibition	% Inhibition
1	12.5	22.31	15.57	24.23	15.23	20.16
2	25	30.11	26.89	30.29	28.08	36.68
3	50	47.54	30.10	46.10	47.92	54.43
4	100	69.90	37.34	52.60	58.21	78.81
5	200	94.67	48.56	60.16	64.22	90.14
6	**IC_{50} value**	**69.83**	**197.88**	**118.83**	**108.84**	**60.11**

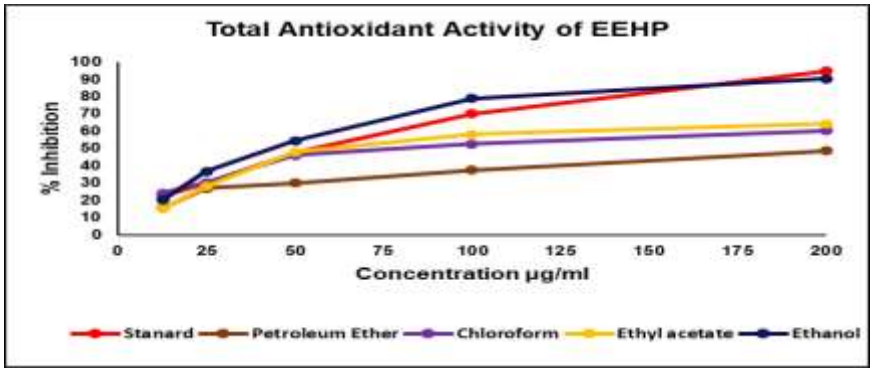

Figure 4. Total antioxidant activity of *Hippeastrum puniceum* bulb extracts

Table 2. % Inhibition and IC_{50} values of total antioxidant activity of *Hippeastrum puniceum* bulb extracts by DPPH Radical scavenging method

S.No	Concentration (µg/ml)	Standard (Ascorbic acid)	Petroleum Ether Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% inhibition	% inhibition	% inhibition	% inhibition	% Inhibition
1	12.5	40.95	13.48	22.45	31.65	24.21

2	25	53.10	27.50	27.20	38.19	37.45
3	50	66.61	31.30	33.76	49.34	51.03
4	100	78.69	38.56	45.01	56.65	68.91
5	200	88.21	49.82	53.13	61.15	79.12
6	**IC_{50} value**	**9.4**	**190.63**	**163.83**	**96.21**	**69.85**

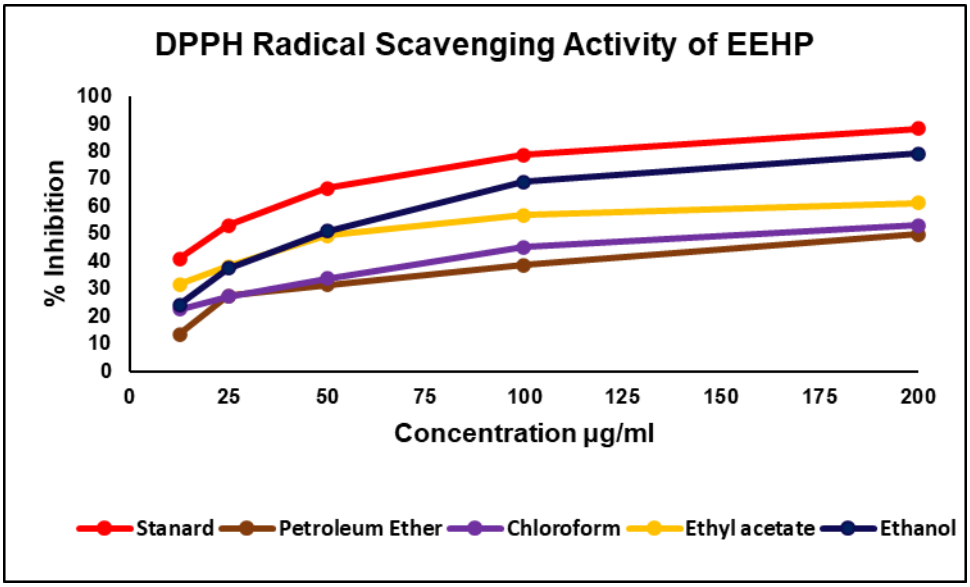

Figure 4. Total antioxidant activity of *Hippeastrum puniceum*bulb extracts by DPPH radical scavenging activity

DISCUSSION

Today many sophisticated modern investigation tools are available for the assessment of plant drugs, but microscopic method is still one of the simplest and cheapest methods to start for establishing the correct identity of the source materials. In our study, the morphological, microscopical and fluorescence analysis of the bulb of *Hippeastrum puniceum* is carried out. Morphological and histological studies enable us to identify the crude drug. The macroscopical characters of the bulb can be taken as diagnostic limitation. The presence of starch grains and polygonal cortical parenchyma cells is studied through the transverse section of the bulb. The powder of the bulb showed the presence of acicular calcium oxalate crystals, pitted vascular strands, and spiral vessels. The fluorescence analysis of the bulb powder is notable and could be used as an indicator to identify the adulteration in the crude drug powder.

Some techniques have been employed to determine the antioxidant activity in "*in-vitro*" to allow express screening of the substances. It is healthy shown that free radicals play a significant part in a wide range of clinical indications. Antioxidants save us from various diseases by fighting free radicals by either scavenging reactive oxygen species or protect the antioxidant defence mechanisms to carry out their effect. The total antioxidant activity is estimated by using the thiocyanate method. The total antioxidant capacity of the ethanol extract of *Hippeastrum puniceum* bulb is found to be higher when compared to other extracts used. The IC_{50} values of total antioxidant capacity of standard ascorbic acid, petroleum ether extract, chloroform extract, ethyl acetate extract, and ethanol extract are found to be 69.83 µg/ml, 197.88µg/ml, 118.83µg/ml, 108.84µg/ml, and 60.11µg/ml respectively. Among the different extracts used in the study, ethanol and chloroform extracts showed significant dose-dependent antioxidant activity and the DPPH radical can be used as a model for a lipophilic radical chain reaction, initiated by the lipid auto-oxidation. EEHP showed significant dose-dependent DPPH radical scavenging activities which are stated in Table 2 and Figure 2. Percentage scavenging activity or percentage inhibition is calculated using the linear regression method. The IC_{50} values of DPPH radical scavenging activity of standard ascorbic acid, petroleum ether extract, chloroform extract, ethyl acetate extract, and ethanol extract are found to be 9.4µg/ml, 190.63µg/ml, 163.83 µg/ml, 96.21µg/ml, and 69.85µg/ml respectively.

CONCLUSION

To conclude, the objective of this investigation was to establish certain criteria that may be used to determine the authenticity of the potential of this plant as a medicine. An important source of information to be concerned in the treatment of many illnesses comes from the microscopic analysis and antioxidant property determinations of this plant. According to the data, the ethanolic extract has a remarkable antioxidant capacity. It is advised that more research could be done with the goal of isolating and characterizing the pure phytoactive components. To examine the safety of the ethanolic extract, toxicology tests should be carried out.

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