

Pharmacognostic, Physicochemical and Antioxidant Screening of *Hibiscus hirtus*

Rajeswari Pullabhatla^{*1}, Saripilli Rajeswari², Padmaja Kota³

¹Maharajah's College of Pharmacy, Vizianagaram, Andhra Pradesh.

²Raghu College of Pharmacy, Vizianagaram, Andhra Pradesh.

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ABSTRACT

Objective: Aim of the present study was to perform pharmacognostic, phytochemical evaluation and antioxidant screening of ethanol extracts of *Hibiscus hirtus*. Methods: The free radical scavenging activity of ethanolic extract of *Hibiscus hirtus* was measured by DPPH (1, 1-diphenyl-2-picryl-hydrazil) employing the method described by Blois, 1958. 0.1 mM solution of DPPH in ethanol was prepared and 1 mL of this solution was added to 3 mL of various concentrations (50, 100, 150, 200, 250, 500, 750 and 1000 µg/mL) of methanol extract. After 30 min, absorbance was measured at 517 nm. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Ascorbic acid was used as a reference compound.

Keywords: *Hibiscus hirtus*, antioxidant activity, DPPH method.

INTRODUCTION

Hibiscus hirtus (*H. Hirtus*) belongs to *Malvaceae* family is an evergreen small herb under shrub. Height of the plant is 1-1.5 m high, stems are erect, pubescent with simple stiff minute stellate hairs, leaves are alternate, flowers are axillary, solitary or seemingly in racemes or panicles by reduction of upper leaves, tap root and branched.

Phyto chemical constituents

The plant leaves and stems contain stigmaterol, γ -sitosterol, β -sitosterol, taraxeryl acetate and 3-cyclopropane compounds and their derivatives.

Flowers contains cyanidin, diglucoside, flavonoids and vitamins, thiamine, riboflavin, niacin, and ascorbic acid. Quercetin-3-diglucoside, 3, 7-diglucoside, cyanidin 3, 5 diglucoside and cyanidin-3-sophoroside-5-glucoside.

Ovary of white flowers all the above compounds and kaempferol-3-xylosyl glucoside.

Medicinal uses

Plant flowers are used as refrigerant, emollient and emmenagogue aphrodisiac and its decoction given for the treatment of bronchial catarrh. Infusion of petals is a refrigerant drink in fever. It is also for demulcent in cough useful in strangury, cystitis and other genito urinary track troubles. Oils from fresh petals and olive oil in equal proportion find application in alopecia.

Leaves are emollient, anodyne, aperients, juice of leaves used for gonorrhoea. Buds have bitter taste, cooling and astringent effect and remove burning of the body, urinary discharges promote the growth of foetus. Leaf extract acts as tolbutamide.

Pharmacological activities

The pharmacological activities of extracts of *H. Hirtus* are anti-inflammatory, anti bacterial, anti-fungal and antioxidant activity.

METHODS

Plant materials

Fresh entair part of *H. Hirtus* were collected from vicinity of vizianagaram district of andhra pradesh, identified and authenticated by Prof. S. B. Padal Msc. Ph D. PGDCA Professor in Botany department, Andhra University, Visakhapatnam.

Macro and Microscopic Evaluation

The macroscopic evaluation involves study of morphological characters and organoleptic studies like colour, odour, taste, texture etc.

In microscopic evaluation, studies were conducted qualitatively. All the microscopic evaluation was performed under microscope. Photomicrographs were taken using digital camera model No. DSC - W510.

Physicochemical and phytochemical studies.

Physicochemical and phytochemical studies like extractive values, total ash, acid insoluble ash, acid insoluble ash, total sugar, starch, tannins and phenols were calculated from the shade-dried and powdered (60 # mesh) plant material.

Extraction of plant material for in vitro Antioxidant activity.

Freshly collected plant material was shade dried at room temperature and coarsely powdered and subjected to extraction with ethanol by simple maceration process for seven days. This process consists of keeping the crude drug in intimate contact with whole of the menstrum in a closed vessel with occasional shaking for seven days,

straining, pressing the marc, mixing the liquids, and finally clarifying by subsidence or filtration. Then

minuted by steam distillation and then dried properly.



Figure 1: Hibiscus hirtus entire plant.



Figure 2: Flower of *H. hirtus*.



Figure 3: Stellate type trichomes.

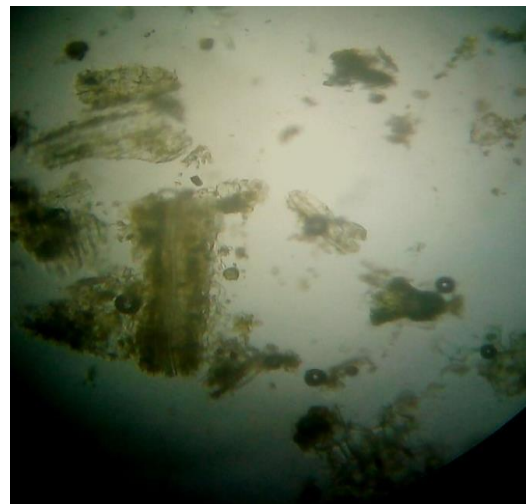


Figure 4: Parenchymatous cells and fibers.

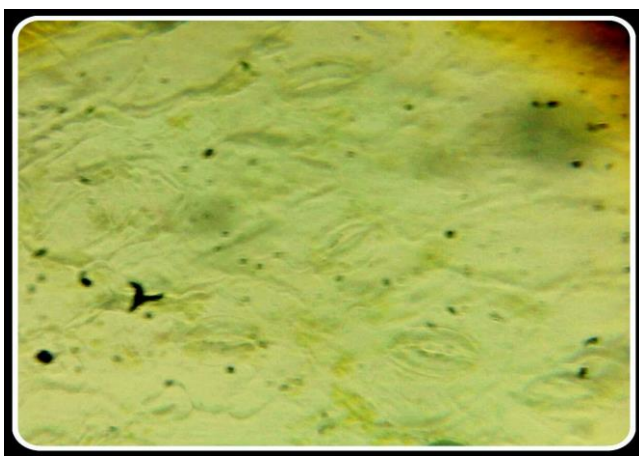


Figure 5: Paracytic stomata.

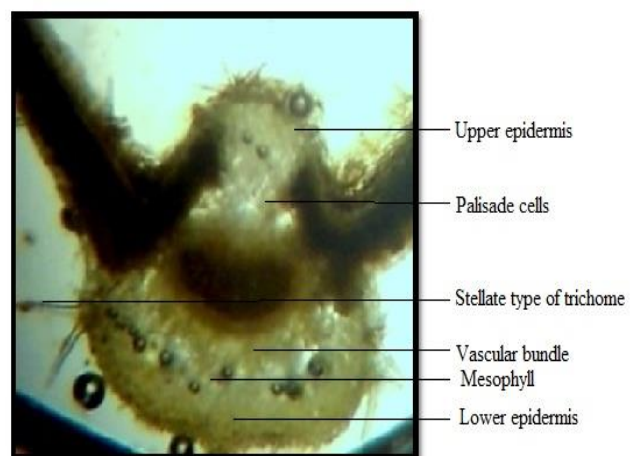


Figure 6: T.S of *H. hirtus* leaf



Figure 7: Tap root system.

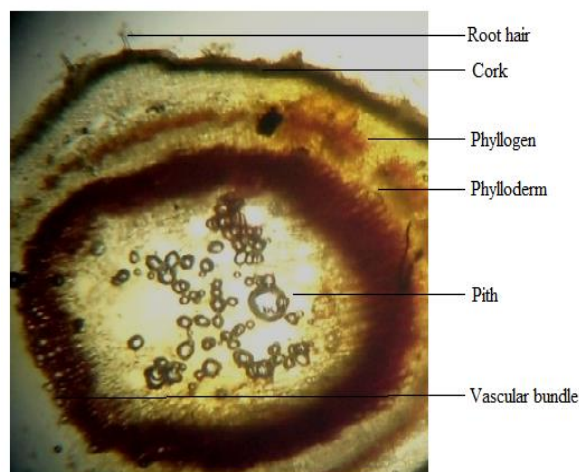


Figure 8: T.S. of *H. hirtus* root

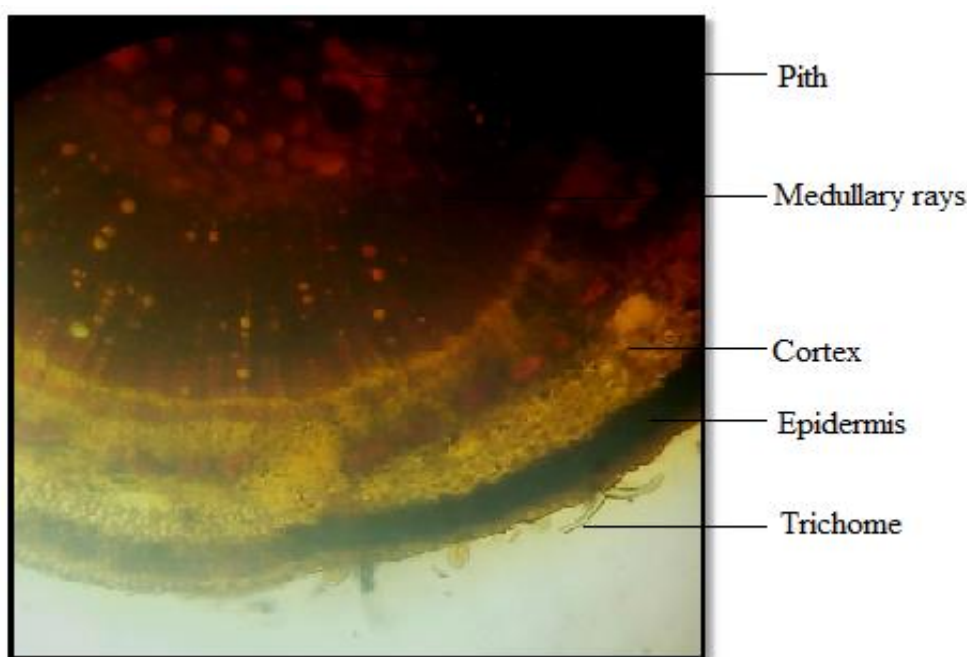


Figure 9: T.S. of *H. hirtus* stem.

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The free radical scavenging activity of ethanolic extract of *H. Hirtus* was measured by DPPH (1, 1-diphenyl-2-picryl-hydrazil) employing the method described by Blois, 1958. 0.1 mM solution of DPPH in ethanol was prepared and 1 ml of this solution was added to 3 mL of various concentrations (50, 100, 150, 200, 250, 500, 750 and 1000 µg/mL) of methanol extract. After 30 min, absorbance was measured at 517 nm. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Ascorbic acid was used as a reference compound. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ DPPH Scavenging activity} = \frac{(A \text{ Control} - A \text{ test})}{A \text{ Control}} \times 100$$

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. The antioxidant activity of the extract was expressed as IC₅₀. The IC₅₀ value was defined as the concentration (µg/mL) of extracts that inhibits the formation of DPPH radicals by 50 %.

Determination of Superoxide Radical Scavenging Activity:

Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical.

Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan.

100 µl of Riboflavin solution [20 µg], 200 µl EDTA

Table 1: Describes the physicochemical properties and its values.

S. No.	Physiochemical Properties	Values
A	Organoleptic Characters	
1	Colour	Dark green
2	Odour	characteristic
3	Taste	Bitter
B	Extractives	
1	Water soluble extractives	0.36g
2	Alcohol soluble extractives	0.28g
3	Ether soluble extractives	0.05g
C	PHYSIOCHEMICAL PROPERTIES	
1	Moisture content	0.1%
2	Bulk density	0.19
3	Tapped density	0.3
4	Carr index	37
5	Hausner Ratio	1.578
6	Ph of the suspension	5.5
D	Ash value	
1	Total ash	0.37gm
2	Acid soluble ash	0.02gm
3	Water soluble ash	0.04gm

Table 2: Chemical tests for identification of chemical constituents present in *H. hirtus*.

S. No.	Name of the test	Alcoholic extract of <i>Hibiscus hirtus</i>
1.	Test for steroids	+
2.	Test for triterpenes	+
3.	Test for saponins	+
4.	Test for steroidal saponin	+
5.	Test for glycosides	+
6.	Test for alkaloids	-
7.	Test for carbohydrates	-
8.	Test for flavonoids	+
9.	Test for tannins	+
10.	Test for phenols	+
11.	Test for irioids	-
12.	Test for cardiac glycosides	-
13.	Test for coumarins	-
14.	Test for fixed oils and fats	-
15.	Test for mucilage	+
16.	Test for resin	-
17.	Test for carboxylic acid	-
18.	Test for quinones	-
19.	Test for xanthoproteins	-
20.	Test for anthocynin	-
21.	Test for leucoanthocyanins turns	-
22.	Test for emodins	-
23.	Test for phlobatannins	-

solution [12 mM], 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution [0.1 mg] were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer [50 mM]. The absorbance of solution was measured at 590 nm using phosphate buffer as blank

Table 3: Concentration dependant percent inhibition on DPPH free radical by *Hibiscus hirtus* extract and ascorbic acid *in vitro* studies.

Concentrations (µg/ml)	Percentage inhibition on DPPH radical quantity of extracts/ascorbic acid in micrograms (µg/ml)	% Inhibition of test	% Ascorbic acid
50	19.9	17.77	
100	31	30.71	
250	40.2	50.22	
500	69	71.74	
750	75.4	82.6	
1000	85.7	90.12	

Where: All values expressed as mean± S.D, (n=3)

IC_{50%} of ascorbic acid = 250 µg/mL and IC_{50%} of ethanolic fraction = 300 µg/mL

after illumination for 5 min. This is taken as control 50 µl of different concentrations of coumarin compounds as well as standard preparation were taken and diluted upto 100 µl with methanol. To each of these, 100 µl riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl. NBT was mixed in test tubes and further diluted up to 3 mL with phosphate buffer.

Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. The IC₅₀ values for each compound as well as standard preparation were calculated⁹⁻¹⁷.

Hydroxyl radical scavenging activity:

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and various extract for hydroxyl radical generated by Fe³⁺ Ascorbate-EDTA- H₂O₂ system (Fenton reaction) according to the method of Kunchandy and Rao *et al.* (1990).

The reaction mixture contained in a final volume of 1.0 mL, 100 µl of 28 mM 2-deoxy-2- ribose in 20 mM KH₂PO₄-KOH buffer of pH 7.4, 500 µl of the selected concentrations of extract (50, 100, 150, 200, 250, 500, 750 and 1000 µg/mL) in KH₂PO₄-KOH buffer (20 mM, pH 7.4), 100 µl of 1.04 mM EDTA, 100 ml 200 mM FeCl₃, 100 µl of 1.0 mM H₂O₂ and 100 µl of 1.0 mM ascorbic acid was incubated at 37 °C for 1 h. 1.0 ml of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8 %) were added to the test tubes and were incubated at 100 °C for 20 min. After cooling, absorbance was measured at 532 nm against control containing deoxyribose and buffer. Ascorbic acid was used as a positive control. Reactions were carried out in triplicate. The percentage inhibition was determined by comparing the results of the test and control compounds.

RESULTS AND DISCUSSION

Macroscopic Evaluation

The organoleptic features of the leaves indicate dark green colour on upper surface and light green colour on lower surface. The powder of leaves appeared green in

colour, coarse in texture, slightly aromatic and unpleasant in odour. The stem was brown in colour with bitter taste.

The morphological characters of leaves were observed as leaves are alternate, lower ones ovate, rounded or cuneate

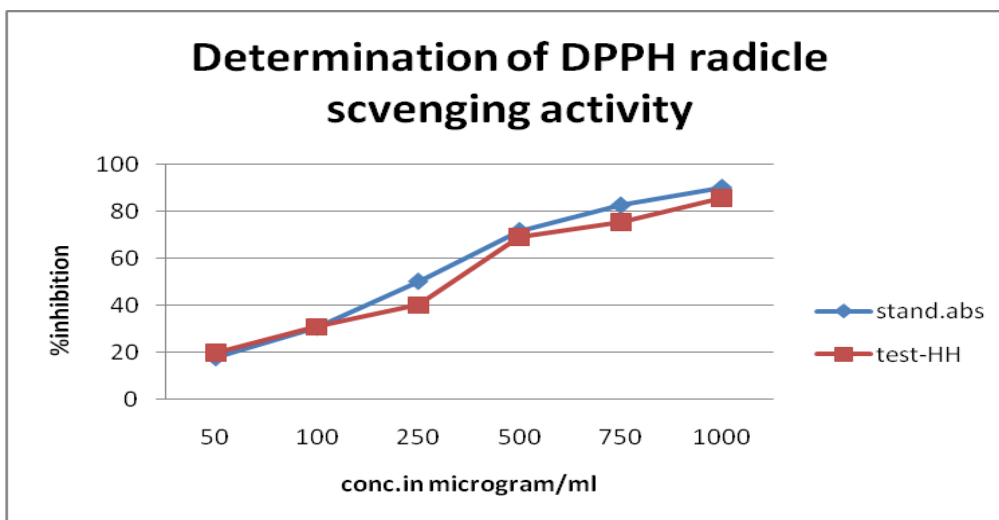


Figure10: Determination of DPPH free radical scavenging activity.

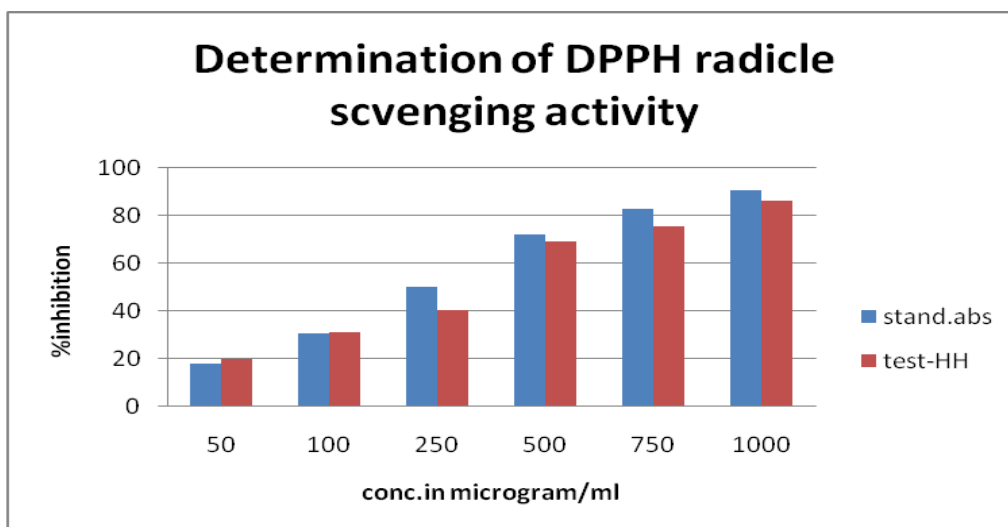


Figure 11: Bar diagram representation of DPPH free radical scavenging activity.

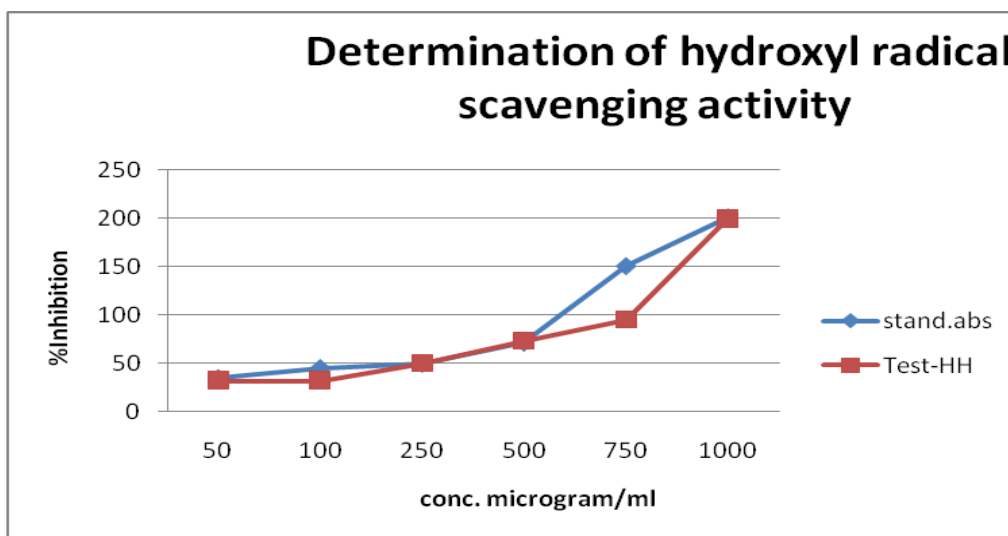


Figure 12: Determination of DPPH hydroxyl radical scavenging activity.

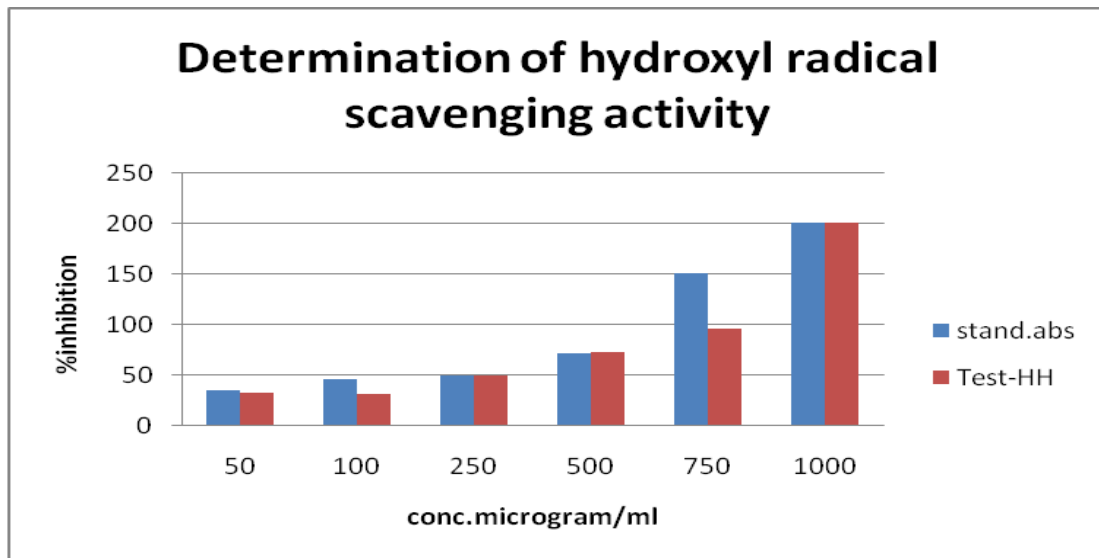


Figure 13: Bar diagram representation of DPPH hydroxyl radical scavenging activity.

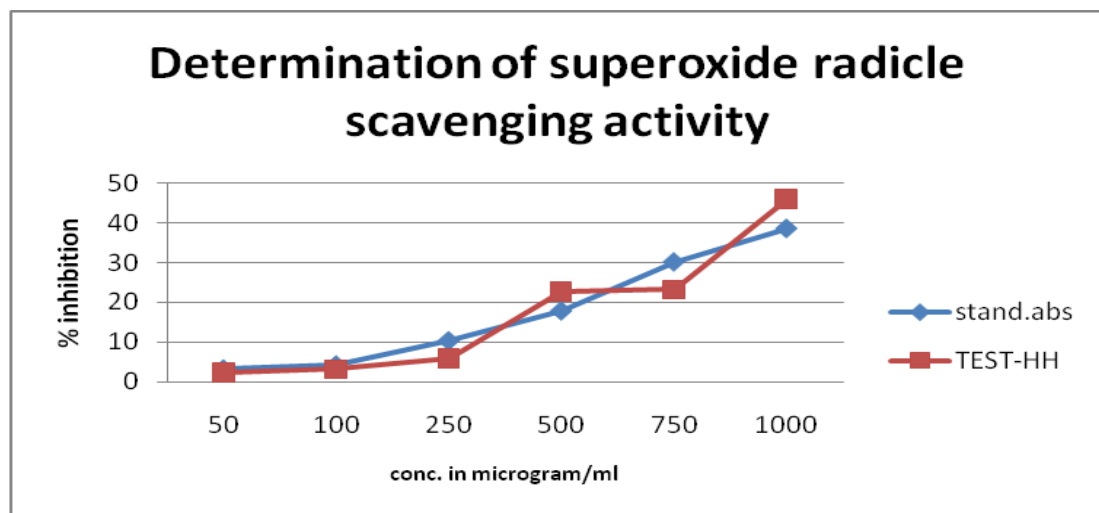


Figure 14: Determination of DPPH superoxide radical scavenging activity.

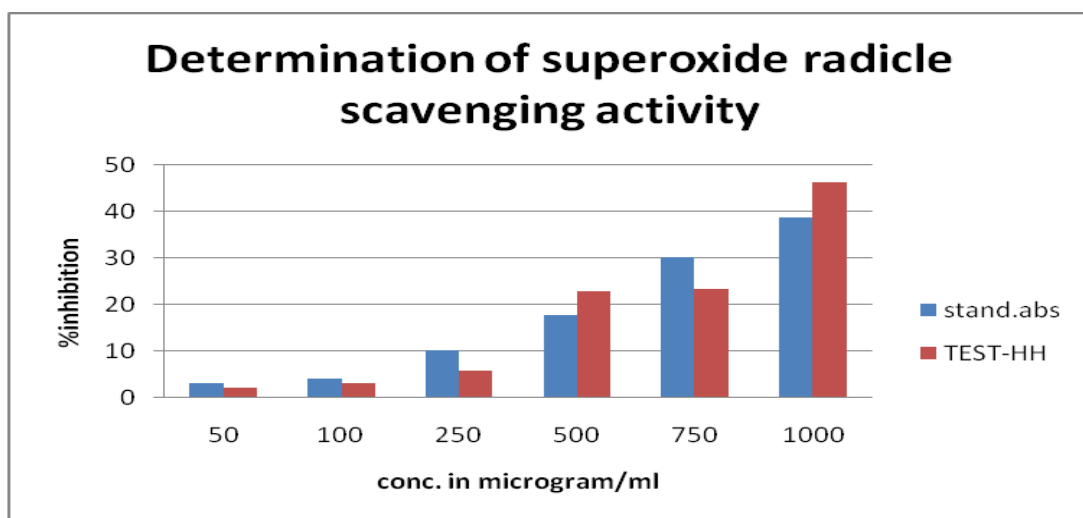


Figure 15: Bar diagram representation of DPPH superoxide radical scavenging activity.

Table 4: *In vitro* concentration dependent percentage inhibition of hydroxyl radical scavenging activity by extracts of *Hibiscus hirtus* and Ascorbic acid as standard.

Concentrations (µg/ml)	Percentage inhibition on Hydroxyl radical scavenging activity of extracts/ascorbic acid in micrograms(µg/ml)	% Inhibition of % of ascorbic acid test
50	32.3	35.5
100	32	45.8
250	49.8	50.22
500	73.2	71.74
750	95.5	150.6
1000	200	200.8

All values expressed as mean+ S.E.M of 3 samples
 $IC_{50\%}$ of ascorbic acid = 200µg/mL and $IC_{50\%}$ of ethanolic fraction =250 µg/mL

at base, crenate-serrate or irregularly toothed at margin, acute to acuminate at apex, 3-6 x 2-3 cm, 3-5 nerved at base, often with an oblong nectary on midnerve beneath, minutely pubescent with stellate hairs; petioles 0.5-1.5 cm long, with long stellate and simple hairs; stipules linear, 2-8 mm long, ciliate, shown in Fig. No. 1.

Flowers: flowers are axillary, solitary or seemingly in racemes or panicles by reduction of upper leaves; pedicels longer than petioles, 0.5-2 cm, accrescent to 5 cm, jointed below or above middle, pubescent. Epicalyx lobes 6-9, free, lanceolate to linear or subulate, acute, 3-8 x 0.5-1 mm. Calyx 1.5 cm across, 5-fid or parted; lobes divided nearly to base, linear-lanceolate, hairy, persistent, shown in Fig. No. 2.

Corolla rotate, pink or white. Petals obovate, 1-1.5 x 0.5-0.8 mm, rounded at apex. Staminal column shorter than or as long as petals. Ovary subglobose, 2 mm across; stigmas capitate. Capsules globose, 7-10 mm, shorter than calyx; seeds 2 or 3 in each locule, reniform, 2 mm across, densely covered with long woolly hairs

3.2 Microscopic Evaluation

To determine the size, shape and relative positions of the different cells and tissues

To determine the chemical nature of the cell walls

To determine the form and chemical nature of the cell contents

To identify the crude drug by microscopical or histological characters

The organoleptic characters like color, odour and taste, external features are useful in the identification of crude drug in addition to the microscopical characters and chemical tests. However identifying the crude drugs only on the basis of organoleptic characters is slightly misleading. So powder analysis by microscopical examination is an important phenomenon in identifying the describing the crude drug.

For systematic study, a set of 3 slides must be prepares.

Powdered crude drug mounted in dilute iodine solution for the identification of starch grains.

Powdered crude drug mounted in either chloral hydrate solution or lactophenol solution for locating the presence of calcium oxalate crystals.

Powdered crude drug mounted in alcoholic solution of phloroglucinol and hydrochloric acid for identification of lignified tissues.

Stellate type of trichome was shown in Fig. No.3

Parenchymatous cell fibres was shown in Fig. No. 4

Stomata type: Paracytic type of stomata was shown in Fig. No. 5

T.S. of leaf: Was described clearly in Fig. No.6.

T.S. of root: Roots are tap root system was shown in Fig. No.7. and T. S. of root was given in Fig. No. 8.

T. S. STEM:

Physico-chemical properties

Organoleptic evaluation

Organoleptic evaluation refers to evaluation of individual drugs and formulations by color, odor, taste, texture, etc.¹. The organoleptic characters of the samples were carried out based on the method as described by Wallis². For determining the odor of an innocuous material, small portion of the sample was placed in the beaker of suitable size, and examined by slow and repeated inhalation of the air over the material. If no distinct odor was perceptible, the sample was crushed between the thumb and index finger, between the palms of the hands, using gentle pressure or if the material was known to be dangerous, by other suitable means such as pouring a small quantity of boiling water onto the crushed sample placed in a beaker. First, the strength of the odor was determined (none, weak, distinct, strong) and then the odor sensation (aromatic, fruity, musty, mouldy, rancid, etc.) was studied. Taste was distinctively classified as aromatic, pungent, sweet, sour, astringent, mucilaginous, or bitter.

Extractive values³

Water soluble extractives: Five grams of coarsely powdered air-dried drug was macerated with 100 mL of water in closed conical flask for 24 hr, shaken frequently for the first 6 hr and allowed to stand for 18 hr. This was filtered through Whatman filter paper grade No.100. Twenty-five milliliters of the filtrate was evaporated to dryness in petri dish, dried at 105 °C and weighed. Percentage of water soluble extractive with reference to air-dried material was calculated.

Alcohol soluble extractives: Five grams of air-dried and coarsely powdered drug was macerated with 100 mL of 70% ethanol in a closed conical flask for 24 hr, shaken frequently during the first 6 hr and allowed to stand for 18 hr. This was filtered rapidly taking precaution against loss of ethanol. Twenty-five milliliters of the filtrate was evaporated to dryness in a petri dish, dried at 105° C and weighed. Percentage of alcohol soluble extractive was calculated with reference to air-dried drug.

Ether soluble extractives: Five grams of air-dried and coarsely powdered drug was extracted with ethyl ether in a soxhlet extractor for 20 hr. The ether extract was transferred in a petri dish and allowed to evaporate. It was dried at 105° C to constant weight. Percentage of ether soluble extractive was calculated with reference to air-dried drug.

FTable 5: *In vitro* concentration dependent percentage inhibition of superoxide radical scavenging activity by extracts of *Hibiscus hirtus* and Ascorbic acid as standard.

Concentrations (µg/mL)	Percentage inhibition on superoxide radical scavenging activity of extracts/ascorbic acid in micrograms (µg/mL)	
	% Inhibition of test	% of ascorbic acid
50	2.3	3.2
100	3.2	4.2
250	5.9	10.3
500	22.7	17.8
750	23.2	30.1
1000	46	38.5

All values expressed as mean+ S.E.M of 3 samples
 $IC_{25\%}$ of ascorbic acid = 600µg/mL and $IC_{25\%}$ of ethanolic fraction =500 µg/mL

Physicochemical properties

Physical characteristics like moisture content, bulk density, tap density, angle of repose, Hausner's ratio and Carr's index were determined for different formulations⁴.

Moisture Content⁵

The shade-dried drug was grounded in a mixer grinder. The powder passed through #40 and retained on sieve No. #120. Accurately weighed 10 g of # 40/120 drug powder was kept in a tared evaporating dish. This was dried at 105°C for 5 hours in tray drier and weighed. The drying was continued and weighing was done at one-hour interval until difference between two successive weighings corresponds to not more than 0.25 percent. Drying was continued until a constant weight was reached with two successive weighings after drying for 30 min and cooling for 30 min in a desiccator was showing not more than 0.01 g difference.

Bulk Density and Tapped Density⁶

In the present study, we had taken the weighed quantity (30 gm) of shade-dried and presieved (#40/120) different drugs, marketed and in-house formulation powders and carefully added them to a cylinder with the aid of a funnel without any losses. The initial volume was noted and the sample was then tapped until no further reduction in volume was noted. The initial volume gave the bulk density value and after tapping the volume reduced, giving the value of tapped density.

Carr's Index

Carr's index has been used as an indirect method of quantifying powder flow ability from bulk density; this method was developed by Carr. The percentage compressibility of a powder is a direct measure of the potential powder arch or bridge strength and stability, and is calculated according to following equation.

$$\text{Carr's Index} = 100 \times \left(1 - \frac{D_b}{D_t}\right) \quad \text{Eq. 2}$$

Where D_b = Bulk density, D_t = Tapped density

Hausner's Ratio: Hausner ratio has been also used as indirect method of quantifying powder flowability from

bulk density. Hausner ratio = D_t / D_b . Where D_b = Bulk density and D_t = Tapped density.

pH of suspension of the drugs: pH of freshly prepared 1% w/v suspension and 10% w/v suspension in distilled water was determined using simple glass electrode pH meter.

Ash values [7]

Total ash

Two grams of grounded air-dried material was accurately weighed in a previously ignited and tared silica crucible. The drug was gradually ignited by raising the temperature to 450°C until it was white. The sample was cooled in a desiccator and weighed. The percentage of total ash was calculated with reference to air-dried drug.

Acid Insoluble ash

The ash was boiled with 25 mL of 2 M hydrochloric acid for 5 min, the insoluble matter was collected on an ash less filter paper, washed with hot water, ignited, cooled in a desiccator and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Water soluble ash

The ash was boiled with 25 mL of water for 5 min, the insoluble matter on ash less filter paper collected, washed with hot water, ignited, cooled in a desiccator and weighed. The weight of the insoluble matter from the weight of the total ash was subtracted; the difference represents the water soluble ash. The percentage of water insoluble ash was calculated with reference to the air-dried drug.

RESULT

The extractive values of water soluble, alcohol soluble and ether soluble were given Table No. 1.

Based on the above result obtained in physicochemical properties:

Organoleptic characteristics

The powder which is formulated in the into mixture form is dark green in colour with characteristic odour but intensely bitter in taste. Hence we have to use additives for which to mask the taste.

Extractive value

Water soluble extractive values play important role in evaluation of crude drugs. In our mixture of crude drug the water soluble part will be more when compared to alcohol soluble part.

The petroleum ether soluble extractive is very less when compared to alcohol soluble portion.

So for better yield of extractive we can go for water soluble part but we have to use preservatives.

Other wise it is better to go for hydroalcoholic or alcoholic extract.

Moisture content

Moisture is one of the major factors responsible for the deterioration of drugs and formulations. Low moisture content is always desirable for higher stability of drugs. The moisture content will be less than 10%.

Bulk Density, Tapped Density, Carr's Index And Hausner Ratio

Study of bulk density and tapped density are important of a powder defines its packing. The tapped density gives

information on consolidation of a powder. A consolidated powder is likely to have a greater arch strength than a less consolidated one and may therefore be more resistant to powder flow.

The Carr's index and Hausners ratio were 37 and 1.5 which indicates good compressibility.

pH of suspension of the drug

The pH value of 1% and 10% suspension of the drugs were in the range of 4-6 which indicates stable for human use.

Ash value

A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the drug or drug combinations for marketing. The drug was found to have total ash is 0.37. The water soluble ash were found is 0.02 and the alcohol soluble ash is 0.04.

Chemical tests

Various chemical tests were performed to identify the chemical constituents present in *Hibiscus hirtus* and are given in Table No. 2.

Antioxidant activity

Calculation of 50% inhibition concentration: The graph was plotted by taking concentration on X-axis and percentage inhibition on Y-axis, the graph was extrapolated to find the 50% inhibition concentration of the sample. DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances, the values were given in Table No. 3 and shown in Fig. No. 10 and 11.

DPPH has been widely used to evaluate the hydroxyl radical scavenging effectiveness of various antioxidant substances, the values were given in Table No. 4 and shown in Fig. No. 12 and 13.

DPPH has been widely used to evaluate the superoxide radical scavenging effectiveness of various antioxidant substances, the values were given in Table No. 5 and shown in Fig. No. 14 and 15.

IC_{50%} of ascorbic acid is 250 µg/ml, IC_{50%} of ethanolic extract is 300 µg/ml. The IC₅₀ values of ethanolic extract and the standard (Ascorbic acid) was compared and here in the study it reveals that ethanolic fraction has nearly equal to the ascorbic acid.

DISCUSSION

Ayurveda and naturopathy the medical system indigenous to India advocates the plant extract/mixture of extracts for treating various disorders apart from others from times immemorial in humans without preclinical evidence, which is required to make the systems popular and scientific. The claimed usefulness of herbs in several disorders might be due to their antioxidant activity.

To support the use of the selected plant extracts in traditional use, in ayurveda and naturopathy, the antioxidant potential of the total ethanolic extract of *Hibiscus hirtus*, was investigated in comparison with the known antioxidant ascorbic acid (AA) following *in vitro* studies. The antioxidant activity of ascorbic acid was well established.

Herbal drugs containing antiradical constituents are gaining importance in prevention and treatment of stress related disorders. The free radical scavengers like poly phenolics are well known for their therapeutic activity in disorder such as cancer, diabetes and skin diseases.

The terpenoids are reported to protect lipids, blood and body fluids against the attack of radicals, some types of reactive oxygen, hydroxyl groups, and peroxide and super oxide radicals. It was found that potential of the extract to scavenge oxygen radicals depends on the type of radicals encountered.

Phytochemical studies on the *Hibiscus hirtus* revealed terpenoids, flavanoids and steroids. The presence of above compounds in various extracts might be responsible for the observed antioxidant activity.

Since reactive oxygen species are involved in stress and stress related disorders, the extract of these plants may be beneficial in preventing the initiation or progression of such disorders.

3.6. Conclusion:

Based on the above discussion, it was found that the formulation prepared with these powdered drug gives good dosage form preparation, which indicates the these powers nearly have the same antioxidant activity shown by ethanolic extract of *Hibiscus hirtus* when compared to ascorbic acid.

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