

EVALUATION OF PHARMACOGNOSTICAL CHARACTERS, PHYTOCHEMICAL SCREENING, AND *IN-VITRO* ANTIBACTERIAL ACTIVITY OF *Clematis buchananiana* DE CANDOLLE (RANUNCULACEAE)

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ABSTRACT

Phytotherapy is used as a complementary approach to the prevention and treatment of many diseases. *Clematis buchananiana*, Family: Ranunculaceae (Buttercup family) possess medicinal value and are used as an anti-inflammatory, for peptic ulcer, treatment of ingestion, applied externally to cuts and wounds and sinusitis. The purpose of the present study is to establish the standardized identification parameters such as pharmacognostic characteristics and phytochemical composition, antioxidant, and antibacterial activities of *Clematis buchananiana* leaves extract. Pharmacognostical analysis indicates that the leaf surface is oval, hairy, and pilose, and has an acute apex. Microscopic characteristics reveal the upper epidermis has a single layer with a thin cuticle and inter-spread with numerous actinocytic stomata and unicellular and multiseriate trichomes. The Stomatal number: 168-192 (lower epidermis), Stomatal index: 14.28 %, Vein- Islet number: 48-54 and Vein termination number: 24-30 respectively. The extractive values for alcoholic and water-soluble were found to be 6.8% w/w and 4.5% w/w respectively. The phytochemical screening of extracts exhibited the presence of alkaloids, carbohydrates, saponins and proteins. The methanolic extracts of leaves of *C. buchananiana* at a concentration of 500µg/ml showed high potency against gram-negative bacteria such as *Salmonella typhi* and *Escherichia coli*. The extract showed maximum scavenging activity of 67.89 and 59.67 respectively against Ascorbic acid with an IC₅₀ value is 48µg/ml. The different phytoconstituents mainly alkaloids, carbohydrates, saponins, amino acids, and proteins could be responsible for the *In-vitro* antibacterial and antioxidant potentials of leaf extract.

Keywords: *Clematis buchananiana*, Standardization, Antioxidant, Phytoconstituent Profiling, Antibacterial, Chromatography.

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INTRODUCTION

Modern medicine has today displaced plants in many countries with many synthetic products, but nearly 30 percent of pharmaceutical preparations are still obtained from plants directly or indirectly. Modern times have seen a decline in the use of medicinal plants and their extracts as therapeutic agents, especially in developing countries, many of which have either been rejected by the medical profession or are now administered in the form of isolated compounds.¹ The strategy of isolating the active principles from the medicinal plants and manufacturing a pharmaceutical preparation then became popular.² In many developing countries, including India, modern medicines and herbal medicines are being used complementarily in healthcare system areas. The interest in plant products emerges worldwide late because of the assumption that many herbal drugs are considered to be free from side effects. It is a fact that the discovery of a new synthetic drug takes time and is a costly affair.³ The effectiveness of the synthetic drug is often followed by its single or multiple adverse effects, and the curatives are not

available in some situations. Herbs have been used throughout history by all cultures but India has one of the oldest, richest, and most complex cultural practices associated with the use of medicinal plants. The market for herbal products is rising exponentially around the world in the current scenario and major pharmaceutical companies are currently conducting comprehensive research on plant materials for their possible medicines.^{4,5} The number of scientific publications focused on herbal drugs is rising in many national and international journals. Plants have provided a wide range of effective medicines for humanity to relieve disease suffering following remarkable developments in synthetic drugs in recent years, some of the drugs used from Plant origin still maintain their use of plant-based medicines is on the increase throughout the world. Given the tremendous advances made in modern medicine, there are still a large number of disorders that need to be found for successful medicinal products.⁶ There is an urgent need today for the development of safer drugs to treat inflammatory disorders, diabetes, liver diseases, and gastrointestinal disorders. Therefore, there is growing interest in the pharmacological evaluation of different plants used in conventional medicinal systems in India. However, the folkloric use of synthetic medicines is mostly observational and is based on observation without scientific support from clinical trials. One cannot overemphasize the need for comprehensive systematic research into indigenous drugs.⁷

EXPERIMENTAL

Methods and Chemicals

Dragendorff's reagent, Hager's reagent, sulphuric acid, sodium hydroxide, Fehling reagent-A and B, zinc dust, ferric chloride, alcohol, acetic acid, ethyl alcohol, chloral hydrate, toluidine blue, phloroglucinol, glycerine, hydrochloric acid and all other chemicals used in this study were of analytical grade.

Collection, Identification, and Authentication of the Plant Material

The leaves of the plant *Clematis buchananiana* are collected in the month of August from the selected geographical area.

Morphological Analysis

The morphological analysis was performed in pharmacobotany and anatomic characteristics of young leaves of *Clematis buchananiana*. The adult leaves were collected in three replicates to accomplish this study.^{8,9}

Microscopic Analysis

Transverse Section (T.S) of the fresh leaf of *Clematis buchananiana* midrib region was cut and fixed in FAA and then dehydrated by employing graded series of ethyl alcohol and tertiary butyl alcohol.¹⁰ Sections were taken using a microtome. A permanent mount was prepared using safranin fast green double staining technique.^{11,12}

Powder Microscopy

The coarse powder of the leaf was used to study the microscopical characteristics of the leaf powder.^{13,14}

Physicochemical Analysis

Extractive values, moisture contents, total Ash value, and leaf constants such as vein islet numbers, vein terminal numbers, stomatal numbers, and stomatal indexes were determined.^{15,16}

Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out to find out the presence of various Phytoconstituents using standard procedure.^{17,18}

Chromatographic Studies

Thin Layer Chromatography (TLC) of Methanolic Extracts

Pre-coated silica gel G plates were used in this study which acts as a stationary phase. 50 mg of the dried methanolic extract was dissolved in 50 ml of methanol. 10 µl of this solution was loaded on TLC plates using 2 µl capillary tubes. The thin layer chromatograms were developed using hexane: ethyl acetate (8:2) as the mobile phase. The thin layer chromatograms were visualized under short UV i.e. UV 254 nm. After the spots were visualized and labeled, their retardation factors (R_f value) were calculated.¹⁹ The R_f values were calculated according to the following formula:

$$R_f = \frac{\text{Distancetravelledbythesample}}{\text{Distancetravelledbythesolventfront}}$$

Pharmacological Study

Antioxidant Activity

3ml of reaction mixture containing 0.2 ml of a 0.004% methanol solution of 1,1,2- DPPH and 2.8 ml of test solution, at various concentrations of the Methanolic extract, was prepared. Absorbance at 517nm was determined after 30 minutes and the percentage inhibition activity was calculated by using the equation.²⁰⁻²⁴

$$\% \text{ Scavenging Activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. The lower the absorbance, the higher the free radical scavenging activity.

Antibacterial Screening

Determination of MIC

Nutrient broth test tubes were prepared and labeled as shown in the Table-1 below. In the first tube (UT), the inoculum was not added which is used for checking the sterility of the medium and as a negative control. Other all test tubes, inoculum (3 to 4 drops) were added to reach the final concentration of microorganisms is 10^6 cells/ml. In all the test tubes, a test sample (methanolic extract) was added ranging from 20.8125 μ l to 2ml. except for uninoculated (negative control) and control (positive) tubes. The positive control tube is used to check the suitability of the medium for the growth of the test microorganism and the viability of the inoculum. Adjust the final volume to 10ml in all the test tubes by using sterile water. All the test tubes are properly shaken and then incubated at 37°C for two days.

Table-1: Determination of MIC by using Broth Dilution Method

Test Tube Number	The volume of Medium (ml)	The volume of the methanolic extract Solution (μ l)	The volume of Sterile water (μ l)	Concentration of methanolic extract solution (μ g/ml)
UT (negative control)	8	0	2000	-
CT (positive Control)	8	0	2000	-
1	8	20.81	1979.19	31.25
2	8	41.625	1958.38	62.5
3	8	83.25	1916.75	125
4	8	166.5	1833.5	250
5	8	333	1667	500
6	8	666	1334	1000
7	8	1000	1000	1500
8	8	1333	667	2000
9	8	1666	334	2500
10	8	2000	0	3000

Determination of Zone of Inhibition (ZOI) by Agar well Diffusion Method

The antibacterial activity was performed by employing Agar well diffusion method.

The following Gram-positive and Gram-negative bacteria were used for antibacterial screening. *Staphylococcus aureus* (S1) NCTC-6571, *Bacillus subtilis* (B1) NCTC-10341, *Escherichia Coli* (E.coli2) NCTC-832, *Salmonella typhi* (SL2) NCTC-59.

Table-2: Preparation of Nutrient Agar Media (500ml)

Peptone	5g
Sodium chloride	2.5g
Agar	10g
Beef extract	5g
Distilled water q.s to	500ml
pH adjust to	7.2 – 7.4

Sodium chloride, peptone, beef extract, and agar were weight out and dissolved in the required amount of distilled water by keeping the media in the steam bath, the agar was melted out and the indicator was added and the volume was made with distilled water. pH was adjusted at 7.2-7.4. Then the flux was plugged and wrapped in paper then autoclave at 15 p.s.i. pressure at 121°C for 15 min.²¹

Standard Drug Solution

Standard drug dilution was prepared in double distilled water.

Gentamicin: 2.8µg/ml.

Preparation of Inoculums

The peptone water medium was sterilized by autoclaving at 15 p.s.i pressure and 121°C for 15 minutes. A loop full of organisms was transferred from a laboratory-maintained mother culture into different Bijou bottles containing sterilized peptone water medium. The Bijou bottles were incubated for 37°C for 48 hours.

Agar Well Diffusion Method

The antibacterial activity of methanolic extract of *Clematis buchananiana* against four pathogenic bacteria was evaluated by using the agar well diffusion method. The Nutrient agar plates were prepared by pouring 15 ml of molten media into the sterile Petri plates. About (108- 109) colony-forming units per ml were used. Wells or cups of 5mm size were made with sterile borer into agar plates containing the bacterial inoculums. 20µL of microbial broth was spread on the surface of nutrient agar plates. The methanolic extracts were introduced into the respective hole of the plates. Sterile water was used as negative control which was introduced into the well instead of methanolic extract. Gentamicin was used as standard. After that, the plates were incubated for 48 hrs at 37°C. After incubation for 48 hrs at 37°C, the plates were observed. The antibacterial activity was present on the plates; it was indicated by an inhibition zone surrounding the well. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded when the radius of the zone of inhibition was greater than 4 mm.^{25,26}

RESULTS AND DISCUSSION

The plant *Clematis buchananiana* has been investigated in a systematic way covering its pharmacognostical, physiochemical, and phytochemical study. In this section, the result of various investigations carried out was compiled.

Macroscopic Examination

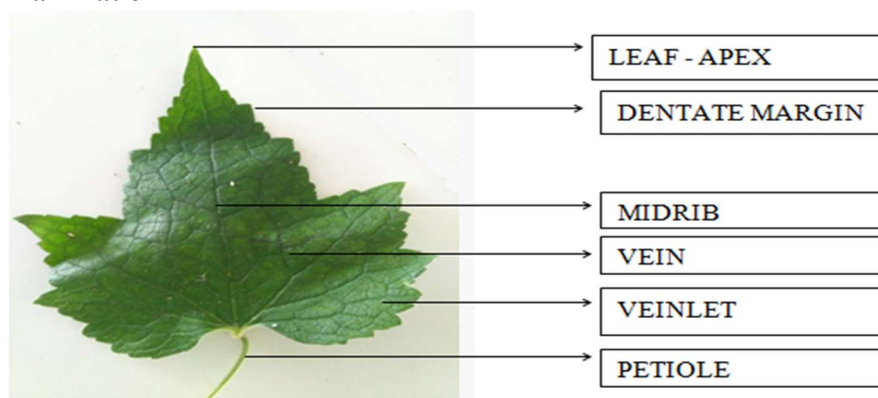


Fig.-1: Leaf of *Clematis buchananiana*

Macroscopic Parameters

A leaf of *Clematis buchananiana* consists of different parts like leaf surface, length, width, taste, odor, and color are summarized below.

Table-3: Macroscopic Observation	
Observation	Results
Surface	Hairy
Length	3-6 cm

Width	2-3 cm
Taste	Bitter
Odor	Pungent
Colour	Greenish

Microscopic Examination

Transverse Section (T.S) of leaves of *Clematis buchananiana*.



Fig.-2: T.S of *C. buchananiana* Leaf

Quantitative Microscopy

The leaves were subjected to the determination of various constants as numerical values using the reported procedure.

Leaf Constant of *Clematis buchananiana*

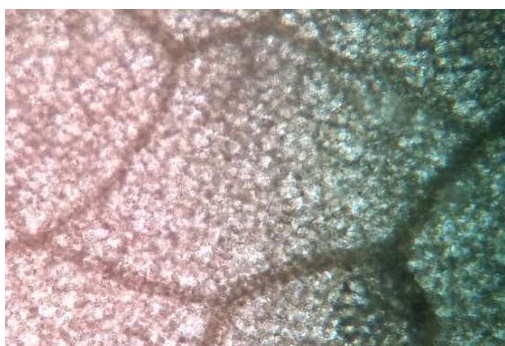


Fig.-3: Lower Epidermis Showing Actinocytic Stomata

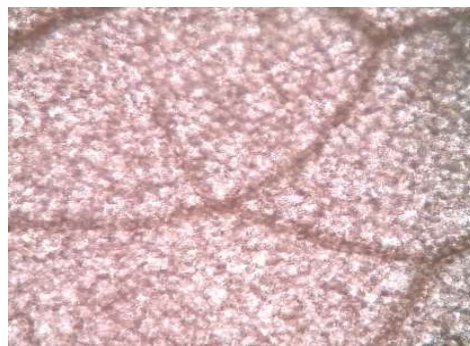


Fig.-4: leaf Vein islet

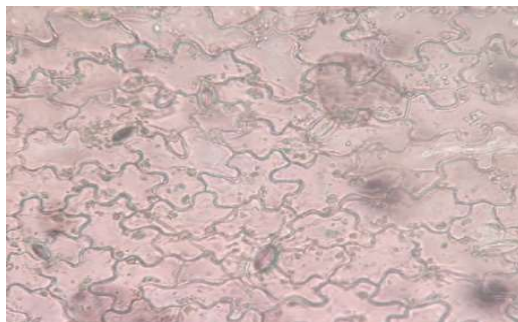


Fig.-5: Leaf Vein Termination

Powder Microscopy

The powdered leaf of *Clematis buchananiana* was mounted with phloroglucinol and HCL (1:1), iodine solution, and sulphuric acid respectively, and the following elements were observed: Trichome, starch grains, and calcium oxalate crystals.

Table-4: Leaf Constant

Determination	Values
Stomatal Number	168- 192 (lower epidermis)
Stomatal Index	14.28 %
Vein- Islet Number	48-54
Vein Termination Number	24-30



Fig. 6: Trichomes



Fig. 7: Calcium oxalate crystals



Fig. 8: Starch grains

Table-5: Extractive Value of *Clematis buchananiana*:

S. No.	Extracts	Extractive Value (%W/W)
1	Alcohol soluble extracts	6.8
2	Water soluble extracts	4.5

Table-6: Moisture Content of *Clematis buchananiana*:

Fresh Weight(g)	Dry Weight (g)	Loss on Drying(g)	Moisture Content (%w/w)
5	4.92	0.08	1.6

Table-7: Ash Value of *Clematis buchananiana*

Total Ash (%w/w)	Acid Insoluble Ash (%W/W)	Water Soluble Ash (%w/w)
6.67	1.2	5.36

Table-8: Phytochemical Profiling

Phytoconstituents	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanolic extract
Alkaloids	-	-	-	+
Glycosides	-	-	-	-
Tannins/Phenolic	-	-	-	-
Phytosterols	-	-	-	-
Carbohydrates	+	+	+	+
Flavanoids	-	-	-	-
Proteins and amino	+	+	+	+

acids				
Fixed oils	-	-	-	-
Saponins	+	+	+	+
Gums and Mucilages	-	-	-	-

Thin Layer Chromatography (TLC) of Methanolic Extracts

Methanolic extract showed four spots using hexane: ethyl acetate (8:2) as the mobile phase when visualized under UV 254 nm. The R_f value of the four spots was found to be 0.34, 0.56, 0.68, and 0.82 respectively.

Table-9: TLC investigation of fractions eluted using column chromatography

S. No.	Test Tube No.	Description	No. of Spots
1	18,19,20,21	Light green band	one
2	32,33,34,35,36	Light yellow band	one

Pharmacological Study

Table-10: Antioxidant activity of methanolic extract of leaves of *Clematis b Buchananiana*: methanolic extract of *clematis b Buchananiana* showing % inhibition at different concentrations. Data given are the mean of three replicates $\pm$ standard error mean, $p < 0.01$.

Concentration	Ascorbic acid	Methanolic extract
20 $\mu\text{g/ml}$	47.47 $\pm$ 0.06	25.07 $\pm$ 0.11
40 $\mu\text{g/ml}$	54.54 $\pm$ 0.32	37.11 $\pm$ 0.09
60 $\mu\text{g/ml}$	66.89 $\pm$ 0.41	48.23 $\pm$ 0.43
80 $\mu\text{g/ml}$	82.00 $\pm$ 0.24	59.67 $\pm$ 0.16
100 $\mu\text{g/ml}$	90.81 $\pm$ 0.03	67.89 $\pm$ 0.20

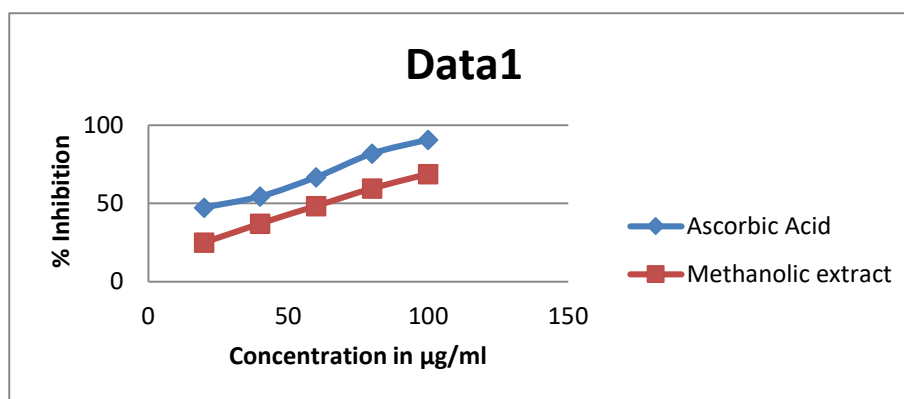


Fig.-9: 1, 1-diphenyl-2-picrylhydrazil (DPPH) Scavenging Activity of Methanolic Extract of Leaves of *Clematis Buchananiana* and the Standard Ascorbic Acid. The Graph Represents The Percentage of DPPH Inhibition. Each Point Represents the values obtained from Three Experiments Performed. The IC_{50} Value of the Methanolic Extract and Standard Ascorbic Acid Were Found To Be 67.5 $\mu\text{g/ml}$ and 48 Mg/ml Respectively

Determination of Minimum Inhibitory Concentration (MIC) by Broth Dilution Method

After incubation, all test tubes are examined for the growth in form of turbidity and colonies, respectively. The MIC was calculated by comparing all results with positive and negative control.

Table-11: MIC of Methanolic Extract of *C. b Buchananiana* against Bacterial Strains

Microorganisms	MIC ($\mu\text{g/ml}$)
<i>B. subtilis</i>	500
<i>S. aureus</i>	125
<i>E. coli</i>	500
<i>S. typhi</i>	500

Determination of Zone of Inhibition by Agar Well Diffusion Method

The Studies of physicochemical constants can serve as a valuable source of information and are usually used in judging the purity and quality of the drug. The extractive values have given an idea about the chemical constitution of the drug and from the study. The extractive value of alcohol was highest followed by water. The ash value determines the earthy matter or inorganic composition and other impurities present along with the drug. The qualitative phytochemical tests on different extracts showed the diverse nature of phytoconstituents. The petroleum ether, chloroform, and ethyl acetate extracts were found to contain carbohydrates, proteins, amino acids, and saponins, whereas the methanolic extract was found to contain alkaloids, carbohydrates, proteins, amino acids, and saponins as compared to previous studies in the same genus.^{27,28} TLC of methanolic extracts of *Clematis b Buchananiana* leaves in solvent system hexane: ethyl acetate (8:2) using pre-coated silica gel G254 plate under UV 254 nm was done which confirms the presence of a diverse group of phytochemicals. This variation in R_f values of the phytochemicals provides a very important clue in the understanding of their polarity. The antioxidant activity of the methanolic extract was performed by the DPPH method.

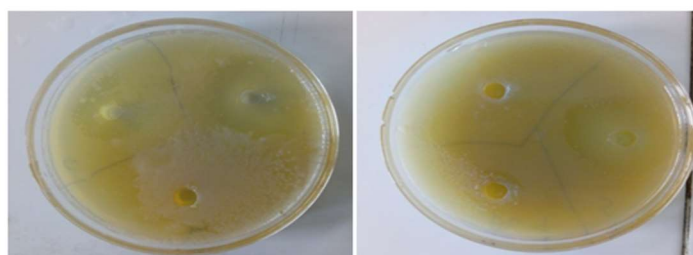


Fig10: ZOI of methanolic extract against *S. typhi*.

Fig11: ZOI of methanolic extract against *B. subtilis*.

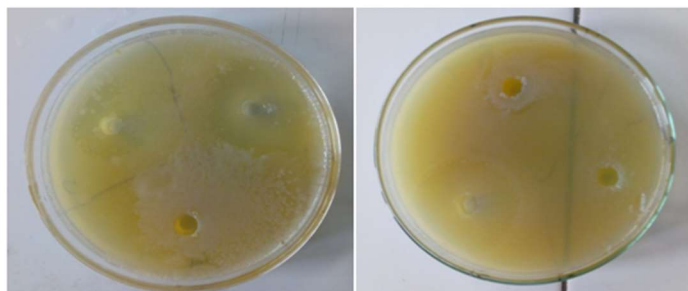


Fig12: ZOI of methanolic extract against *E. coli*.

Fig13: ZOI of methanolic extract against *S. aureus*.

Table-12: Determination of Zone of Inhibition by Agar Well Diffusion Method

Microorganisms	Zone of Inhibition (mm)		
	Methanolic extract (500µg/ml)	Gentamicin (2.8 µg/ml)	Sterile water
<i>B. subtilis</i>	13mm	33mm	-
<i>E. coli</i>	17mm	34mm	-
<i>S. typhi</i>	22mm	36mm	-

Table-13: Determination of Zone of Inhibition by Agar Well Diffusion Method

Microorganism	Zone of Inhibition (mm)		
	Methanolic extract (125µg/ml)	Gentamicin (2.8µg/ml)	Sterile water
<i>S. aureus</i>	14mm	34mm	-

The methanolic extract has shown good antioxidant activity ($IC_{50} = 58.5 \mu\text{g/ml}$). The same values were found in specie.²⁹ The antibacterial activity of the methanolic extract was determined by using the broth dilution method (for MIC) and agar well diffusion method (for ZOI). The antibacterial activity of methanolic extract of *Clematis buchananiana* leaves was broad spectrum as it inhibited both gram-positive and gram-negative bacteria. The methanol extract contains secondary metabolites such as alkaloids, carbohydrates, proteins, amino acids, and saponins. Some of these bioactive compounds may serve as potential antibacterial agents. Still, it was found that the methanolic extract is more active toward gram-negative bacteria than gram-positive bacteria.³⁰

CONCLUSION

The pharmacognostic standard and phytochemical evaluation for the leaves of *Clematis buchananiana* have been laid down for the first time in this study. In conclusion, it can be said that the study could be used as a diagnostic tool for the standardization of this medicinal plant. *In vitro* antioxidant and antibacterial activity showed that the extract has good antioxidant as well as antibacterial potential. So it can be concluded that the methanolic extract of the leaves of *Clematis buchananiana* possesses good antibacterial activity. Further study is required to isolate the compound from the methanolic extract of the leaves of *Clematis buchananiana* which is responsible for the antioxidant and antibacterial activities.

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LIST OF ABBREVIATIONS

1. TLC=Thin layer chromatography
2. TS= Transverse Section
3. FAA= Formaldehyde Alcohol Acetic Acid
4. UV=Ultra Violet
5. DPPH= 2,2-diphenyl-1-picrylhydrazyl

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