

**ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF  
*BRYOPHYLLUM CALYCINUM* SALISB LEAF**

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**Summary**

The development of bacterial resistance to presently available antibiotics has necessitated the search of new antibacterial agents. Plants and plant products are known to possess excellent antioxidant properties and play a significant role in preventing the conditions due to the excessive free radicals. The present study was aimed to evaluate the antimicrobial and antioxidant activities of the alcoholic and aqueous extracts from leaves of *Bryophyllum calycinum* Salibs. Aqueous and alcoholic extracts prepared and were tested on Gram positive and Gram negative bacteria. Agar cup plate test was used to determine the sensitivity of the tested samples well micro-dilution method was used to determine the minimum inhibitory concentration. The DPPH and Nitric oxide free radical scavenging method were used to detect oxidative activity. The results of antimicrobial assays showed that aqueous extract were active against all tested microbial strains. The aqueous extract showed antimicrobial activity against all tested microorganism with minimum inhibitory concentration ranging between 0.26 to 2.08 mg/ml, while alcoholic extract showed 1.04 to 8.32 mg/ml, respectively. The results of DPPH method showed 50% inhibition rate at the 144.23µg/ml and 117.42µg/ml with aqueous and alcoholic extract, respectively. Nitric oxide scavenging inhibition showed 50% inhibition rate at the 525.92µg/ml and 460.48µg/ml with aqueous and alcoholic extract, respectively. The overall results of this study indicates that the extract from leaves have interesting antimicrobial and potential free radical scavenging activity for treatment of diseases.

**Key words:** Antimicrobial activity, *Bryophyllum calycinum* Salibs, DPPH method, Nitric oxide scavenging method.

### Introduction

In developing countries, infectious diseases remain the main cause of the high mortality rates recorded; the majority of rural people has limited access to formal and adequate health services and thus heavily resources to traditional healers (1). Indigenous herbal remedies are widely used against many infectious diseases, but only few of them have been studied chemically and biologically in order to identify their active constituents (2). In modern medical practice, the alarming worldwide incidence of antibiotic resistance causes a need for new compounds that can act either by a direct antimicrobial activity or by inhibiting resistance mechanisms of microorganisms of medical importance. Medicinal plants represent a valuable source for this kind of compounds (3).

*Bryophyllum calycinum* Salisb (BCS) (Family - Crassulaceae) is an erect, succulent, glabrous, 0.3-1.2 m height, perennial herbs. It is also known as air plant, miracle leaf native to Madagascar. Etymologically it is also known as "Pasanabheda" means which breaks or destroys stones. It is also known as "Agnigarbha" (pregnant with fire) probably due to its property of producing blisters (4, 5). Various synonyms of plants are *Kalanchoe pinnatum*, *Bryophyllum pinnatum*. are commonly known as Panfutti, Ghayamari, Miracle leaf, Mexican love plant, Air plant. Various species of BCS are used medicinally in Indo-china and Philippines islands; where as BCS (Syn. *Bryophyllum pinnatum*) is naturalized throughout the hot and moist part of India. The leaves and bark is bitter tonic, astringent to the bowels, analgesic and carminative, useful in diarrhea and vomiting. Leaves powder used as wound dressing and sold as 'Jakhmehayat'. It is used as pulp on bruised wound and insect bites. Leaves are used as astringent, styptic, antiseptic and bitter. Internally juice is given in diarrhoea, dysentery and lithiasis. Toasted leaves are applied to wounds, bruises, boils and bites of venomous insects. In the form of poultice and powder they are applied to sloughing ulcers. In traditional medicine, *Bryophyllum* species have been used to treat ailments such as infection, rheumatism and inflammation (6). Flavanoids glycoside and phenolic component have been reported in the leaves of *Bryophyllum* species (7). *Bryophyllum* is a part of the extensively medicinal plant utilized by the tradipraticians (8).

The juice from fresh leaves is used to treat smallpox, otitis, cough, asthma, palpitations, headache, convulsion and general debility. Phytochemical investigations have reported the presence of alkaloid and saponins in the aqueous and alcoholic extract of *Kalanchoe crenata* leaves (9). Phytochemical analysis gave positive test for alkaloids and flavonoids in *Bryophyllum pinnatum* leaves extract.

The plant selected for study was based on its availability and its various therapeutic activities in various ailments mentioned in Ayurveda. In the present work, we have reported for the first time results of the combined investigations on in-vitro antibacterial and antioxidant activities of the extracts of BCS.

## **Materials and Methods**

### **Plant Material**

The proposed material of *Bryophyllum calycinum* Salisb leaf was procured from South Gujarat region, India, with the help of local tribal and field botanist. The species for the proposed study was identified *Bryophyllum calycinum* Salisb leaf, by Dr. Minoo H. Parabia, Botanist, Department of Bioscience, Veer Narmad South Gujarat University, Surat, Gujarat, India, the voucher specimen number is VCJ/03/25032005.

### **Preparation of Extracts**

Preparation of the extract of BCS powdered leaves is done using alcohol and distilled water. The shade dried coarse powder of the leaves (500 gm) was packed well in soxhlet apparatus and was subjected to continuous hot extraction with 90% alcohol until the completion of the extraction. The extract was filtered while hot and the resultant extract was distilled in vacuum under reduced pressure in order to remove the solvent completely. It is dried and kept in a desiccator till experimentation. Similarly, aqueous extract was prepared (10, 11, 12).

### **Antimicrobial Activity**

**Microbial strains** - The microorganisms used in the antimicrobial tests were Gram-positive (*Staphylococcus aureus* ATCC 25925, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermis* ATCC 12228 and *Micrococcus luteus* ATCC 10240) and Gram-negative (*Enterobacter aerogens* ATCC 13048, *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 51812 and *Shigella dysenteriae* ATCC 25931) organisms have been selected. All strains were obtained from C. G. Bhakta institute of Biotechnology, Tarsadi, Bardoli, Gujarat, India.

**Culture media** - Nutrient agar (NA) (Himedia Laboratories Ltd., Mumbai, India) medium and Nutrient broth medium were used for the growth of bacteria. Suspend the ingredients in 1000 ml distilled water and boil to dissolve it completely. Adjust the pH of media  $7.4 \pm 0.2$  (at  $25^{\circ}$  C) and sterilized it by autoclaving at 15 lbs pressure ( $121^{\circ}$  C) for 15 min. The solution of the test extracts was prepared at the concentration of 50 mg/ml by dissolving in DMSO in stopper specific gravity bottle & stored in refrigerator. The solution was removed from the refrigerator one hour prior to use & was allowed to warm up to room temperature.

**Chemicals** - Gentamycin (Alkem Laboratories Ltd., Mumbai, India) was used as reference antibiotic (RA) against bacteria and dimethylsulphoxide (DMSO) (Himedia, India) was used as solvent for tested samples. Other chemicals were used of analytical grade of S. D. Fine Chemicals, Mumbai, India.

**Sensitivity test: Cup Plate Method (Zone of Inhibition)** This method depends on the diffusion of the various extracts from a cavity through the solidified agar layer of petri dish, to an extent such that growth of the added micro-organism is prevented entirely in circular area or zone around the cavity containing the extracts. 0.2 ml of each of the seeded broth containing  $10^{-6}$ – $10^{-7}$  cfu/ml test organism was inoculated on the two plates of solidified agar & spread it uniformly. Sterilize the cup-borer of 10 mm diameter by dipping it in alcohol followed by flaming it and make four wells, one in each quadrant, at equal distance in nutrient agar plate previously seeded with culture. Add 0.2 ml of reference antibiotic Gentamycin (50  $\mu$ g/ml) in to the first well, in second well control solvent – DMSO and in third and fourth well test extracts solution

was added. The plates were kept in refrigerator at 4 - 5°C for 30 min after addition to allow diffusion of the solution into the medium & then incubated the plate in upright position at 37°C ± 1°C for 24 hrs. After the incubation period the diameter of the zone of inhibition in mm obtained around the well was measured (13).

**Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC)** - The stock solution of different test extracts were prepared in concentrations of 50 mg/ml in DMSO and used for study. Similarly the reference antibiotic concentration of 50µg/ml was prepared. Prepare 8 ml of broth containing 50 mg/ml concentration of extract for the first tube in row. Mix the content of universal bottle, using a pipette and transfer to the first tube in row. Add 4 ml of broth to the remaining 4 ml of the universal bottle, mix and transfer 2 ml to the second tube in row, prepare dilutions up to the 10<sup>th</sup> tubes. Place 2 ml of extract free broth in the last tube. All tubes were placed in incubator at 37°C ± 1°C for 24 h. Inoculate tube containing 2 ml broth with the organism and keep at 4°C in a refrigerator overnight, to be used as standard for the determination of complete inhibition. The lowest concentration of test extract and reference antibiotic which caused apparently a complete inhibition of growth of organism was taken as minimum inhibitory concentration (MIC). The assay was repeated thrice (14, 15).

For the determination of MMC, a portion of a liquid (5µl) from each well that showed minimum zone was again plated on NA media and incubated at 37°C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC.

### **Antioxidant study**

**DPPH Scavenging Method** - The test samples (3ml extract solution in water) were mixed with 1ml (0.1 mM solution of DPPH in methanol) and different concentrations (40-200 µg/ml) were prepared. After 30 min at room temperature, the absorbance values were measured at 517nm and converted into percentage of antioxidant activity. Ascorbic acid was used as a standard. Each assay was repeated thrice and recorded as mean of the triplicate.

Capacity to scavenge DPPH radical was calculated by using following equation (16, 17, 18, 19).

$$\% \text{ Scavenging Effect} = [1 - \text{Abs. (s)} / \text{Abs. (c)}] \times 100$$

Where, Abs. (s) = Absorbance of sample, Abs. (c) = Absorbance of control.

**Nitric Oxide Radical Scavenging Method** - Nitric oxide scavenging activity was determined (20) using sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide, which interact with oxygen to produce nitrite ions, which can be determined by the use of Griess Illosvoy reaction (21). Mix 2 ml (10 mM Sodium nitroprusside) in 0.5ml phosphate buffer saline (pH 7.4) with 0.5ml of extract at various concentrations (100-600 µg/ml) and was incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1ml sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1ml naphthylethylenediamine dihydrochloride (NEDA) (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilic acid subsequent coupling with NEDA was read at 540nm. Ascorbic acid was used as a positive control. Experiment was done in triplicate. Capacity to scavenge the nitric oxide radical was calculated by using following equation (22, 23, 24, 25).

$$\% \text{ Inhibition} = [1 - \text{Absorbance of sample} / \text{Absorbance of control}] \times 100$$

## **Results and Discussion**

**Results of Antibacterial activity** - The results of antibacterial sensitivity test of the plant extract are shown in (Table 1). The most active extract was aqueous extract followed by the alcoholic extract. These results indicated that most of the active constituents (responsible for exerting antibacterial action) in these plants are expected to be soluble in polar solvent. It authenticates that the entire tested microorganism are susceptible to aqueous extract and degree of susceptibility is given below in the decreasing order:

*Enterobacter aerogenes* > *Escherichia coli* > *Shigella dysnteries* > *Salmonella typhi* > *Staphylococcus aureus* > *Bacillus subtilis* > *Staphylococcus epidermis* > *Micrococcus luteus*. On the other hand, the susceptibility to alcohol extract is in the order: *Staphylococcus aureus* > *Salmonella typhi* > *Shigella dysenteries* > *Enterobacter aerogenes* > *Escherichia coli* > *Bacillus subtilis*. Aqueous extract of BCS leaves have equal antibacterial activity to gentamycin i. e., effective against all tested Gram positive and Gram negative bacteria, while alcoholic extract was effective against all Gram negative bacteria and *Staphylococcus aureus* and *Bacillus subtilis*.

**Table 1.** Antibacterial activity of the alcoholic and aqueous extracts from leaves of BCS and reference antibiotics determined by the cup plate method.

Microorganisms	Aqueous Extract	Alcoholic Extract	RA*
<b>Gram positive bacteria</b>			
<i>Staphylococcus aureus</i> ATCC 25925	+	+	+
<i>Bacilus Subtilis</i> ATCC 6633	+	+	+
<i>Staphylococcus epidermis</i> ATCC 12228	+	-	+
<i>Micrococcus luteus</i> ATCC 10240	+	-	+
<b>Gram negative bacteria</b>			
<i>Enetrobacter aerogens</i> ATCC 13048	+	+	+
<i>Escherichia coli</i> ATCC 25922	+	+	+
<i>Salmonella typhi</i> ATCC 51812	+	+	+
<i>Shigella dysenterie</i> ATCC 25931	+	+	+
(-): Not active, (+): Active			

\*RA = Reference antibiotic – Gentamycin (50 µg/ml),

Result shows that the aqueous extract is most active extract with less MIC 0.26 mg/ml while with alcoholic extract is 1.04 mg/ml, which was further supported by observed MMC value. MMC value of aqueous extract is 0.52 while with alcoholic extract is 2.08, which is about 4 times higher than the MMC value of aqueous extract (Table 2). All the strains were more susceptible to aqueous extract, our result indicate the presence of chemical compound in both extract with antibacterial activity against all strains comparable to gentamycin (26, 27, 28).

Table 2. Minimum inhibitory concentration (mg/ml) and Minimum microbicidal concentration (mg/ml) of aqueous and alcoholic extract from leaves of BCS and reference antibiotic ( $\mu\text{g/ml}$ ).

Microorganisms	Minimum inhibition concentration			Minimum microbicidal concentration		
	Aqueous extract	Alcoholic extract	RA*	Aqueous extract	Alcoholic extract	RA*
<b>Gram positive bacteria</b>						
<i>Staphylococcus aureus</i> ATCC 25925	2.08	8.32	9.55	4.16	8.32	9.55
<i>Bacillus Subtilis</i> ATCC 6633	0.52	2.08	4.16	1.04	4.16	9.55
<i>Staphylococcus epidermis</i> ATCC 12228	1.04	-	4.16	2.08	-	9.55
<i>Micrococcus luteus</i> ATCC 10240	2.08	-	9.55	4.16	-	9.55
<b>Gram negative bacteria</b>						
<i>Enetrobacter aerogens</i> ATCC 13048	0.52	4.16	2.08	2.08	> 16.64	8.32
<i>Escherichia coli</i> ATCC 25922	1.04	4.16	4.16	2.08	8.32	8.32



<i>Salmonella typhi</i> ATCC 51812	0.52	2.08	16.64	1.04	4.16	4.16
<i>Shigella dysenteriae</i> ATCC 25931	0.26	1.04	8.32	0.52	2.08	2.08

(-): not tested because samples were not active by diffusion.

\*RA = Reference antibiotic (Gentamycin)

**Results of *In- vitro* antioxidant activity** - In today's environment, hyper physiological burden of free radical causes imbalance in homeostatic phenomenon between oxidants and antioxidants in the body. The imbalance leads to oxidative stress that is being suggested as the root cause of aging and various human diseases like arteriosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Thus free radical scavenging is very essential for preventing organ injury associated with shock, inflammation & ischemia or reperfusion. Therefore research in recent past have accumulated enormous evidence advocating enrichment of body system with antioxidants to correct vitiated homeostasis and prevent onset as well as treat the disease caused due to free radical and related oxidative stress. Stress, smoking, drugs & diet generates excessive free radicals in human body. Plants & plant products are known to possess excellent antioxidant properties & play a significant role in preventing the conditions due to the excessive free radicals. The results of absorbance and % inhibition showed decrease in the concentration of DPPH radical due to the scavenging ability of extract and standard ascorbic acid, as a reference standard (19, 23, 29, 30, 31).

Fig 1 shows in vitro antioxidant activity of BCS leaves extract by DPPH method while Fig 2 shows by Nitric oxide method. The aqueous extract of BCS leaves presented more antioxidant activity than alcoholic extract. Aqueous extract, alcoholic extracts and ascorbic acid exhibits 74.7, 58.4 and 88.6 % inhibition and the EC<sub>50</sub> (µg/ml) –144.23, 117.42 and 96.15 µg/ml, respectively.

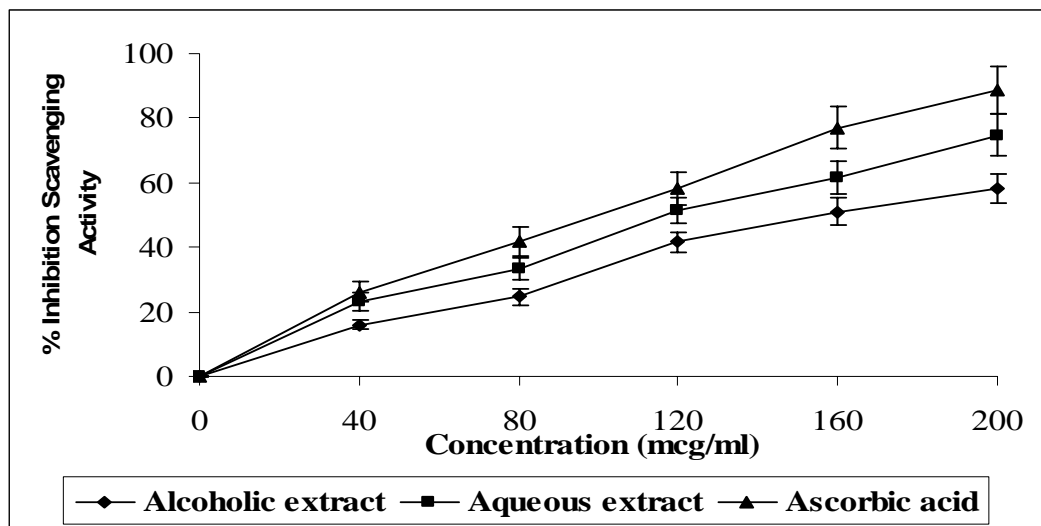


Figure 1. Graphical representation of *In vitro* Antioxidant activity of BCS leaves extract by DPPH method.

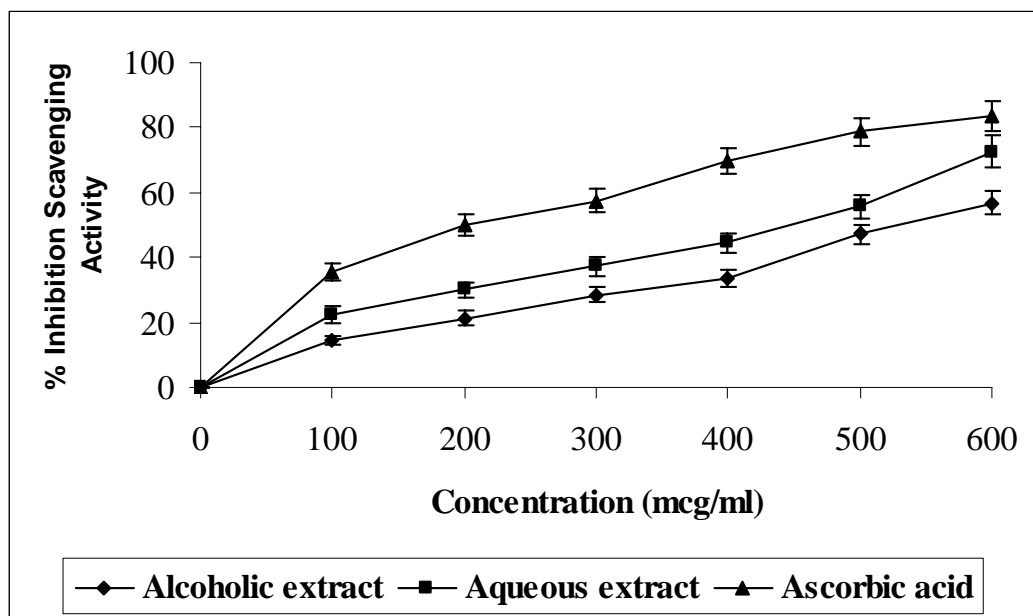


Figure 2. Graphical representation of *In vitro* Antioxidant activity of BCS leaves extract by Nitric oxide method.

In Nitric oxide method the results showed that aqueous extract has more % inhibition and less EC<sub>50</sub> value. The % inhibition of aqueous extract, alcoholic extract and ascorbic acid were 72.5, 56.9 and 83.5% and EC<sub>50</sub> value were 525.92, 460.48 and 200.76µg/ml, respectively. The aqueous extract exhibited more antioxidant activity with low EC<sub>50</sub> value in these two methods. The Phytochemical analysis indicated the presence of flavanoids in extracts. Several such compounds were known to possess potent antioxidant activity (22, 32, 33, 34). Hence, the observed activity may be due to the presence of any of these compounds.

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