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# Antioxidant and antibacterial activities of leaves extract of *Hygrophila auriculata* (Schumach.) Heine

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**Abstract**
*Hygrophila auriculata* is one of the important medicinal plants found in India, which is Acanthaceae family and is distributed in tropical and subtropical region of India. The plant is used in cancer and tubercular fistula. Root and seeds used as tonic for asthama and dysentery. The leaves, roots and seeds of this plant are traditionally used for the treatment of inflammation, jaundice, hepatic obstruction and urinary infection. The aim of the present study was to evaluate the antioxidant and antibacterial activities of methanol leaves extract of *H. auriculata*. Antioxidant assays such as DPPH$^{\cdot}$ radical, superoxide ($O_2^{\cdot-}$) radical, ABTS$^{\cdot+}$ radical cation, $Fe^{3+}$ reducing power and phosphomolybdenum reduction assays were carried out for evaluating antioxidant activities. The maximum DPPH$^{\cdot}$ radical scavenging activity of methanol leaves extract was 72.82±0.19% at 120 µg/mL concentration and the $IC_{50}$ was 83.88 µg/mL concentration. The maximum superoxide radical scavenging activity of methanol leaves extract was 81.72±0.28% at 120 µg/mL concentration and the $IC_{50}$ was 20.60 µg/mL concentration. The maximum ABTS$^{\bullet+}$ radical cation scavenging activity of methanol leaves extract was 88.93±0.23% at 30 µg/mL concentration and the $IC_{50}$ was 11.75 µg/mL concentration. The maximum of $Mo^{6+}$ reduction and $Fe^{3+}$ reduction of methanol leaves extract were 79.68±0.41% and 47.88±0.47% at 120 µg/mL concentrations and the $RC_{50}$ were 56.35 µg/mL and 125.31 µg/mL concentrations respectively. The antibacterial activity was carried out by well diffusion method and showed maximum zone of inhibition of 16 mm for *Proteus vulgaris* at 500 µg/mL concentration.

**Keywords:** *Hygrophila auriculata*, DPPH, ABTS$^{\cdot+}$, superoxide ($O_2^{\cdot-}$), antibacterial activity

## 1. Introduction
*Hygrophila auriculata* belongs to the family Acanthaceae and has been traditionally used for the treatment of inflammation, pain, edema, gout and as a diuretic. It is described in ayurvedic literature as Ikshura, Ikshugandha, and Kokilasha having eyes like the Kokila or Indian Cuckoo. The plant is widely distributed throughout India, Sri Lanka, Burma, Malaysia and Nepal. Various folk claims as a cure for numerous diseases, efforts have been made by researchers to verify the efficacy of the plant by scientific biological screening. The literature revealed some notable pharmacological effects like anti-nociceptive, antitumor, antioxidant, hepatoprotective, hypoglycemic, haematinic, diuretic, anti-inflammatory, antipyretic and androgenic activities. The present study is an attempt to evaluate various ethanobotanical and traditional uses as well as phytochemical and antioxidant activities of *Hygrophila auriculata*.
Herbal medicines have been used in medical practice for thousands of years and are recognized especially as a valuable and readily available healthcare resource [1]. Botanical drugs and dietary supplements may be obtained from a broader variety of plants than those normally present in the human diet. Botanicals or phytopharmaceuticals are very suitable for prophylactic use in order to prevent diseases and also to maintain our normal wellbeing. The screening and evaluation of medicinal plants is very dependent on the proper cultivation and collection of the plant materials followed by their extraction and isolation of the phytochemical entities to enable optimized bioactive compound production and subsequent therapeutic applications. This is very important for multi-component drugs and their standardized extracts to ensure high quality and batch-to-batch consistency [2]. The Indian subcontinent is the site of one of the oldest civilizations, and it has seen the development of many traditional healthcare systems. Their development was supported by the great biodiversity in flora and fauna due to variations in geography and climate [3].

## 2. Materials and methods
### 2.1. Collection of leaves and preparation of extract
The leaves of *H. auriculata* were collected from Madurai, Tamil Nadu, India.

The leaves were washed, shade dried for 10 days and powdered in mechanical blender. About 10 g of leaves powder was soaked in methanol for 72 h. The greenish supernatant liquid was filtered by filter paper and condensed in a hot plate at 50°C, which yields gummy extract.

**Fig 1:** Habitat of *H. auriculate*

### 2.2. Qualitative phytochemical analysis
The methanol leaves extract of *H. auriculata* was subjected to different classes of phytoconstituents, using specific reagents and following standard methods [4, 5].

### 2.3. Estimation of total phenols
Folin-Ciocalteau reagent method was used to determine the total phenolic compounds [6] with slight modifications. One hundred µL of methanol leaves extract of *H. auriculata* (1mg/mL) was mixed with 900 µL of methanol and 1 mL of Folin Ciocalteau reagent (1:10 diluted with distilled water). After 5 min, 1 mL of 20% (w/v) of $Na_2CO_3$ solution was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured at 765 nm in UV-Vis spectrophotometer. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

### 2.4. Estimation of total flavonoids
The total flavonoid content was determined using aluminium chloride reagent method with slight modification [7]. Five hundred µL of methanol leaves extract of *H. auriculata* (1mg/mL) was mixed with 500 µL of methanol and 500 µL of 5% (w/v) sodium nitrite solution followed by 500 µL of 10% (w/v) aluminium chloride solution was added and incubated for 5 min at room temperature. Then 1 mL of 1 M NaOH solution was added and the total volume was made up to 5 mL with distilled water. Absorbance was measured at 510 nm in UV-Vis spectrophotometer. The result was expressed as (µg/mg of extract) quercetin equivalent.

### 2.5. *In vitro* antioxidant assays
#### 2.5.1. DPPH˙ radical scavenging assay
The antioxidant activity of methanol leaves extract of *H. auriculata* was measured on the basis of stable DPPH free radical reduction method [8]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (20-120 µg/mL) of leaves extract. The mixture was then allowed to stand for 30 min incubation in dark. One mL methanol and 1 mL DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis Spectrophotometer at 517 nm. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\%\ \text{of DPPH}^{\cdot}\ \text{radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \text{x } 100$$

#### 2.5.2. Superoxide radical ($O_2^{\bullet-}$) scavenging assay
Superoxide radical scavenging activity was carried out by the method of Ravishankara *et al*. [9]. Different concentrations of leaves extract (20-120 µg/mL) of *H. auriculata* was mixed with 50 mM of phosphate buffer (pH 7.8), 1.5 mM of riboflavin, 12 mM of EDTA and 50 mM of NBT solutions and added in that sequence. The reaction was started by illuminating the reaction mixture for 15 min. After illumination, the absorbance was measured at 590 nm in UV-Vis Spectrophotometer. Ascorbic acid was used as standard reference. The percentage of inhibition was calculated as:

$$\%\ \text{of superoxide radical } (O_2^{\bullet-})\ \text{inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

#### 2.5.3. $ABTS^{\bullet+}$ radical cation scavenging assay
The antioxidant capacity was estimated in terms of the $ABTS^{\bullet+}$ radical cation scavenging activity [10]. $ABTS^{\bullet+}$ was obtained by reacting 7 mM ABTS solution in 5 mM of phosphate-buffered saline (pH 7.4) with 2.45 mM potassium persulfate and the mixture was left to stand in dark at room temperature for 12-16 h before use. The ABTS solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) till to reach an absorbance of 0.70±0.02 at 734 nm. To the various concentrations (20-120µg/mL) of methanol leaves extract of *H. auriculata*, 500 µL of diluted $ABTS^{\bullet+}$ solution was added. The absorbance was measured after 10 min incubation at 734 nm. Ascorbic acid was used as the standard reference. The $ABTS^{\bullet+}$ radical cation scavenging activity was expressed as:

$$\%\ \text{of ABTS}^{\bullet+}\ \text{radical cation inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \text{x } 100$$

#### 2.5.4. Phosphomolybdenum reduction assay
The antioxidant capacity of methanol leaves extract of *H. auriculata* was assessed by $Mo^{6+}$ reduction method [11]. The leaves extract with concentrations ranging from 20-120 µg/mL was combined with 1 mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in water bath at 95ºC for 90 min. The absorbance of the coloured complex was measured at 695 nm in UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\%\ \text{of phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \text{x } 100$$

#### 2.5.5. Ferric ($Fe^{3+}$) reducing power assay
The reducing power of methanol leaves extract of *H. auriculata* was determined by $Fe^{3+}$ reduction method with slight modification [12]. One mL of leaves extract of different

concentrations (20 - 120 µg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1 % w/v). The mixtures were then incubated at 50°C in water bath for 30 min. One mL of trichloroacetic acid (10 % w/v) was added to each mixture. Then 1 mL of freshly prepared $FeCl_3$ (0.1% w/v) solution was added and the absorbance was measured at 700 nm in UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\% \text{ of } Fe^{3+} \text{ reduction} = \left[ \frac{\text{Sample} - \text{Control}}{\text{Sample}} \right] \times 100$$

### 2.6. Antibacterial activity

#### 2.6.1. Microbial strains

The microorganisms of Gram positive strains such as *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* as well as Gram negative strains such as *Escherichia coli*, *Proteus vulgaris* and *Shigella flexneri* were used for the evaluation of antibacterial activity.

#### 2.6.2. Reference and control

Tetracycline was used as the standard for bacteria. The controls consist of solidifying agar onto which was solvent, and the extract was soluble in it.

#### 2.6.3. Aseptic conditions

The aseptic chamber which consist of a wooden box (1.3m x 1.6m x 0.6m) with a door, was cleaned with 70% ethanol and irradiated with short wave UV light (from lamp).

#### 2.6.4. Nutrient broth agar medium

Nutrient broth agar medium was prepared (peptone-5 g; yeast extract-3 g; NaCl-5 g; distilled water-1000 mL; pH-7.0±0.2; Agar-20 g) according to the standard method and required amount was weighed, suspended in required volume of 200 mL distilled water in a 500 mL conical flask, stirred and then autoclaved at 15 LBS and at 121ºC for 15 min. The hot medium was poured in sterile petriplates which were kept in the aseptic Laminar chamber. The medium was allowed to solidify for 15 min.

#### 2.6.5. Agar well-diffusion method

The potential antibacterial activity of methanol leaves extract of *H. auriculata* was analyzed by agar well diffusion method [13]. The solidified nutrient agar in the petriplates was inoculated by dispensing the inoculums using sterilized cotton swabs and spread evenly onto the solidified agar medium. Five wells were created in each plate with the help of a sterile well-borer of 8 mm diameter. The extract was then poured into each well to get desirable concentrations. Tetracycline was used as the standard with the concentration of 25 µg. All the plates containing sample loaded wells were incubated for 24 h at 37°C. After the incubation period, zone of inhibition in each plate, for each concentration of extract and standard were measured by calculating the diameter of zone of inhibition.

## 3. Results and discussion

### 3.1. Qualitative phytochemical analysis

The qualitative phytochemical analysis showed the presence of alkaloids, terpenoids, steroids, phenols, flavonoids, tannins, glycosides and saponins in the methanol leaves extract of *H. auriculata* (Table 1).

**Table 1:** Qualitative phytochemical analysis of methanol extract of leaves of *H. auriculata*

| S. No | Phytochemicals | Tests | Results |
|---|---|---|---|
| 1 | Alkaloids | Mayer's test | + |
| 2 | Terpenoids | Salkowski test | + |
| 3 | Steroids | Libermann-Burchard test | + |
| 4 | Phenols | Ferric chloride (0.5%) test | + |
| 5 | Flavonoids | Sodium hydroxide test | + |
| 6 | Tannins | Lead acetate test | + |
| 7 | Glycosides | Legal's test | + |
| 8 | Saponins | Foam test | - |

### 3.2. Total phenols and flavonoids

Phenolic compounds are predominantly distributed in plants and they have gained much attention, due to their antioxidant activity and free radical scavenging ability with potential benefits for human health [14]. Natural antioxidants available in medicinal plants have been alternative source for synthetic antioxidants. Free radicals are formed naturally in the body and play an important role in many normal cellular processes. At high concentrations, however, free radicals can be hazardous to the body and damage all major components of cells, including DNA, proteins, and cell membranes. The damage to cells caused by free radicals, especially the damage to DNA, may play a role in the development of cancer and other health conditions. Antioxidants such as phenols and flavonoids offer resistance against oxidative stress by preventing free radicals which are damaging biomolecules such as proteins, DNA, and lipids, thus prevent disease progression [15]. Phenolic compounds such as flavonoids, phenolics acid, and tannins possess diverse biological activities including antiinflammatory, anticarcinogenic, and antiatherosclerotic activities (Table 1). The phenols and flavonoids were quantified in the methanol extract of *H. auriculata* seemed to be responsible for antioxidant activity. The total phenolic content was 289.72 µg/mg of GAE and the total flavonoid content was 48.82 µg/mg of QE in the extract (Table 2).

**Table 2:** Quantitative estimation of methanol extract of leaves of *H. auriculate*

| S. No | Phytochemical | Amount (µg/mg) |
|---|---|---|
| 1 | Phenols | 289.72±0.28 |
| 2 | Flavonoids | 48.82±0.18 |

### 3.3. DPPH˙ radical scavenging assay

DPPH˙ (1,1-Diphenyl-2-picrylhydrazyl) is a stable nitrogen-centered free radical which has an unpaired valence electron at one atom of nitrogen bridge. Scavenging of DPPH free radical is one of the popular antioxidant assays. DPPH radical scavenging assay is a decolorization assay that will measure the capacity of antioxidants to directly scavenge DPPH˙ radicals by monitoring its absorbance using spectrophotometer at wavelength of 517 nm [16]. The DPPH assay provided rapid and an easy way to evaluate the antioxidant activity of most of the plant extracts. The methanol leaves extract of *H. auriculata* to scavenge free radicals was assessed by using DPPH˙ radical as the substrate, which measures the hydrogen or electron donating ability of leaves extract. The leaves extract of *H. auriculata* reducing the stable purple colour DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical to the yellow coloured 1,1-diphenyl-2-picrylhydrazine and the reduction capacity increases with increasing concentration of the extract. The maximum DPPH˙ radical scavenging activity was

72.82±0.19% at 120 µg/mL concentration (Table 3 and Fig 2). The $IC_{50}$ was 83.88 µg/mL concentration and was compared with standard ascorbic acid ($IC_{50}$ = 6.31 µg/mL concentration).

### 3.4. Superoxide ($O_2^{\cdot-}$) radical scavenging assay

Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents [17]. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. Superoxide anions derived from dissolved oxygen by the riboflavin-light-NBT system will reduce NBT in this system. In this method, superoxide anion reduces the yellow dye ($NBT^{2+}$) to blue formazan, which is measured at 590 nm in UV-Vis spectrophotometer. Antioxidants are able to inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The maximum superoxide radical scavenging activity of *H. auriculata* was 81.72±0.28% at 120 µg/mL concentration (Table 3 and Fig 2) and the $IC_{50}$ was 20.60 µg/mL concentration. It was compared with the standard of ascorbic acid ($IC_{50}$ = 9.65 µg/mL concentration).

**Table 3:** DPPH$^{\cdot}$ radical and superoxide ($O_2^{\cdot-}$) radical scavenging activity of methanol leaves extract of *H. auriculata*

| S. No | Concentration (µg/mL) | % of inhibition* | |
|---|---|---|---|
| | | DPPH$^{\cdot}$ radical | Superoxide radical ($O_2^{\cdot-}$) |
| 1 | 20 | 10.11±0.11 | 48.55±0.40 |
| 2 | 40 | 16.64±0.18 | 52.42±0.46 |
| 3 | 60 | 29.19±0.39 | 59.04±0.24 |
| 4 | 80 | 47.69±0.30 | 64.51±0.49 |
| 5 | 100 | 61.69±0.26 | 78.95±0.14 |
| 6 | 120 | 72.82±0.19 | 81.72±0.28 |
| | | $IC_{50}$ = 83.88 µg/mL | $IC_{50}$ = 20.60 µg/mL |

(* - Average of three replicates)

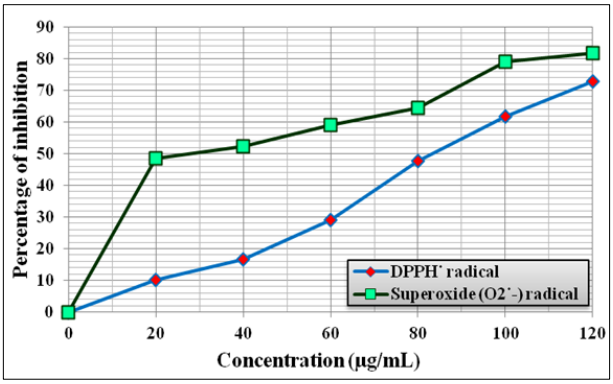


**Fig 2:** DPPH$^{\cdot}$ radical and Superoxide ($O_2^{\cdot-}$) radical scavenging activity of methanol leaves extract of *H. auriculata*

### 3.5. ABTS$^{\bullet+}$ radical cation scavenging assay

ABTS$^{\bullet+}$ is a blue-green chromophore produced by the reaction between ABTS and potassium persulfate. The blue-green chromophore radical cation gets reduced while reacting with an antioxidant in the leaves extract of *H. auriculata* and the remaining radical cation concentration was quantified with the loss of colour [18]. The antioxidant reduces ABTS$^{\bullet+}$ to ABTS and decolorize the blue-green chromophore. The maximum ABTS•+ radical cation scavenging activity was 88.93±0.23% at 30 µg/mL concentration (Table 4 and Fig 3) with the $IC_{50}$ of 11.75 µg/mL concentration and was compared with standard ascorbic acid ($IC_{50}$ = 4.21 µg/mL concentration).

**Table 4:** ABTS$^{\cdot+}$ radical cation scavenging assay of methanol leaves extract of *H. auriculata*

| S. No | Concentration (µg/mL) | % of inhibition* |
|---|---|---|
| | | ABTS$^{\bullet+}$ radical cation |
| 1 | 5 | 28.02±0.12 |
| 2 | 10 | 42.54±0.46 |
| 3 | 15 | 55.68±0.47 |
| 4 | 20 | 73.88±0.21 |
| 5 | 25 | 79.6±0.44 |
| 6 | 30 | 88.93±0.23 |
| $IC_{50}$ = 11.75 µg/mL | | |

(* - Average of three replicates)

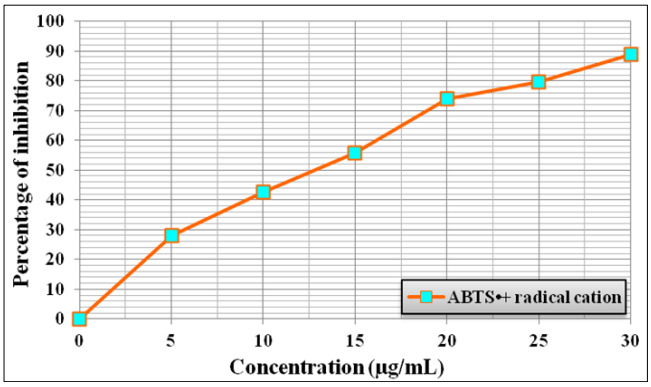


**Fig 3:** ABTS$^{\bullet+}$ radical cation scavenging assay of methanol leaves extract of *H. auriculata*

### 3.6. Phosphomolybdenum reduction assay

Metal-Catalyzed Oxidation (MCO) systems catalyze the reduction reaction, which alters the nature of proteins at the metal-binding site and cause DNA and protein damage [19]. The total antioxidant activity of methanol leaves extract of *H. auriculata* was measured by phophomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum phosphomolybdenum reduction was 79.68±0.41% at 120 µg/mL concentration with the $RC_{50}$ of 56.35 µg/mL concentration (Table 5 and Fig 4). It was compared with the standard ascorbic acid ($RC_{50}$ =6.34 µg/mL).

**Table 5:** Phophomolybdenum reduction and $Fe^{3+}$ reducing power assay of methanol leaves extract of *H. auriculata*

| S. No | Concentration (µg/mL) | % of reduction* | |
|---|---|---|---|
| | | Phosphomolybdenum reduction | $Fe^{3+}$ reduction |
| 1 | 20 | 26.85±0.22 | 20.91±0.29 |
| 2 | 40 | 34.57±0.40 | 24.49±0.45 |
| 3 | 60 | 53.24±0.35 | 24.62±0.42 |
| 4 | 80 | 65.61±0.46 | 30.72±0.33 |
| 5 | 100 | 76.64±0.43 | 43.37±0.38 |
| 6 | 120 | 79.68±0.41 | 47.88±0.47 |
| | | $RC_{50}$ = 56.35 µg/mL | $RC_{50}$ = 125.31 µg/mL |

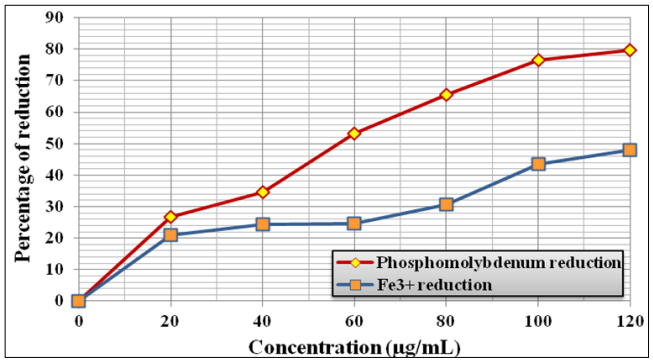


**Fig 4:** Phophomolybdenum reduction and $Fe^{3+}$ reducing power assay of methanol leaves extract of *H. auriculata*

### 3.7. Ferric ($Fe^{3+}$) reducing power assay

The reducing power assay was carried out by the reduction of $Fe^{3+}$ to $Fe^{2+}$ by the methanol leaves extract of *H. auriculata* and the subsequent formation of ferro-ferric complex. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action [20]. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom or by neutralizing the free radicals by donating an electron and become lone pair of electrons instead of odd electron. The reduction ability increases with increase in concentration of the extract. The maximum $Fe^{3+}$ reduction was 47.88±0.47% at 120 µg/mL concentration with the $RC_{50}$ of 125.31 µg/mL concentration (Table 5 and Fig 4) and was compared with the standard ascorbic acid ($RC_{50}$ =7.72 µg/mL).

### 3.8. Antibacterial activity by agar well diffusion method

The antibacterial activity was carried out for methanol extract of leaves of *H. auriculata* against Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus and Staphylococcus aureus*) and Gram-negative bacteria *(Escherichia coli*, *Proteus vulgaris and Shigella flexneri*). The antibacterial sensitivity of the crude extract and their potency were assessed quantitatively by measuring the diameter of clear zone in cultures in petriplates. The antibacterial activity of the extract could be correlated to the presence of secondary metabolites such as alkaloids, terpenoids and phenolic compounds that adversely affect the growth and metabolism of microbes. The maximum zone of inhibition of 16 mm for *Proteus vulgaris* and of 15 mm for *Bacillus subtilis* were showed at 500 µg/mL concentration of the extract (Table 7 and Fig 6). The molecular components and morphology of membranes from Gram-positive bacteria are fundamentally different from those of Gram-negative bacteria. Gram-negative bacteria are surrounded by two membranes, the cytoplasmic cell membrane and the outer membrane. The outer monolayer of the membrane contains lipopolysaccharide (LPS) as the major lipid component, a lipid species unique to Gram-negative bacteria [21].

**Table 7:** Antibacterial activity of methanol leaves extract of *H. auriculate*

| Bacterial pathogens | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | 250 µg/mL | 375 µg/mL | 500 µg/mL | Standard (Tetracycline) |
| *Bacillus subtilis* | 14 | 15 | 15 | 15 |
| *Micrococcus luteus* | 10 | 12 | 12 | 20 |
| *Staphylococcus aureus* | 10 | 11 | 11 | 21 |
| *Escherichia coli* | - | 12 | 14 | 23 |
| *Proteus vulgaris* | 14 | 16 | 16 | 23 |
| *Shigella flexneri* | 12 | 12 | 13 | 28 |

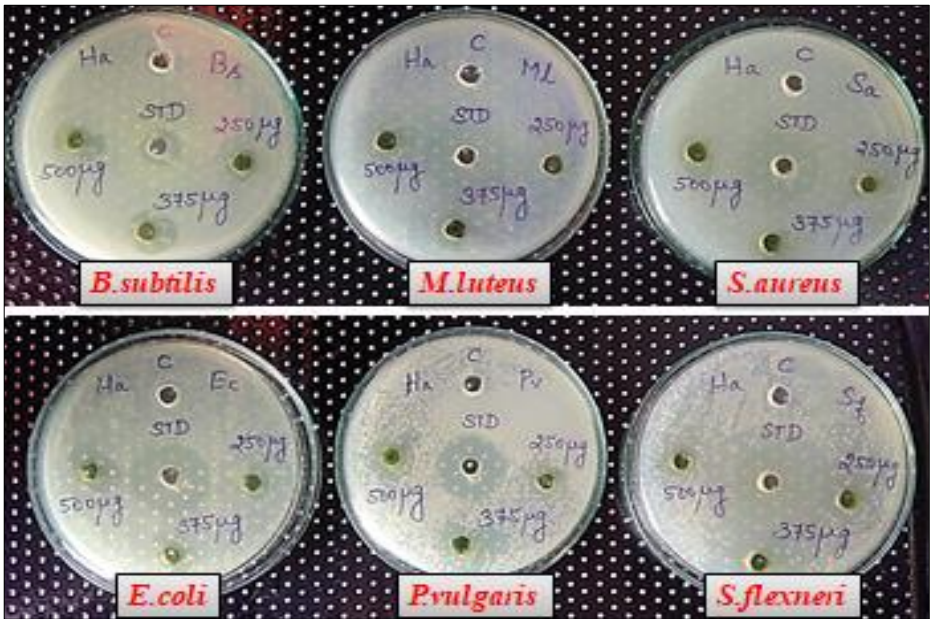


**Fig 6:** Antibacterial activity of methanol leaves extract of *H. auriculata*

## 4. Conclusion

The results of present study indicate that methanol leaves extract of *H. auriculata* has significant antioxidant activities to scavenge free radicals as well as to protect cells from oxidative damage. Further studies are necessary to isolate the active compounds present in methanol leaves extract of *H. auriculata* and study their pharmacological activities against cancer cells.

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