

The *in-vitro* Antimicrobial and Antioxidant Potential of ethanolic leaf extracts of *Anisochilus carnosus*

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Abstract: In the present study, the *in vitro* Antimicrobial and Antioxidant potential of ethanolic extract of Indian habitants *Anisochilus carnosus*, which is an annual shrub belonging to the family Lamiaceae which is also known as mint family were evaluated. The current study terminated the antibacterial activity of the extract of *Anisochilus carnosus* against three different bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* and Antifungal activity against *Candida albicans*, *Aspergillus niger* and *Trichoderma viridae*. Among the bacterial strains *Bacillus subtilis* showed the maximum zone of inhibition of 15 mm at 800 µg/disc and among the fungal strains *Candida albicans* showed the maximum zone of inhibition of 9.5 mm at 800 µg/disc. The Antioxidant Potential by radical-scavenging activity of the extracts was measured as decolorizing activity followed by the trapping of the unpaired electron of DPPH. The percentage decrease of 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) standard solution was recorded maximum for extracts was at a concentration of 400 µg/ml at IC₅₀ of 29.23%. Total phenolic and Total Antioxidant activity contents in the ethanol extract were µg/ml gallic acid equivalents and 100 mg/ml ascorbic acid equivalents respectively. The reducing power increased sharply as concentrations increased. The present study confirmed that the ethanol extract of *Anisochilus carnosus* leaves is a potential source of natural antioxidants.

Keywords: *Anisochilus carnosus*, Antimicrobial activity, DPPH free radical scavenging, Reducing Power, Total Antioxidant activity, Total Phenolic content and Zone of Inhibition.

I. INTRODUCTION

Anisochilus carnosus belongs to the family Lamiaceae, which is otherwise known as thick-leaved lavender in English and karpuravalli in Telugu and Tamil. It is an annual erect herb, stems quadrangular, sparsely pubescent, brownish from prolonged exposure to sun. Leaves fleshy, broadly ovate, deeply crenate, obtuse or acute, base rounded, verrucose above, and pubescent beneath. The whole plant is used as diaphoretic, stimulant, expectorant, liver disorders, cough and cold. Leaves are used for cough, dropsy, indigestion and sores in the leg fingers [1].

The discovery and development of antibiotics is the most powerful achievements of modern science and technology for the control of infectious diseases. However, the rate of resistance of pathogenic microorganisms to conventionally used antimicrobial agents is increasing at an alarming rate [2], [3] and [4]. Isolation of microbial agents less susceptible to regular antibiotics and recovery of isolates that are resistant during antibacterial therapy is increasing throughout the world [5] and [6].

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes; as a result, plants are one of the bedrocks for modern medicine [7]. Plant based antimicrobials represent a vast source of medicine. Plant based antimicrobials have great therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. Further continued exploration of plant derived antimicrobials is needed today [8].

Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century [9]. It is estimated that today, plant materials have provided the models for 50% Western drugs [10]. The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents [11], [12] and [13]. Numerous experiments have been carried out to screen natural products for antimicrobial property [14]-[18]

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The term 'antioxidant' refers to numerous vitamins, minerals and other phytochemicals to protect against the effects caused by reactive oxygen species (ROS). The ROS are highly capable of damaging nucleus and cell membranes by reacting with various vital intracellular molecules like DNA, protein, carbohydrates and lipids. Free radicals and other ROS are derived by two process namely normal metabolic process in the human body and external sources such as exposure to X-rays, ozone, cigarette smoking, air-pollutants and industrial chemicals. The inhibition of free radicals can serve as model for evaluating the activity of Antioxidants [19].

The medicinal plants selected for the present investigation of Antimicrobial and Antioxidant Potential were *Anisochilus carnosus* collected from Tirupathy, Andhrapradesh.

II. RELATED WORK

Medicinal plant extracts prepared with selected ethanol concentrations from eight species, *Peumusboldus* (boldo leaf), *Agathosma betulina* (buchu leaf), *Echinacea angustifolia* (echinacea root), *Humulus lupulus* (hops strobile), *Glycyrrhiza glabra* (licorice root), *Mahonia aquifolium* (Oregon grape root), *Usnea barbata* (usnea lichen), and *Anemopsis californica* (yerba mansa root), were screened for antibacterial activity against four Gram-positive and four Gram-negative pathogens. The antibacterial activity of the extracts (50, 70, and 90% ethanol) was evaluated using a standard well assay and microbroth dilution method. Minimum bactericidal concentrations were also determined for each extract. Plant extracts showed strong antibacterial action against Gram-positive bacteria, *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* and *Streptococcus pyogenes*, while negligible to no inhibitory activity against Gram-negative bacteria; *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella enteritidis* was observed. Among the plant extracts, the boldo, hops, licorice and yerba mansa exhibited a strong antibacterial action at all three ethanol concentrations. Of these four, hops showed the strongest activity at 90% ethanol. *Echinacea angustifolia* extracts did not show any considerable antibacterial activity, while usnea showed strong activity only at 90% against *S. epidermidis*. Except *Echinacea angustifolia* and usnea, the plant extracts were strongly inhibitory towards the MRSA strain. Buchu, yerba mansa and Oregon grape showed higher activity at 50% or 70% on MRSA. The results suggest that the extracts of boldo, hops, licorice and yerba mansa could be considered as potentially effective antibacterial agents against Gram-positive bacteria including MRSA. For hops, buchu, Oregon grape and usnea, the activity is independent on the concentration of ethanol used in the extraction procedure. The ratio of ethanol/water mixture used for extraction of medicinal plants is an important factor to obtain optimum antibacterial [28].

The present work was conducted aiming to evaluate the effect of different solvent extracts on the antioxidant and antibacterial activities of *Annona squamosa* L. leaves. Four solvents were chosen for the study namely; methanol 80%, acetone 50%, ethanol 50% and boiling water. Acetone and boiling water gave the highest extraction yields as compared to methanol and ethanol. Total phenolic contents of the four extracts were significantly different with acetone being the most efficient solvent and water being the least efficient one. Correlation coefficient between the total antioxidant and total phenolic content was found to be $R^2 = 0.89$ suggesting the contribution of phenolic compounds of the extract by 89% to its total antioxidant activity. The extracts were capable of scavenging H_2O_2 in a range of 43–54%. Reducing power of the extracts increased by increasing their concentration. The extracts were found to exert low to moderate antibacterial activity compared to a standard antibacterial agent. The bacterial inhibition of the extracts was found to positively correlate with their phenolic contents [29].

Thus the present study was carried out using *Anisochilus carnosus* for Antimicrobial and Antioxidant potential activities.

III. MATERIALS AND METHODS

Collection of Plant

The fresh plant was collected from Tirupathy is authenticated by Dr. Madhavachetty, Srivenkateswara university, Tirupathy, Andhrapradesh, India, comparing with the spotter (specimen) Rapinat herbarium of St. Joseph College at Trichy in Tamil Nadu. [Specimen No: RHT 563, 10567].

Preparation of organic solvents based extract:

The air dried dissected plant materials were prepared as extracts using ethanol, chloroform, acetone & diethyl ether appropriately. The different extracts were prepared using Soxhlet method and incubated for 7 hours. The ethanol

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based leaf extract, showed very high activity. So the concentrated extract of ethanol was used for the further studies. Healthy leaves of *Anisochilus carnosus* were collected from the tissue cultured saplings invitro. The leaves were screened for contamination and thoroughly washed. It was then dried, grounded in a mechanical mixer-grinder, and extracted with ethanol by maintaining the powder: solvent ratio as 1:6 using a Soxhlet apparatus. The crude extract obtained was concentrated at 40°C under reduced pressure (72 mbar) with a Rotavapor. The dried extract was weighed to determine the yield of soluble constituents and stored in a vacuum desiccator at room temperature until further use [21].

Antimicrobial and Antifungal activity

Pure bacterial culture were collected from the Microbial Type of Culture Collection (MTCC), MTCC *E.coli*, MTCC 424 *Pseudomonas aeruginos* and MTCC 441 *Bacillus subtilis*, The organism for the study *Candida albicans* 183 *Aspergillus niger* (MTCC 282), and *trichoderma viridae* was obtained from M.T.C.C, Chandigarh, India and subjected for screening studies. Bacterial and fungal strains to be tested were streaked in nutrient agar plates to obtain the pure culture. The pure cultures were streaked on Luria agar slant and stored at 4°C.

The agar well diffusion method was used to determine the growth inhibition. Sterile Muller Hinton agar plates were prepared. Three well of 6mm diameter were prepared with the help of a sterile well puncher. The 6 hour culture broth was taken and swabbed over the plate using sterile cotton swab to obtain a uniform lawn culture. The wells were filled with 10µl of the prepared dilutions of the plant extract using dimethyl sulphoxide (DMSO). DMSO was used as the control. Then the plates were incubated at 37 °C for 24 hr. After incubation, diameters of the inhibition zones were measured and tabulated. The ethanol extract was subjected to microbiological screening using different concentrations like 200µg, 400µg, 600µg, 800µg employing disc diffusion method, Diameter was measured (mm) & recorded [22].

Minimum inhibitory concentration:

Minimum inhibitory concentration of *A. carnosus* was found using broth dilution technique. Seven test tubes containing 1 ml of sterile Sabouraud's Dextrose broth were prepared. For assaying plant extract, the starting concentration kept at 8mg/ml in the first tube containing 1 ml of sterile Sabouraud's dextrose broth. The plant extract were serially diluted at the concentration 8, 4, 2, 1, 0.5, 0.25, 0.125 mg/ml. To each of this test tube, 0.1 ml of 6 hr culture of *bacterial and fungi* was added. The tubes were incubated at 30 °C for 24-48 hr. The test tubes were examined for visible turbidity. 1ml of the above mentioned tube was transferred to a microfuge and centrifuged at 5,000 rpm for 4 min. The supernatant was completely removed using micropipette and the pellet was suspended in 0.1 ml sterile distilled water. The resulting bacterial suspension was serially diluted and plated on Sabouraud's dextrose agar plates. The end point of complete inhibition was defined as the minimum inhibition concentration of the test compound in the original tube which fails to yield discernible growth when sub cultured. The Nalidixic acid was used as the reference standard [23].

Determination of total phenols by spectrophotometric method

Estimation of total phenolics

Quantification of polyphenolics:

Total phenolic content of the extract was determined using the Folin-Ciocalteu reagent method described by Lister and Wilson (2001). To the 50 µl of each extract concentrations (25, 50, 100, 200 and 400 µg), 2.5 ml of Folin-Ciocalteu reagent (1/10 dilution) and 2 ml of 7.5% Na₂CO₃ (w/v) were added and mixed well. The mixture was incubated at 45°C for 15 min. The absorbance were measured at 765 nm using a ELICO SL-150 UV-Vis spectrophotometer with Na₂CO₃ solution (2 ml of 7.5% Na₂CO₃ in 2.55 ml of distilled water) as blank. Gallic acid was used as a standard, and results were expressed as GAE (gallic acid equivalence) in µg [24].

DETERMINATION OF ANTIOXIDANT POTENTIAL

The antioxidants play an important role in nutritional by lengthening the self life of food and reducing nutritional losses and formation of harmful substances. Antioxidants inhibit and scavenge radicals, thus providing protection against infections and degenerative diseases. They can either directly scavenge or prevent generation of ROS (Reactive Oxygen Species).

Reducing power of extract:

The ability of the extract to reduce Fe³⁺→ Fe²⁺ was analysed. 25, 50, 100, 200 and 400 µg of the extract were mixed with 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of 1% potassium ferricyanide followed by incubation at 50°C for 30 min. 2.5 ml of

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10% trichloroacetic acid was later added and the tubes were centrifuged at 3000 rpm for 10 min. 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated increased reducing power.

Free radical scavenging activity:

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of free radical scavenging activity of the extract. Different concentrations (25, 50, 100, 200 and 400 µg) of the leaf extract were added, at an equal volume, to the ethanolic solution of 3 mL of DPPH* (0.1mM). After 30 min of incubation in the dark in room temperature, the absorbance was recorded at 517 nm using a UV-Vis Spectrophotometer. Ascorbic acid was used as a standard. The percentage inhibition (I %) was calculated using the formula,
 $I \% = \frac{[Abs (Control) - Abs (Sample)]}{Abs (Control)} \times 100$. [25].

Total Antioxidant Activity

The total antioxidant activity was measured using the extract of different concentrations and the absorbance was noted at 695 nm using Ascorbic acid as standard curve, from which the total antioxidant activity can be measured.

The total antioxidant capacity was measured by spectrophotometric method. At different concentration ranges, aqueous extracts were prepared in their respective solvents and combined in an Eppendorf tube with 1ml of reagent solution (0.6M H₂SO₄, 28mM sodium phosphate, 4mM ammonium molybdate mixture). The tubes were incubated for 90min at 95°C. The mixture was cooled to room temperature and the absorbance was read at 695nm against blank. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicates and values are expressed as equivalents of ascorbic acid µg per mg of extract [26,27].

IV. RESULTS

Antimicrobial & Antifungal - Zone of Inhibition at Various Concentrations

Table 1

Concentration of ethanol extract (µg/Disc) / Zone of inhibition(in mm)							
S. No	Name of the organisms	+ve control	-ve control	200 µg/ Disc	400 µg/Disc	600 µg/Disc	800 µg/Disc
1	<i>Escherichia coli</i>	10	-	12	13.2	13.7	14
2	<i>Pseudomonas aeruginosa</i>	11	-	8	9.7	11	11.4
3	<i>Bacillus subtilis</i>	12	-	10	11.5	13.8	15
4	<i>Candida albicans</i>	6	-	8	7.2	7.4	9.5
5	<i>Aspergillus niger</i>	2	-	6.2	6.9	8.4	9
6	<i>Trichoderma Viridae</i>	1	-	6	5.7	6.4	7.2

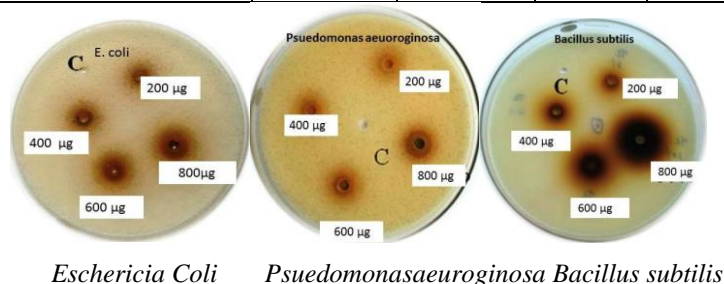
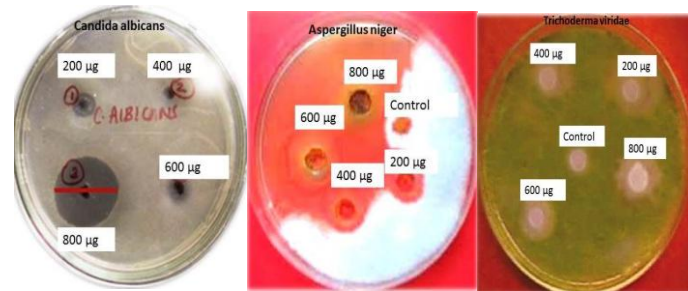


Fig.1 Antimicrobial – Zone of Inhibition

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Candida albicans *Aspergillus niger* *Trichoderma viridae*

Fig. 2 Antifungal – Zone of Inhibition

Among the Bacterial strains the maximum zone of inhibition was obtained from *Bacillus subtilis* of 15mm at 800 µg/ disc and the minimum zone of inhibition with *Pseudomonas aeruginosa* of 8mm at 200 / disc. Among the Fungal strains used *Candida albicans* showed maximum zone of inhibition of 9.5mm at 800 µg/disc and minimum inhibition was obtained using *Trichoderma viridae*, 6mm at 200 µg/ disc.

Total phenolics

Table 2

CONCENTRATION (µg/ml)	OPTICAL DENSITY at 517 nm	PHENOL CONTENT
25	1.2321	220 µg/ml
50	1.9634	360 µg/ml
100	2.1523	390 µg/ml
200	2.7577	490 µg/ml
400	2.9861	540 µg/ml

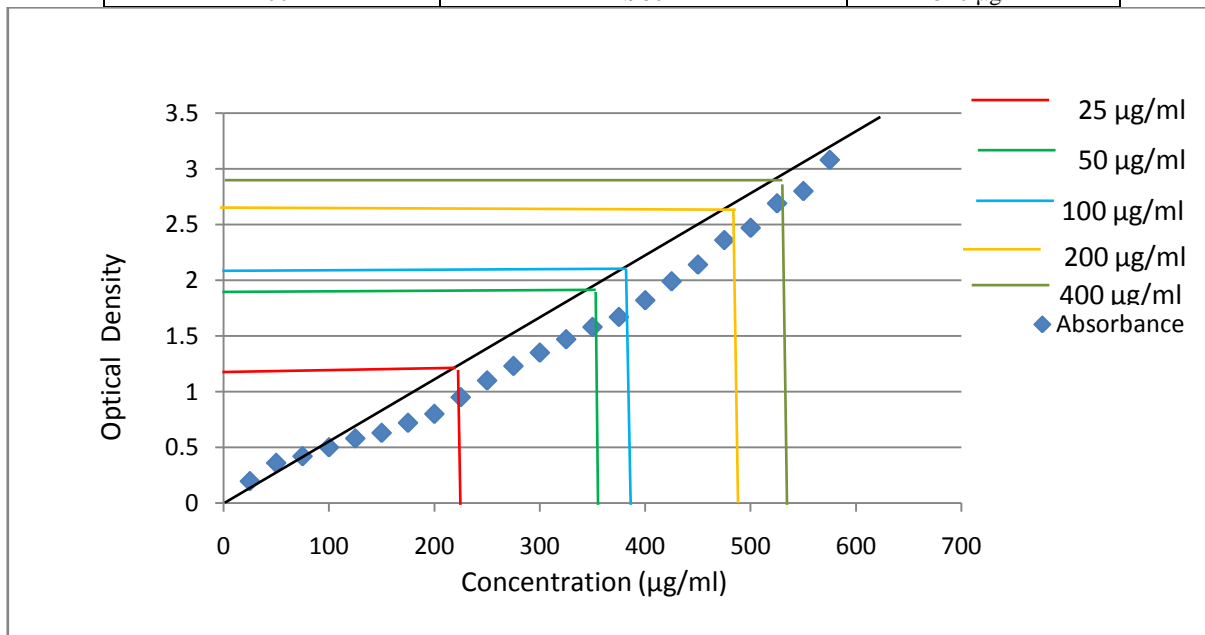


Fig. 3 Estimation of Total Phenolic Content

The Total phenol content at concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml are found to be 220 µg/ml, 360 µg/ml, 390 µg/ml, 490 µg/ml and 540 µg/ml respectively. High Phenol content was found to be present in 400 µg/ml.

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REDUCING POWER OF EXTRACT:

The ability of the plant extract to reduce Fe³⁺ to Fe²⁺ was analysed by taking different concentrations of the extract and its absorbance was measured.

Effect of Reducing Power

Table .3

CONCENTRATION (µg/ml)	OPTICAL DENSITY (OD) @ 700 nm
25	0.0908
50	0.1381
100	1.3228
200	1.3613
400	1.4868

It was observed that for the reducing power of extract sharply increased as the concentration was increased.

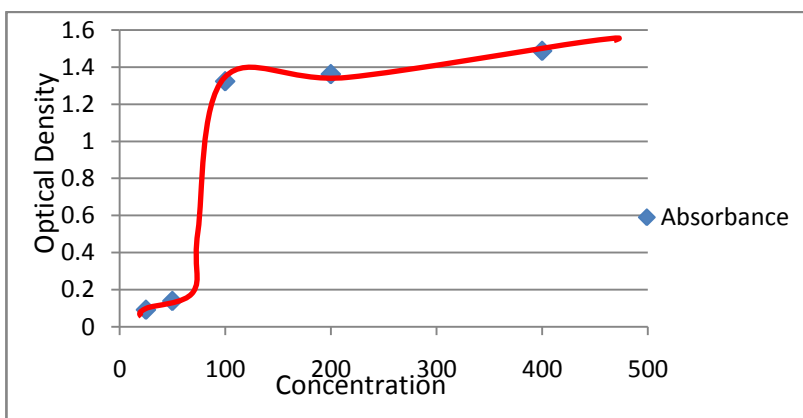


Fig. 4 Graph showing the effect of reducing power

FREE RADICAL SCAVENGING ACTIVITY

The ability of the plant extract to inhibit the DPPH amount was calculated at 517 nm.

Effect Of Free Radical Scavenging Activity

Table. 4

CONCENTRATION (µg/ml)	OPTICAL DENSITY at 517 nm
25	2.9782
50	3.0291
100	3.1771
200	3.7314
400	3.8767
Control	3.0000

It was observed that the Free Radical Scavenging activity for 25 µg/ml inhibited 0.72 %DPPH, for 50 µg/ml was 0.97%, for 100 µg/ml was 5.90%, for 200 µg/ml the inhibition was found to be 24.38% and in 400 µg/ml the highest inhibition was found as 29.23%.

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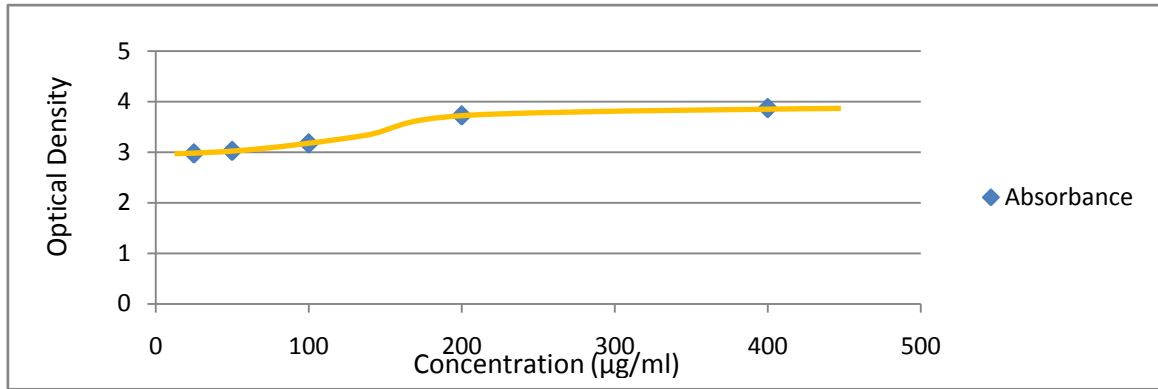


Fig. 5 Graph showing the effect of Free Radical Scavenging Activity

Total Antioxidant Activity

The Total Antioxidant Activity was estimated at 695 nm using ascorbic acid as standard curve for five different concentrations.

Table. 5
Estimation Of Total Antioxidant Activity

Concentration (ppm)	Absorbance (695 nm)	Estimation of total antioxidant activity (mg/ml)
0.2	0.1323	10
0.4	0.2064	14
0.6	0.3506	19.5
0.8	1.5359	80.2
1.0	1.9734	100

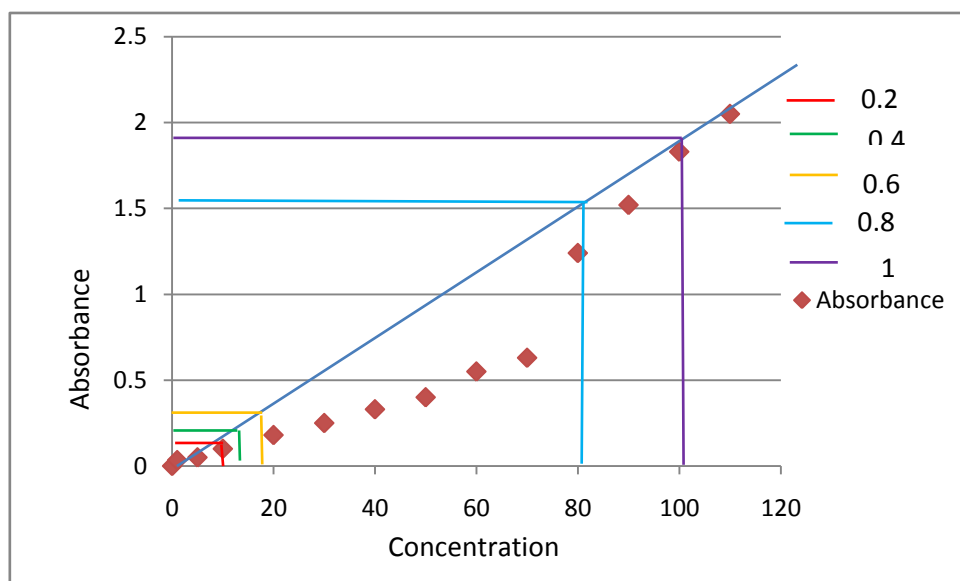


Fig. 6 Graph showing the Total Antioxidant Activity

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V. DISCUSSION

The preliminary studies proved the Antimicrobial activity of the leaves ethanolic extract against three bacterial and three fungal strains. The plant extracts are considered as best source of bioactive compounds particularly for traditional healers as they contain components of therapeutic values. The bioactive compounds have been detected for either bacteriostatic have very minimum or no toxicity to host. The Zone of Inhibition for *E. Coli* was found to be maximum at 800 µg/ disc of 14mm and minimum at 200 µg/ disc of 12mm, *B. subtilis* was found to be maximum at 800 µg/ disc of 15mm and minimum at 200 µg/ disc of 10mm and *P. aeruginosa* was found to be maximum at 800 µg/ disc of 11.4mm and minimum at 200 µg/ disc of 8mm. The Antifungal activity against *Candida albicans* showed maximum Zone of Inhibition at 800 µg/ disc of 9.5mm and minimum at 200 µg/ disc of 8mm, *Aspergillus niger* showed maximum Zone of Inhibition at 800 µg/ disc of 9mm and minimum at 200 µg/ disc of 6.2mm and *Trichoderma viridae* showed maximum Zone of Inhibition at 800 µg/ disc of 7.2mm and minimum at 400 µg/ disc of 5.7mm. Antioxidant Potential of the leaf extract was carried out for the estimation of Total Phenolics, Free Radical Scavenging, Reducing Power and Total antioxidant activity. The study proved that the Total Phenolics activity was maximum at concentration of 400 µg/ml of 540 µg/ml. The Free Radical IC₅₀ was 29.23%. The reducing Power of the extract increased as the concentration increased. In the numerous methods of antioxidant assay, DPPH radical scavenging activity, xanthine oxidase inhibition assay and nitric oxide radical scavenging assay was followed for the evaluation of antioxidant activity of the reported plants.

V. CONCLUSION

Antibacterial and Antifungal screening was carried out against various strains using the agar well diffusion method. Against all the extracts used the ethanolic leaf extract was proved to have very high activity. The study proves that the Antibacterial and Antifungal actions in the leaves extract is due to the bioactive compounds present in them. The antioxidant assays such as DPPH radical scavenging activity, Total Phenolics, Reducing Power and Total Antioxidant assay were used for the evaluation of antioxidant activity of the *Anisochilus carnosus*. It was concluded that the leaves of *Anisochilus carnosus* has potent medicinal importance as reported by us for its antimicrobial and antioxidant activity.

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