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Original Research Article

Studies on Endophytes, Phytochemicals, Antioxidant and Antimicrobial Property of *Vitex altissima*

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ABSTRACT

Background and objectives: *Vitex altissima*is medicinal plant, traditionally used for treating various diseases. Plant extracts shows the good antioxidant, antimicrobial activity and endophytes. Plant extracts prepared with methanol exhibited maximum antioxidant activity when compared to other extracts. To analyse the phytochemical constituents in leaf and endophytic extracts. The aim of this study was to determine the Antioxidant potential, antifungal and antibacterial activity of various extracts and compare the property of leaf and endophytic extracts.
Materials and Methods: A total 5 different extracts of *Vitex altissima* were taken for study, i.e, antioxidant, antimicrobial activity and phytochemical analysis. The radical scavenging activity was carried out by two sensitive methods1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethybenzothiazoline-6-sulphonic acid (ABTS)and antimicrobial activity by DMSO (Dimethysulfoxide).
Results: Methanol and chloroform extracts showed good Antioxidant property then other plant extracts. Methanol extracts showed the highest total phenolic content and reducing power compared to other extracts. Antimicrobial activity of this plant extracts indicated that the plant did not effective against the bacteria, Salmonella Species, E. coli and P. aeruginosa Species. The hot water extract showed high activity in *salmonella specie*s, *Enterobacteria specie*s and *Staphylococcus species*. While the methanol extracts demonstrated the least activity.
Conclusion: According to the results of this study, *Vitex altissima* could be used as a potential source for new drugs development for many disease and disorders. However, further studies need to be carried out to utilize this plant as powerful source for pharmaceutical drugs.

Keywords: Medicinal plant, antioxidant, endophytes, pseudomonas aeruginosa, Phytochemicals, *Vitex altissima*

INTRODUCTION

Plants are the source of different constitutive which supports to the modern medicinal system. The phytochemical are the plant derived chemical and they compound, flavonoids, tannins act as

antimicrobial ,antifungal, antihypertensive, antitumor, antioxidant activity. These bioactive compounds formed the foundation of the modern drugs as we know today. Plants are a good source of biologically active compounds known as phytochemicals. The phytochemicals have been found to act as antioxidants by scavenging free radicals, and many have therapeutic potential for free radical associated disorders . It is well known that free radicals are the major cause of various chronic and degenerative diseases such as coronary heart disease, inflammation, stroke, diabetes mellitus and cancer (Meena,et.al.,2011).

Antioxidants are substances that protect cells from the cellular damage caused by unstable molecules known as free radicals induced oxidative stress. Antioxidants neutralize free radicals as a natural by-product of normal cell processes. Phenolic compounds, which are widely distributed in many fruits, vegetables, and tea are believed to account mainly for the antioxidant capacity of many plants (Jocab and walker, 2008).

The phytochemical are the plant derived chemical and they compound, flavonoids, tannins act as antimicrobial, antifungal, antihypertensive, antitumor, antioxidant activities. These bioactive compound formed the foundation of the modern drugs as we know today (Elizabeth, et.al.,2005).All plants in natural ecosystems appear to be symbiotic with fungal endophytes. Endophytes are the plant-associated microorganisms that live within the living tissues of their host plants without causing any harm to them. Almost all groups of microorganisms have been found in endophytic association with plants may it be fungi, bacteria or actinomycetes (Strobel,2010).

Mutualism interaction between endophytes and host plants may result in fitness benefits for both partners. The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine. Bioactive natural compounds produced by endophytes have been promising potential usefulness in safety and human health concerns, although there is still a significant demand of drug industry for synthetic products due to economic and time-consuming reasons (Strobel,2010).Antioxidants neutralize free radicals as a natural by-product of normal cell processes. Phenolic compounds, which are widely distributed in many fruits, vegetables, and tea are believed to account mainly for the antioxidant capacity of many plants (lee,2004).

Chemicals Used

Chemicals used for the reagents and media were produced from Hi-media laboratory Pvt Ltd, and Merk India limited, Mumbai:
Hexane, Ethyl acetate, Methanol, Ethanol, Con-Sulphuric acid, tannic acid, ascorbic acid, disodium phosphate, sodium dihydrogen phosphate, Sodium Hydroxide (NaOH), lead acetate, Hydrochloric acid (HCl), Sodium hypochlorite, silica gel, Folin-Ciocalteu (FC) reagent, potassium ferric cyanide, Potassium Iodide (KI), Formic acid, Chloroform, Hydrogen peroxide, Dextrose, Sodium citrate, Citric acid, Sodium chloride (NaCl), Dimethyl sulfoxide (DMSO), 2,2-azino-bis (3-ethybenzothiazoline-6-sulphonic acid (ABTS),1,1-diphenyl-2-picrylhydrazyl (DPPH), Benedicts reagent , DNS , CBB-250 , Methylene Blue.

Instruments used

Soxhelt apparatus, Spectrophotometer, Laminar Air Flow, Incubator, Auto clave, pH meter, Centrifuge, Water bath, Thin layer chromatography (TLC) set, Glass

wears , Ultra Violet – Spectrophotometer , Ultra Violet – cabinet ,Microscopes.

Collection of leaf sample

The leaves of the *V. altissima* was collected from Chiklihole Reservoir Dam near Dubare Coorgkodagu district, Karnataka, India in the month of January,2013 used for further study in the Department of Biochemistry , Mangalore university, India.

Preparation of plant extract

The fresh leaves were washed with tap water 3 to 4 times for removal of soil and dust particles. Then washed with 0.5% Sodium Hypochlorite, this was followed by deionosed water 3 to 4 times .The washed leaves were shade dried for a week then powdered. The leaf powder samples were first extracted with hexane, and filtrate was collected the resultant residue was re-extracted with Chloroform and resultant supernatant was collected. The collected residue was again re- extracted using methanol. The resultant supernatant was preserved and the residue was discarded. Three extracts collected from various solvent extractions were evaporated in the oven at 40˚C to obtain the dry paste. The final past or powder was stored at 8^0c for the further studies.

Preparation Of Water Extract

Cold water extract

The fresh leaves were washed with tap water 3 to 4 times for removal of soil and dust particles Then washed with 0.5% Sodium Hypochlorite, this was followed by deionised water 3 to 4 times. Then leaves were grained with distilled water, then filtered using muslin cloth Filtrate was centrifuge at 5000rpm for 15 minutes. Then filter by using What'sman No. 1 filter paper. Filtrate was collected and stored in refrigerator at 10^0c for further study.

Hot water extract

The fresh leaves were washed with tap water 3 to 4 times for removal of soil and dust particles . Then washed with 0.5% Sodium Hypo chloride, this was followed by deionised water 3 to 4 times. Then leaves were grained and boiled with distilled water, with constant stirring for 30minutes. Solution was allowed to cool to room temperature, then filtered using muslin cloth. Filtrate was centrifuge at 5000rpm for 15minutes. Then filter by using Whattman No. 1 filter paper. Filtrate was collected and and stored in refrigerator at 10^0c for further study.

Preparation Of Endophytic Extract

Isolation of endophytes

Surface sterilization of the plant material

The surface sterilization most frequently utilized to detect and quantify endophytes involves isolation from surface-sterilized host plant tissues. Endophytic isolation was carried out under aseptic conditions. Different symptomless parts of the selected plants such as leaves and stem were used for the isolation of endophytes.

The collected plant material used for the isolation was first surface sterilized following the method of Sadananda et.al. (2011) with few modifications. Plant material was first cleaned by washing several times under running tap water and then cut into small segments. Surface sterilization was performed by sequentially rinsing the plant material with 70% ethanol (C_2H_5OH) for 30 seconds, then with 0.01% mercuric chloride ($HgCl_2$) for 5 minutes followed by 0.5% sodium hypochlorite for 2-3 minutes and finally with sterile distilled water for 2-3 times. Plant materials were place under aseptic conditions for shade dried.

Isolation of the endophytes

After proper drying, the surface sterilized plant material i.e. leaves were cut into smaller pieces and each piece was placed on potato dextrose agar (PDA) medium. Similarly, stem were cut vertically into small segments to expose the inner surface and then placed on the PDA plates.

All the plates were incubated at 28ºC to promote the growth of endophytes and were regularly monitored for any microbial growth. On observing the microbial growth, sub culturing was done. Each endophytic culture was checked for purity and transferred to freshly prepared PDA plate.

Determination of Total phenolic content

The total phenolic or tannic acid content of different extracts of the was determined by the method (Silva,et.al.,2005) with slight modifications. The standard tannic acid and plant extract was taken in the range of 0.0 to 1 ml (100µg/ml) and made up to 1 ml with water. Then add 500µl of the foline-ciocateu reagent and 1250µl of the 7% sodium carbonate were added and the same procedure was carried out for different leaf extracts and absorbance was taken at 725nm and phenolic content of the extracts was expressed as the tannic acid equivalents.

Free Radical Scavenging Assay by DPPH Method

The DPPH radical scavenging activity of "*V. altissima*" extracts and ascorbic acid (Standard) were determined according to the method (Latha,et.al.,2007) with slight modification. 0.1mM DPPH reagent was prepared by using ethanol and standardize to 1.9 OD at 517nm. The standard ascorbic acid and Plant extract (concentration range between 0 to 500µg) was taken separately in different aliquots in the range of 0.0 to 1 ml and made up to 1ml using ethanol. To this 2ml of the 0.1mM DPPH was added and incubated in dark for 30 minutes at room temperature. Absorbance was read at 517nm and percentage of the inhibition was calculated using formula.

Radical scavenging Assay= A DPPH - A Sample/A DPPH *X* 100

A DPPH is the absorbance of DPPH radical + ethanol

A sample is the absorbance DPPH radical + sample or standard.

Free radical scavenging activity by ABTS method

Determination of antioxidant activity by ABTS assay, the procedure followed the method of Aiyegoro, et al. (2010) with some modification. The reagent solutions included 7.5mM $ABTS^{\cdot+}$ and 3mM potassium persulfate. The working solution was prepared by mixing equal volume of two reagents and allowing them to react for 12-16 hrs. at room temperature in dark. The reagent was then diluted by mixing 1ml $ABTS^{\cdot+}$ solution with 60ml of methanol to obtain an absorbance of 1.1±0.02 at 734nm. Fresh $ABTS^{\cdot+}$ was preferred for each assay. The standard ascorbic acid and Plant extract (concentration range between 0 to 500µg) was taken separately in different aliquots in the range of 0.0 to 1 ml and made up to 1ml using ethanol. 1ml of freshly prepared $ABTS^{\cdot+}$ was added and incubated in dark for 2h. Then the absorbance was read at 734nm using a spectrophotometer. The % of inhibition was calculated.

Total reducing power

The reducing power of *"V. altissima* "was determined according to the method of Nabavi,et.al.,2008with some modifications . The aliquots of standard ascorbic acid in range of 0.0 to 1ml (10mgµg/ml) and plant extract in the range of 0.0 to 1ml (10mg/ml) were taken and volume was made up to 1ml by ethanol, this was followed by addition of 2ml phosphate buffer, 2.5ml 1% potassium ferric cyanide and incubated at 50˚c for 20 minutes. After incubation 2.5ml 10% TCA was added and mixed well. Then 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride was added. Mixed well and absorbance was taken at 700nm and total reducing power of the extract was expressed in ascorbic acid equivalents.

Anti Microbial Study Of The Plant And Endophyts

Determination of Antibacterial activity was performed using *Salmonella*

sps, Escherichia coli, Enterobacteria sps and *Staphylococcus sps* For antibacterial evaluation agar cup diffusion method was followed (Elizabeth,2005). On Nutrient Agar plates above mentioned broth culture were evenly spread by using cotton swab and agar cups were prepared by scooping out the media with cork borer. The wells were filled with 10 - 30µl of crude extract dissolved in Water (1mg/ml).Water was used as control. Standard Ampicillin (1mg/1ml). Plates were incubated at 37^{O} C and zone of inhibition were measured and recorded.

RESULT AND DISCUSSION

Phenolic Content

All the plant extract had Phenolic content. among them , Methanolic extract had High phenolic content and Hexane and cold water extract had very less phenolic content (Fig.no.01).

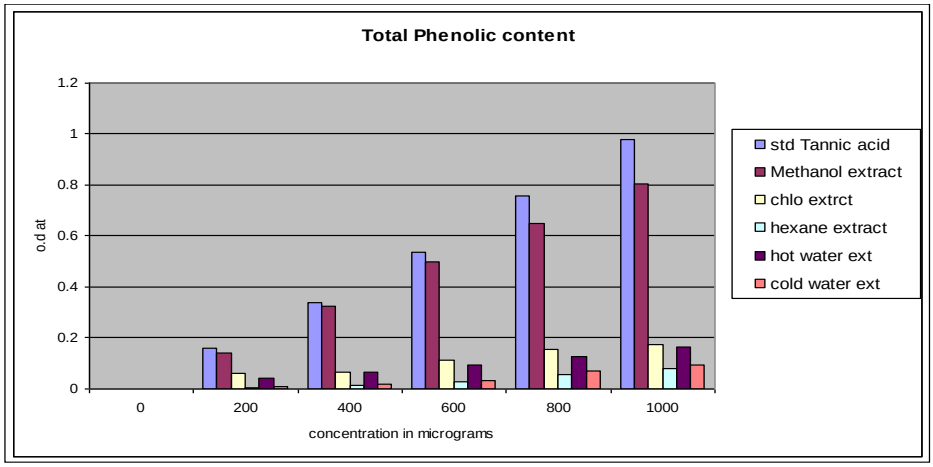

Fig.no.01showing amount of phenol present in different extracts of plant

Phenolic compounds are present in plant and have multiple biological effects. The antioxidant property of phenolic compound is mainly because of their redox properties which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Rivas,et.al.,2000).

Free radical Scavenging Assay by DPPH

Out of five plant extracts, Methanol, chloroform, Hot water and Cold water were showed the free radical scavenging activity (Fig.02 and 03). However, among them, Methanolic extract had highest antioxidant activity. (Latha, et,al.,2007) The antioxidant activity expressed by *A.niger*. endophytic extract less when compared to Methanol extract of *V. Altissima.* Least activity was recorded in the Hexene and cold extract. The result of the present study confirmed the antioxidant activity of endophytes of plant as well as in the plant extract with methanol.

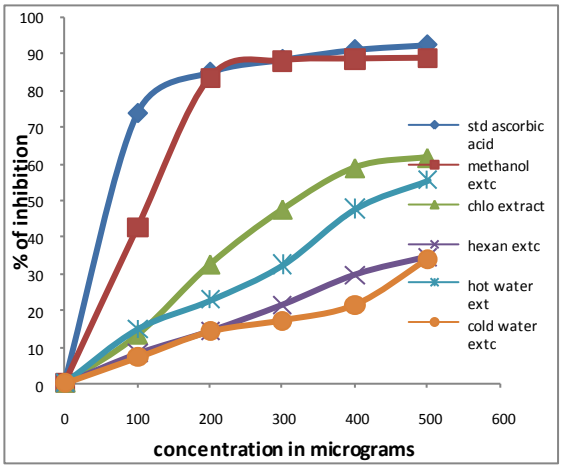

Fig.no.02 showing free radical scavenging assay of plant extract.

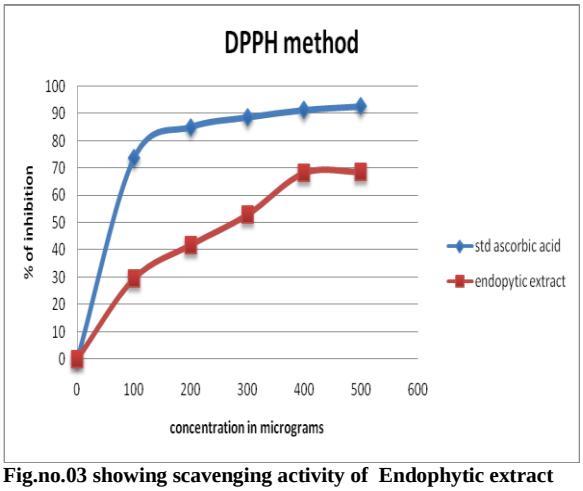

Fig.no.03 showing scavenging activity of Endophytic extract

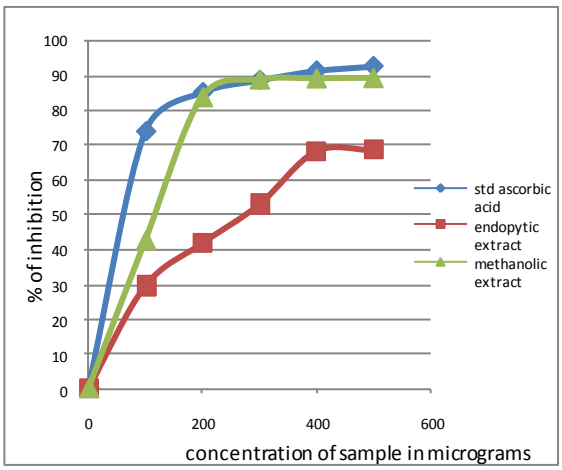

Fig.no.04 showing scavenging activity of plant methanolic extract and endophytic extract.

Antioxidants are thought to be highly oxygen specific mediated tissue impairments. It has been reported that many antioxidants compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, anti-mutagenic, ant carcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Rivas,et al.,2000).

Methanol extract of *Vitex altissima* had showed highest antioxidant activity compared to *A. niger*as shown in the (Fig.no.04).Some antioxidant compounds isolated from endophytic fungi and antioxidant activities have also been reported (Harbane,1988). The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability (Baumann et al.,1979).

Free radical scavenging activity by ABTS method

In this method the methanol, chloroform, hot water extract, expressed maximum activity and hexane and cold water extract had least activity (Fig.no.05)

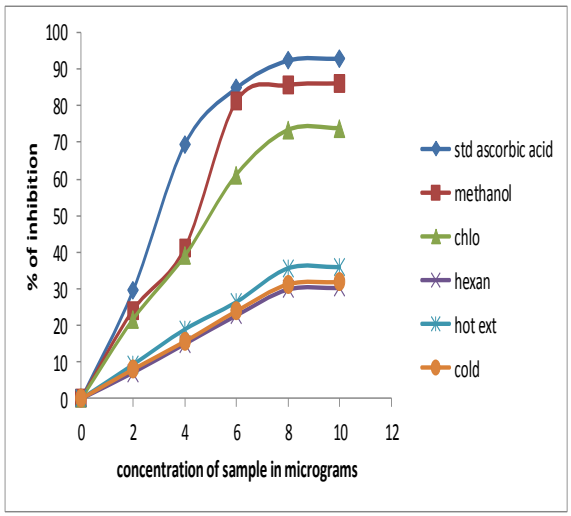

Fig.no.05,Scavenging activity of different plant extract

The ABTS radical cation is reactive to words most antioxidant including phenolic, thiols and vitamin C (Sadananda, et,al.,2009). During this reaction the blue ABTS radical cation is converted back to its colourless neutral form. ABTS scavenging assay of *V. altissima* also supports antioxidant activity in different extract of plant.

Total reducing power:

The total reducing power in five plant extract support to the Antioxidant activity of the plant and out of five extracts. Methanol extract had higher reducing power as given below (Fig.no.06) followed by chloroform extract (Harbane,1988). The results confirmed the antioxidant activity in different extract of plant.

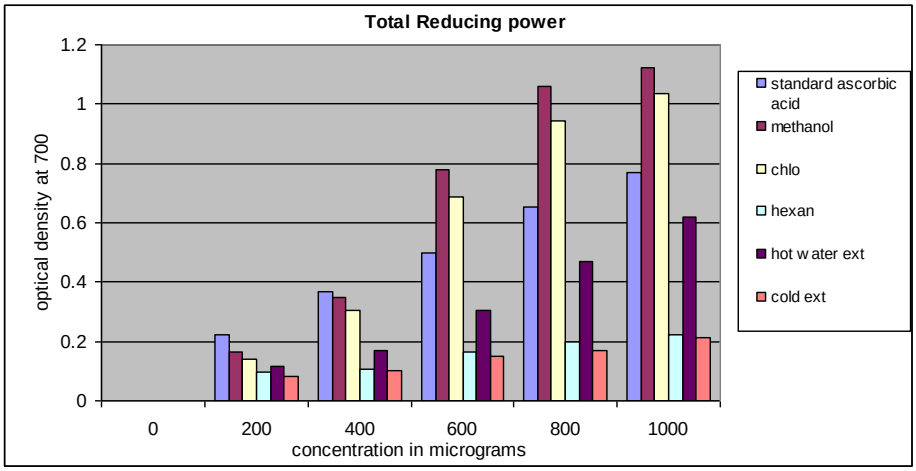

Fig.no. 06. Total reducing power of plant extract

Antimicrobial Activity

In Antibacterial study, Methanol extract showed inhibition zone against *E. coli*(fig.no.07).Hot water extract showed inhibition zone for *Salmonella (fig.no.10)* , *Enterobacteria sps* (fig.no.08) and *Staphylococcus sps (fig.no.*09). However, Maximum inhibition zone was recorded against *Salmonella spp*. Followed by, *Enterobacteria sps* (Table 01).The endophytic extract showed inhibition activity against *E. Coli* only.

Table.01 inhibition zone of different plant extracts against some pathogenic bacteria

Inhibition zone (mm)				
Bacterial strains	*E.Colisps*	Staphylococcus sps	*Salmonella sps*	*Enterobacteriasps*
Water	-	-	-	-
Ampicillin	13	14	11	12
Methanol extract (ME)	06	-	-	-
Chloroform extract(CE)	-	-	-	-
Hexane extract(HE)	-	-	-	-
Hot water extract(HWE)	-	09	12	11
Cold water extract(CWE)	-	-	-	-
Endophytic extract(EE)	06	-	-	-

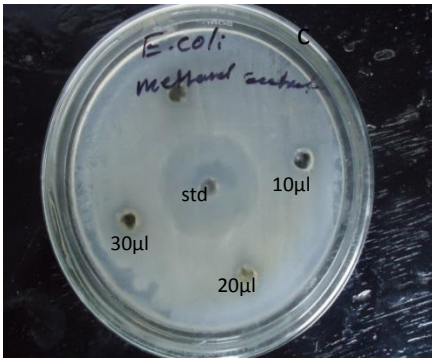

Fig.no.07,ME- *E.coli.*

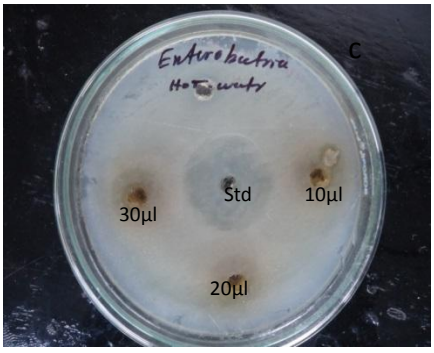

Fig.no.08 HWE- *Enterobacteria*sp

Zongo,et. al.,(2010) reported that the antimicrobial activity of endophytic extract of *A. niger* showed significant effect on different Gram positive and negative bacteria and on different fungi. The antibacterial activity of *Vitex nigundo* (leaves) against dermatophytic bacteria, *Staphylococcus aureus was reported by*

Agrawal,et.al.,(*2011).* The result the present study confirmed that the plant *V. altissima* had antibacterial activity against wide range of pathogenic bacteria.

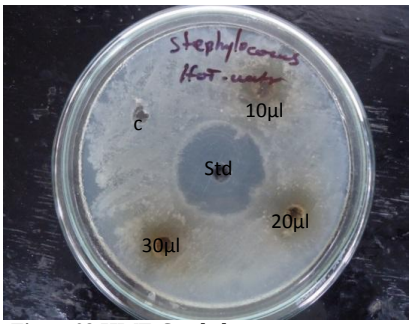

Fig.no.09,HWE-*Staphylococus*

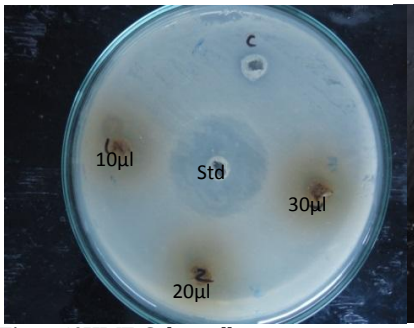

Fig.no.10HWE-*Salmonella*sp

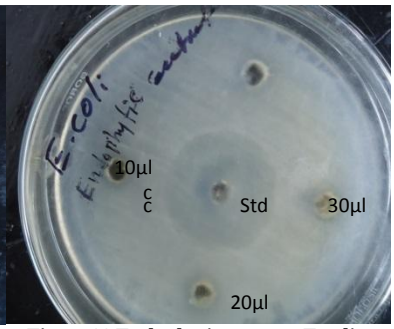

Fig.no.11.Endophytic extract- E.coli

DISCUSSION

Antioxidants are the first line of defence against free radical damage, and are critical for maintaining optimum health and wellbeing *(*Wang et al., 2009). The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Iwu et al., 1999).In this study no work has done even those study for various medicinal properties. So not report regarding the phytochemical analysis, antioxidant activity and antimicrobial activity. Leaves has taken for the present study and aimed that scientific validation of traditional uses. The total phenolic content was high in the methanol extract. After the phytochemical analysis the plant extract was studied for antioxidant activity and antimicrobial activity .Free radical scavenging activity was evaluated using DPPH and ABTS free radical and antimicrobial activity was evaluated using four different bacteria species. The overall antioxidant activity of *Vitex altissima* was the strongest, the present study of in vitro antimicrobial evaluation of plant extract (hot water extract) demonstrated antimicrobial activity at high level against three bacteria species then the other extracts .The wells were filled with 10-30µl of crude extract dissolved in water(1mg/ml). The hot water extract showed activity (12 mm zone diameter of inhibition) in *salmonella specie*s, (11 mm zone diameter of inhibition) in *Enterobacteria specie*s and (09 mm zone diameter of inhibition) in *Staphylococcus sps*. while the methanol extracts demonstrated the least activity (06 mm zone diameter of inhibition) shown in the (Figure 07).The three extracts did not demonstrate any reasonable activity against all the clinical isolates bacteria species , *Salmonella sps, Enterobacteria sps, staphylococcus sps, E. coli sps*. However, Maximum inhibition zone was recorded against *Salmonella spp*. Followed by, *Enterobacteria sps. The endophytes* Methanol extract of *Vitex altissima* had showed highest antioxidant activity compared to *A. niger*as shown in the (Fig.no.04).Some antioxidant compounds isolated from endophytic fungi and antioxidant activities. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. Further studies at the molecular level and in vivo may help to unravel the mechanism of action of the lead molecules present in this plant.

CONCLUSION

Vitex altissima plant have a variety of phytochemicals and rich sources of endophytes which possess a high antioxidant properties activity, hence the plant could be used as a potential source for new drugs development for many disease and disorders. However, further studies need to be carried out to utilize this plant as powerful source for pharmaceutical drugs.

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