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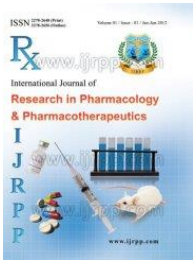

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# Evaluation of anti-tubercular activity of leaves of *vitex negundo* by *in –vitro* method

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## ABSTARCT

One third of the world's population is thought to be infected with tuberculosis. As the searches are going on anti-tubercular natural flavonoids. The leaves of *Vitex negundo* were found to be rich in phytochemical constituents with may have immense pharmacological actions. The literature survey revealed the presences of alkaloids, glycosides, flavonoids, tannins, steroids and terpenoids, carbohydrates, proteins in the entire plant in which flavonoids are responsible for anti-tubercular activity. The results of the present study indicates that different extracts of leaves of *Vitex negundo* exhibited anti-tubercular activity against M.tuberculosis (H37RV Strain) at the doses of 100µg/ml and 50 µg/ml shows the inhibition of growth among all 8 concentrations such as 0.8,1.6,3.12,6.25,12.5,25,50,100 µg/ml by using Alamar blue assay method for *in-vitro* evaluation of anti-tubercular activity.

**Keywords:** *Vitex negundo*, Anti-tubercular activity, Alamar blue assay method,

## INTRODUCTION

Majority of the traditional medicines used in health care are obtained from the plants [1]. The world is gradually turning to herbal formulations which are known to be effective against a large repertoire of disease and ailments .The active component , most often a secondary metabolite varies in quality and quantity for a given plant species for growing in different location . In 2014, there were 1.5 million deaths, more than 95% of death occurred in developing countries. The number of new cases each year has decreased since the year 2000 [2]. About 80% of people in many Asian and African countries test positive while 5-10% of people in the US population test positive by the tuberculin test. Tuberculosis has been present in human since ancient times. Natural products including plants, animals and mineral have been the basis of treatment of human disease. Many effective medicines, including as morphine, aspirin, atropine, ephedrine, reserpine and digitoxin were developed from natural products .In Ayurveda tuberculosis is known tuberculosis is known as rajayakshma, yakshma, shosha, kshaya. Prevention of TB involves screening those at high risk, early detection and treatment of cases and vaccination with the Bacillu Calmette-Guerin vaccine [3]. Treatment requires the use of multiple antibiotics over a long period of time. Antibiotic resistance is a growing problem with increasing rates of multiple

drug resistant tuberculosis (MDR-TB). Tuberculosis is the second cause of death from infectious diseases [4].

*Vitex negundo* belongs to the family called *verbena* family, a small slender tree with quadrangular branchlets densely whitish, tomentose branchlets present throughout India [5]. It includes 77 genera and 3020 species. The species used in medicine are *v.angnuis- castus* and *v.negundo.*Fruits of *v.agus-castus* been used in the treatment of many female conditions, including menstrual disorders, hyperprolactinaemia, infertility, acne, inflammatory conditions, disrupted lactation. The families have great medicinal and economic importance. The roots of clerodendron are used in asthma and cough.

**Figure 1**

## Taxonomical classification

***Kingdom****-Plantae*
***Sub kingdom****-Tracheobionta*
***Division****- Magnoliophyta*
***Class****- Magnoliopsida*
***Subclass****- Asteridaea*
***Order****-Lamiales*
***Family****- Verbenaceae*
***Genus*** *– Vitex*
***Species****- negundo*

## Vitex negundo activities [6]

1. Pet-ether extract: has antifungal activity against Cryptococcus neoformans, acute anti-inflammatory activity.
2. Chloroform extract : cytotoxicity in human cancer cell line, anti feedant activity , anti-bacterial activity
3. Methanolic extract: anti-hyperglycemic effect, anti-oxidative property, growth inhibitory activity human lung and colon cancer cells.
4. Aqueous extract: anti-sciatica activity, spina stenosis
5. Ethanolic extract : Hepatoprotective against anti-tubercular drugs [7]

**Table 1: phytochemical constituents of different parts of *Vitex negundo* [8]**

| PART | PHYTOCHEMICALCONSTITUENTS |
|---|---|
| **Leaves** | igninl-3,6,7,3′,4′-pentamethoxyflavone<br>6′-p-hydroxybenzoyl mussaenosidic acid; 2′-p-hydroxybenzoyl mussaenosidic acid<br>5, 3′-dihydroxy-7,8,4′-trimethoxyflavanone; 5,3′-dihydroxy-6,7,4′- trimethoxyflavanone<br>viridiflorol; β-caryophyllene; sabinene; 4-terpineol; gamma-terpinene; caryophyllene oxide; 1-oceten-3-ol; globulol<br>betulinic acid [3β-hydroxylup-20-(29)-en-28-oic acid]; ursolic acid [2β –hydroxyurs-12-en-28-oic acid]; n-hentriacontanol; β-sitosterol; p-hydroxybenzoic acid<br>protocatechuic acid; oleanolic acid; flavonoids<br>angusid; casticin; vitamin-C; nishindine; gluco-nonitol; p-hydroxybenzoic acid; sitosterol |
| **Seeds** | 3β –acetoxyolean-12-en-27-oic acid; 2α, 3α-dihydroxyoleana-5,12-dien-28-oic acid; 2β,3α diacetoxyoleana-5,12-dien-28-oic acid; 2α, 3β-diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid |

| | |
|---|---|
| | vitedoin-A; vitedoin-B; a phenylnaphthalene-type ignin alkaloid, vitedoamine-A; five other ignin derivatives |
| | 6-hydroxy-4-(4-hydroxy-3- methoxy-phenyl)-3-hydroxymethyl-7-methoxy-3, 4-dihydro-2-naphthaldehyde |
| | β-sitosterol; p-hydroxybenzoic acid; 5-oxyisophthalic acid; n-tritriacontane, n-hentriacontane; n-pentatriacontane; n-nonacosane |
| **Roots** | 2β, 3α-diacetoxyoleana-5,12-dien-28-oic acid; 2α,3α-dihydroxyoleana-5,12-dien-28-oic acid; 2α,3β –diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid; vitexin and isovitexin negundin-A; negundin-B; (+)-diasyringaresinol; (+)-lyoniresinol; vitrofolal-E and vitrofolal-F |
| | acetyl oleanolic acid; sitosterol; 3-formyl-4.5-dimethyl-8- oxo-5H-6,7-dihydronaphtho (2,3-b)furan |
| | Acerosine-5-o-glucoside mono acetate |
| | Vanillic acid |
| | p-hydroxy benzoic acid |
| **Stem** | leuteolin |
| | 3-flavone glycoside |
| | 3,6,7,3’4’-penta methoxy-5-o- gluco pyrono sulrhamnoside |
| | Leucoanthocyanidins |
| | Flavonoid -6-c-glucosyl-5-o-rhamnopyronosys tri methoxy wogonin |
| **Essential oil of fresh leaves, flowers and dried fruits** | δ-guaiene; guaia-3,7-dienecaryophyllene epoxide; ethyl-hexadecenoate; α-selinene; germacren-4-ol; caryophyllene epoxide; (E)-nerolidol; β-selinene; α-cedrene; germacrene D; hexadecanoic acid; p-cymene and valencene. |

Anti-tubercular activity can be determined by Alamar blue assay method. Alamar blue is a reagent, which accompanying assay used to quantify cellular metabolic activity. It can be used to determine the concentration of viable cells in a sample. The reagent solution contains the non-fluorescent blue-colored molecules resazurin which when chemically reduced (a metabolic activity of cells) turns into the highly fluorescent red-colored resorufin. The color change and change in fluorescence allow for the direct measurement of cell metabolic activity via absorbance or fluorescence measurements in a plate reader [9]

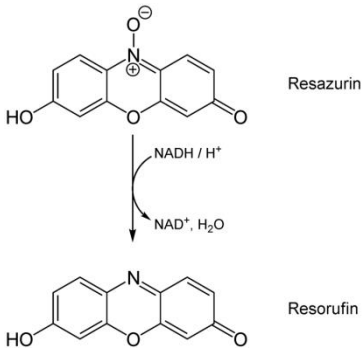


**Figure 2**

## MATERIALS AND METHODS

### Identification and authentication of selected plant species

The leaves are collected from Guntur district, A.P and it was authenticated by professor Dr.S.M.Khasim, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur.

### Collection of plant material

Fresh plant material was collected in bulk, washed under running tap water to remove adhering material, dried under shade and pulverized in a mechanical grinder. The coarse powder was passed through sieve no. 60#. Care was taken to select healthy plants and for normal organs.

### Chemicals and other products used

Pet Ether, Chloroform, Ethanol and Water

### Preparation of extracts [10]

The techniques commonly used in the field of photochemistry were extraction, isolation and structural elucidation of natural products, as well as chromatographic techniques. The solvent extraction of any botanical materials may yield very less quantity of volatile oils and a large yield of non-volatile components like resins, pigments, waxes and fatty acids.

Continuous extraction was done with Petroleum Ether, Chloroform**,** Methanol, and Aqueous by Soxhlet apparatus. About 50gm of dried leaves powder was taken in a whatmann filter paper bag. The various solvents were passed through the tube where the powder bag was kept. The solvents were passed through siphon tube to reach the round bottom flask in which porcelain chips were provided. The vapor's containing the constituents pass through the condenser and reach the tube containing powder bag and the process was repeated. This was continued for 24hrs. Then the round bottom flask containing extract was transferred to a beaker and was allowed to evaporate in a water bath. This concentrated extract was used for further studies.

### Recovery of Solvent

For purification of solvents the distillation apparatus was first settled. Then 40% of solvents were added to the bottom flask kept in electrical mantle. The respective solvents were allowed to distill and pure solvents were collected in a vessel from the adapter attached to the condenser.

## PHYTOCHEMICAL SCREENING

### Preliminary phytochemical identification [11]

The following chemical tests were performed to identify the phytochemical constituents present in petroleum ether, chloroform, ethanol and methanol extracts of *Vitex negundo*

## TEST FOR ALKALOIDS

### Dragendroff's reagent

The extract was shaken with dilute Hcl and filtered. To 2-3ml of filtrate, a few drops of Dragendroff's reagent were added. Orange brown precipitate was formed.

### Mayer's test

The extract was shaken with dilute Hcl and filtered. To 2-3ml of the filtrate, Mayer's reagent was added. Cream precipitate was formed.

### Hager's test

The extract was shaken with dilute Hcl and filtered. To the 2-3ml of filtrate, Hager's reagent was added. Yellow precipitate was formed.

### Wagner's test

The extract was shaken with dilute Hcl and filtered. To the 2-3ml of filtrate, Wagner's reagent was added. Reddish brown precipitate was formed.

## TEST FOR FLAVANOIDS

### Shinoda test

To the extract 5ml of 95% ethanol, few drops of conc. Hcl and 0.5 g magnesium turnings were added. Pink color was observed.

### Alkaline test

10mg of extract was dissolved in 2 ml of water and treated with 1ml of 10% ammonium hydroxide and was observed for coloration. 2 drops of dilute Hcl was added and again observed for discoloration. It was observed that the formation of intense yellow color turned to colorless on addition of dilute acid which indicated the presence of flavonoids.

## TEST FOR CARDIAC GLYCOSIDES

### Legal's test

To the extract 1ml of pyridine and 1ml of sodium nitro prusside was added. Pink to red color appeared.

### Liebermann's test

3ml extract was mixed with 3ml acetic anhydride. Heated and cooled. Few drops of conc. sulphuric acid were added. Blue color appeared.

## TEST FOR ANTHRAQUINONE GLYCOSIDES

### Borntragers test

To 3ml of extract, dilute sulphuric acid was added. Boiled and filtered. To cold filtrate add equal volume of benzene or chloroform was added and shaken well. The organic solvent was separated. Ammonia was added. Ammonical layer turned to pink.

### Modified borntragers test

To 5ml of extract 5ml of 5% ferric chloride and 5ml dilute Hcl were added and heated for 5min in boiling water bath. Equal volume of chloroform or benzene was added and shaken well. The organic solvent was separated and ammonia was added. Ammonical layer showed pinkish red color.

## TEST FOR SAPONIN GLYCOSIDES

### Foam test

The drug extract was shaken vigorously with water. Persistent foam was produced.

### Haemolytic test

The drug extract was added to one drop of blood placed on a glass slide. haemolytic zone appeared.

## TEST FOR TANNINS AND PHENOLIC COMPOUNDS

### Lead acetate solution

To 2-3ml of extract few drops of lead acetate solution was added. A white precipitate was formed.

### 5% ferric chloride solution

To 2-3ml of the extract few drops of 5% ferric chloride solution was added. A deep blue color appeared.

## TEST FOR STEROIDS

### Salkowski reaction

To 2ml of extract 2ml chloroform and 2ml conc. sulphuric acid was added. Shake well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

### Liebermann's test

3ml extract was mixed with 3ml acetic anhydride. Heated and cooled. Few drops of conc. sulphuric acid were added and blue color appeared.

## TEST FOR CARBOHYDRATES

### Molischs test

To 2-3 ml of extract few drops of alpha naphthol solution was added in alcohol, shaken and concentrated sulphuric acid was added from sides of the test tube. Violet ring was formed at the junction of 2 liquids.

### Fehling's test

About 50gms of extract was hydrolyzed with 10ml of dilute hydrochloric acid and neutralized with alkali. The mixture was heated with 1ml of Fehling's solution A and B and observed for precipitate. Formation of red precipitate indicated the presence of reducing sugars.

### Benedicts

To 0.5ml of filtrate 0.5ml of Benedict's reagent was added. The mixture was heated on water bath for 2 minutes and observed for precipitate. Formation of orange red precipitate indicated the presence of reducing sugars.

## TEST FOR PROTEINS

### Xanthoprotein test

3ml test solution was mixed with 1ml of conc. sulphuric acid. White precipitate was formed. It was boiled and precipitate turned to yellow. Ammonium hydroxide was added, and then precipitate turned to orange.

### Biuret test

To 3ml test solution 4% ammonium hydroxide and few drops of 1% copper sulphate solution was added. Violet color appeared.

## TEST FOR FATS AND OILS

### Solubility test

Oils are soluble in ether, benzene and chloroform, but insoluble in 90% ethanolic and water.

### Sudan red III Test

A thin section of drug was placed on glass slide. A drop of Sudan Red III reagent was adhered. After 2 minutes it was washed with 50% alcohol. Glycerin was mounted and observed under microscope. Oil globules appeared in red**.**

## TEST FOR GUMS

Test solution was hydrolyzed and treated with Benedict's reagent. Red color was developed. **ANTI-TB ACTIVITY USING ALAMAR BLUE DYE: [12, 13]**

- The anti-mycobacterial activity of compounds was assessed against M. tuberculosis using micro-plate Alamar Blue assay (MABA).
- This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method.
- Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation.
- The 96 wells plate received 100 µl of the Middle brooks 7H9 broth and serial dilution of compounds were made directly on plate.
- The final drug concentrations tested were 100 to 0.2 µg/ml.
- Plates were covered and sealed with parafilm and incubated at 37ºC for five days.
- After this time, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs.
- A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth.
- The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

## RESULTS

**Table 2: phytochemical constituents present in various leaf extracts of *Vitex negundo*.**

| Phyto – chemical constituents | Pet. Ether | chloroform | Ethanol | Aqueous |
|---|---|---|---|---|
| **Alkaloids** | ++ | ++ | ++ | ++ |
| **Glycosides** | -- | -- | ++ | ++ |
| **Flavonoids** | -- | -- | ++ | ++ |
| **Tannins** | -- | -- | ++ | ++ |
| **Steroids and terpenoids** | ++ | ++ | ++ | -- |
| **Carbohydrates** | ++ | ++ | ++ | -- |
| **Proteins** | -- | -- | ++ | ++ |
| **Fixed oils, fats & waxes** | ++ | ++ | -- | -- |

(++) Presence of phytochemical constituents in particular extract
(--)Absence of phytochemical constituents in particular extract

**Table 3: Anti-TB results of various leaf extracts of *Vitex negundo***

| S.NO | SAMPLE | 100µgm/ml | 50µgm/ml | 25µgm/ml | 12.5µgm/ml | 6.25µgm/ml | 3.12µgm/ml | 1.6µgm/ml | 0.8µgm/ml |
|---|---|---|---|---|---|---|---|---|---|
| **1** | TS9 | S | S | R | R | R | R | R | R |
| **2** | TS10 | S | S | R | R | R | R | R | R |
| **3** | TS11 | S | S | R | R | R | R | R | R |
| **4** | TS12 | S | S | R | R | R | R | R | R |

## Note

S - Sensitive
R - Resistant
Strain used: **M.tuberculosis** (H37 RV strain): ATCC No- 27294.

Here are the **standard values** for the Anti-Tb test which was performed.
Pyrazinamide- 3.125µg/ml
Streptomycin- 6.25µg/ml
Ciprofloxacin-3.125µg/ml

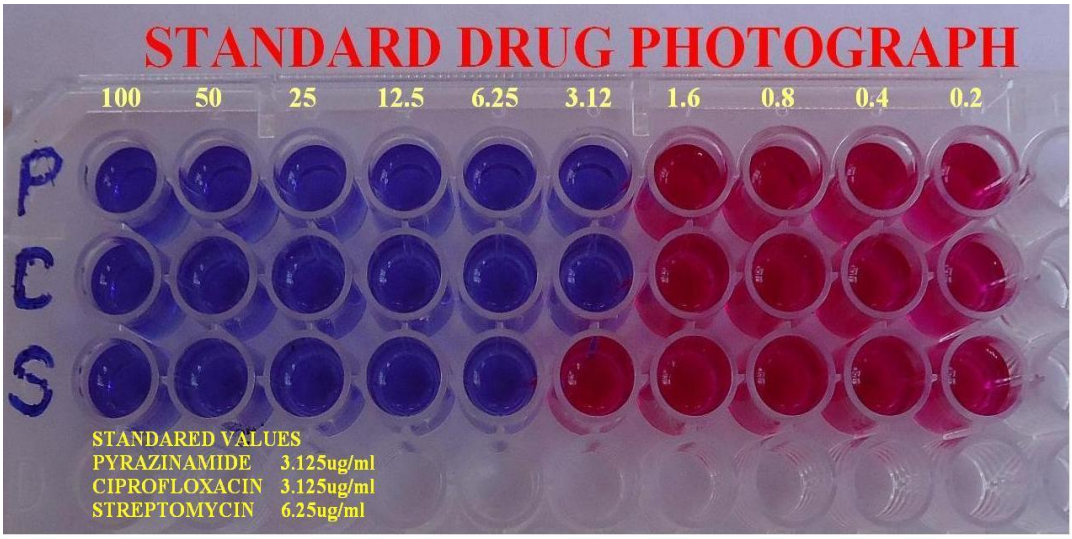


**Figure 3: Standard drug used in Alamar blue assay**

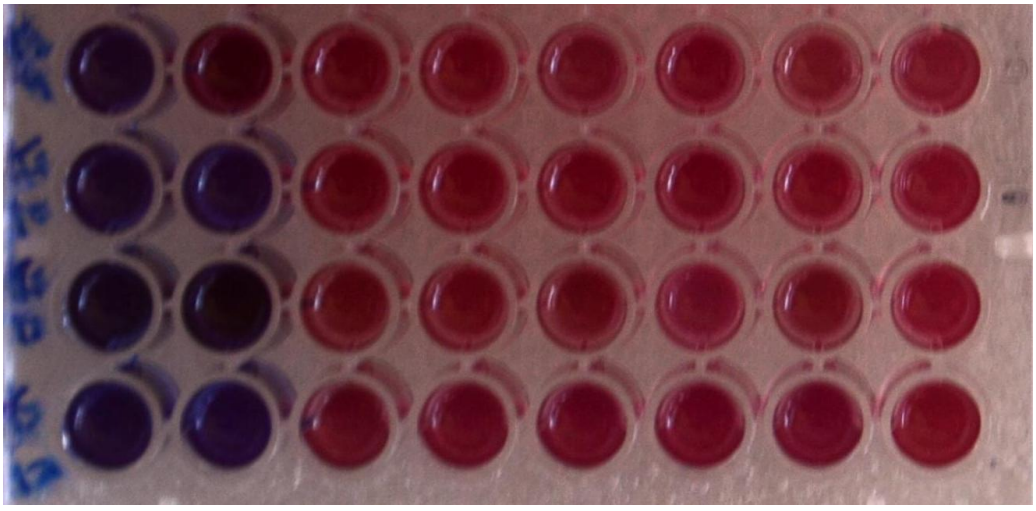

**Figure 4: Test Results of Various Leaf Extracts of *Vitex Negundo***

## DISCUSSION

The leaves of *Vitex negundo* were found to be rich in photochemical constituents which may have a variety of pharmacological actions. The literature survey revealed the presence of Alkaloids, Glycosides, Flavonoids, Tannins, Steroids and terpenoids, Carbohydrates, Proteins, Fixed oils, fats & waxes, in the entire plant. The result of the present study indicates that different extracts of leaves of *Vitex negundo* exhibited anti-tubercular activity against M.tuberculosis *(*H37 RV strain) at the doses of 100µg/ml and 50µg/ml shows the growth of inhibition.

## CONCLUSION

*Vitex negundo* was extracted with solvents like pet ether, chloroform, ethanol, aqueous. Extract were investigated for phytochemical constituents. The secondary metabolites of various extracts include alkaloids, glycosides, flavanoids, tannins, steroid and terpenoids in pet ether, chloroform, ethanol, aqueous. The extracts were evaluated *invitro* for anti tubercular activity using M.tuberculosis *(*H37 RV strain). In which TS9, TS10, TS11, TS12 extract concentration 25µgm/ml, 12.5 µgm/ml, 6.25µgm/ml found to be having effective anti-tubercular activity.

## REFERENCE

[1]. Medicinal and aromatic plants trade programme: traffic. org. retrieved. 20, 2017.
[2]. www.who.int/tb/publication/.../gtbr14_ executive _summary.pdf
[3]. Hawn TR, Day TA, Scriba TJ, M Hankekam WA, Evans TG,Churchyard GJ,Kublin JG, Bekker LG, Self SG. Tuberculosis vaccines and prevention of infection: microbiology and molecular biology review. MMBR. 78(4), 2014, 650-71,doi: 10.1128/MMBR.00021-14.
[4]. Kumar p, L.S.Chauhan, V.H.Balasangameshwara, S.Shyni and S.shiju.Blinded rechecking of sputum AFB smears in RNTCP, India: analysis of lot quality assurance sampling data of nine states submitted to national reference laboratory for the year, SAARC.J.Tuberc.Lung Dis:4(1), 44-50.
[5]. PL.Ladda,CS.Magdum, vitex negundo linn. Ethanobotany ,phytochemisrty and pharmacology ; A review. International journal of advances in pharmacy, biology and chemistry, 1(1), 2012.
[6]. Anita rani, Anupam Sharma, The genus *Vitex*, A Review ,pharmacogn rev, 7(14), 2013, 188-198.
[7]. Vishal R.Tandon, V. Khajuria,B.Kapoor,D.Kour,S.Gupta. Hepatoprotective activity of *Vitex negundo* leag extract against anti-tubercular drugs induced hepatotoxicity, 78(7-8), 2008, 533-538.
[8]. K.Srinivas,M.B.V.Raju, chemistry and pharmacology of *Vitex negundo*, Review, Asian journal of chemistry: 14, 2002, 565-569.
[9]. http://www.invitrogen.com/site/us/en/home/brands/molecular-probs/key-molecular-probes-products/alamarblue-rapid-and-accurate-cell-health-indicator.html≠how.
[10]. Pharmacopeia of India 1996: Delhi, Published by the manger of publication, 1970,650.
[11]. V.Siva parvathi, B. Sri Jyothi, T. Lakshmi, P. Srinivasa Babu, R. Karthikeyan. Morpho–anatomical and physicochemical studies of *Jatropha gossypifolia (*L.). Der Pharmacia Lettre, 4(1), 2012, 256-262.
[12]. N.D.satyanaryana, Wadhah Ahmed Al-Baadani,S.P.Soul sekhar, Harishkumar.S. anti tubercular activity of various solvent extracts *ACALTPHAINDICAL*. Against drug susceptibile H37RV Strain. World journal of pharmacy and pharmaceutical sciences, 5(8), 957-965.
[13]. Maria C. S. Lourenco, Marcus V. N deSouza, Alessandra C Pinheiro, Marcelle de L.Ferreira, Rasnisb B, Goncalves, Thais Cristina M Nogneira, Monica A Peralta. Evaluation of anti-Tubercular activity of nicotinic and isoniazid analogues. ARKIVOC 2007, 181-191.