



PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF VARIOUS SOLVENT EXTRACTS OF ANNONA RETICULATA LEAVES

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ABSTRACT

The leaves of the plant *Annona reticulata* were collected and extracted using different ranges of polar organic solvents like low (Ethyl acetate), medium (Butanol) and high (Methanol). Qualitative analysis and antimicrobial activity was investigated. The phytochemical screening of the leaf extract revealed that the presence of alkaloids, tannins, steroids, terpenoids and coumarins. The Ethyl acetate and Methanol extracts showed better antibacterial activity, the significant inhibitory effect against *Escherichia coli*, *Pseudomonas putida* and *Lactobacillus acidophilus*, and thus displayed highest inhibitory zone of 19.5mm, 19mm and 19mm when compared to Butanol. FT-IR spectroscopic analysis of the Ethyl acetate, Butanol and Methanol extract of *A. reticulata* revealed the presence of -CH, -OH, CH-OH and -NH₂ bond stretching. The clinical isolates were collected from patients suffered from different microbial infections. The antibacterial and antifungal activity was determined by using leaf extracts.

Keywords: *Annona reticulata*, leaf extract, Phytochemical screening, clinical isolates, antimicrobial activity.

INTRODUCTION

Natural remedies from medicinal plants are found to be safe and effective. Many plant species have been used in folkloric medicine to treat various ailments. Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries (Gajalakshmi.S et al., (2011).

According to the WHO survey 80% populations depends upon the traditional medicines for primary health care needs. It suggested in improving the technologies for cultivation of medicinal plants (Holiman.A (1989)). It has been reported that there has been an alarming increase in number of diseases and disorders caused by synthetic drugs prompting a switch over to traditional herbal medicine (Ghule. S.T et al.,(2001) &Nithya.T.G et al.,(2011)).

Annonareticulata (Custard apple) is semi-deciduous tree grown up to 10 meters tall native to West Indies, acts as astringent, sweet and useful in blood complaints. It is also used as anti-dysentric, antidiarrhoeic, anti-helminthic and enlarged liver (Sunilpareek et al., (2011)). Annonareticulata is referred as bullock's heart having a smooth skin fruit that becomes dull red when ripe. Less volatile substances such as alkaloids, diterpenoids, and acetogenins have been identified.

Annonaceousacetogenins are a group of potential anti-neoplastic agents isolated from Annonaceae plants (Alali et al.,(1999)).Recently, Annonaceousacetogenins have emerged as potentially promising anti-cancer drugs for multidrug resistant (MDR) cancers .

Phytochemicals are secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases, stimulation of the immune system, modulation of hormone metabolism and antibacterial and antiviral effect. (James W et al.,(1983) &Nithya T.G et al.,(2011)).The most important of these bioactive constituents of plants are Alkaloids, Tannins, Flavonoids ,Cardiac glycosides, Steroids and Saponins. (Taylor. L (2000) &Nithya.T.G et al., (2011)).Our work was to extract Annonareticulata plant leaves using different solvents and study their antimicrobial activity.

MATERIALS AND METHODS

Sample Collection:

Healthy, disease free, mature leaves of Annonareticulata were collected from Thiruvattar, Kanyakumari District of Tamilnadu, India, in the month of January 2013.

Solvent Extraction:

Washed mature leaves were shaded dried and then powdered. 25g of the powder was filled in the thimble and extracted successively with Ethyl acetate, Butanol and Methanol using a Soxhlet extractor with 500ml of solvent for 16 hours. All the extracts were subjected to phytochemical screening and antimicrobial activity assay.

Phytochemical Screening:

The Phytochemical Screening tests were carried out in the extracts using standard procedure to identify the constituents as described by Trease and Evans et al., (1989).

Test for Alkaloids:

1ml of extract was added with 2-3 drops of Mayer's reagent (dissolve 1.36g of mercuric chloride in 60ml of H₂O and pour into the solution of 5g of potassium iodide in 100ml of H₂O). The appearance of cream colour precipitate or pale yellow colour precipitate indicates the presence of alkaloids.

Test for Terpenoids (salkowski test):

1ml of the extract was mixed with 2ml of chloroform and concentrated H₂SO₄ (3ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to indicate positive results for the presence of terpenoids.

Test for Flavonoids:

1ml of extract was dissolved in diluted NaOH and HCl was added. A yellow solution that turns colourless, indicates the presence of flavonoids.

Test for Phlobatanins:

1ml of extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate indicates the presence of phlobatanins.

Test for Tannins:

1ml of extract was added to few drops of 1% lead acetate. A yellowish precipitate indicates the presence of tannins.

Test for Fattyacids:

1ml of extract was mixed with 10ml of ether. These extract was allow it for evaporation on filter paper and dried the filter paper. The appearance of transparence on filter paper indicates the presence of fatty acids.

Test for Leucoanthocyanins:

1ml of aqueous extract was added to 1ml of isoamyl alcohol. Upper layer appears red in colour indicates for presence of leucoanthocyanins.

Test for Coumarins:

3ml of 10% NaOH was added to 2ml of aqueous extract formation of yellow colour indicates the presence of coumarins.

Test for Steroids:

1ml of the extract was dissolved in 10ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

CHARACTERISATION OF THE LEAF EXTRACT

FT-IR Spectroscopy:

FT-IR spectra were recorded with a FT-IR Shimadzu FT-IR 8400S spectrometer. The technique used was KBr pelleting techniques. The FT-IR spectra were recorded in the middle infrared (4000cm⁻¹ to 400cm⁻¹).

High Performance Liquid Chromatography:

HPLC (Shimadzu, LC-10AT VP) was used for the analysis. The concentration of Sample was estimated on C18 column. The HPLC conditions were as follows; column temperature 37°C. Mobile phase consisted of acetonitrile and water. Before use, the mobile phase was degassed by an ultrasonic bath and fitered using 0.4µm membrane filter before use. The system was run at a flow rate of 1.0 ml/min. Sample detection was achieved at 254nm and injection volumes were 0.02 ml. Chromatographic peaks of incubation samples were identified.

ANTIMICROBIAL ACTIVITY

Microbial Strains: *Escherichia coli* MTCC 40, *Pseudomonas putida* MTCC 7173, *Streptococcus mutans* MTCC 497, *Lactobacillus acidophilus* MTCC 10307 strains were procured from MTCC, Chandigarh, India.

Agar Well Diffusion Method:

The agar well diffusion method recommended by the National Committee for Clinical Laboratory Standards (NCCLS 1999) .Using a sterile swab, a 24 hour old culture were spreaded on solidified nutrient agar plates. A well was made by using sterile gel puncture and 20 μ l of plant extract was added into the well and incubated at 28-37 $^{\circ}$ c. Plates inoculated with culture were incubated for 24-48hrs and zone of inhibition around the wells were measured, which was compared with oxytetracycline hydrochloride, as reference drug.

Clinical isolates:

Different microbes were isolated from patients suffered from dermatitis, tooth decay, foot cracks and blisters. The clinically isolated microbial infections were swabbed on a sterile plate containing nutrient agar. The agar well diffusion was made by injecting 20 μ l of Ethyl acetate, Butanol and Methanol extracts of the plant leaves. The antibacterial and antifungal activity was done for these clinical isolates using leaf extracts.

RESULT AND DISCUSSION

Phytochemical screening:

The phytochemical screening of *A. reticulata* Ethyl acetate, Butanol and Methanol extract showed the presence of alkaloids, tannins, terpenoids and coumarins (Table 1).

S NO.	Plant Constituent	Ethyl Acetate	Butanol	Methanol
1.	TERPENOIDS	+	+	+
2.	FLAVONOIDS	-	-	-
3.	COUMARINS	-	-	+
4.	PHLOBATANINS	-	-	-
5.	TANNINS	-	-	+
6.	FATTY ACIDS	-	-	-
7.	STEROIDS	+	+	+
8.	ALKALOIDS	+	+	-
9.	LEUCOANTHOCYANINS	-	-	-

Table 1: Phytochemical screening of the various extracts of the plant leaves.

NOTE: + INDICATES PRESENCE, - INDICATES NEGATIVE

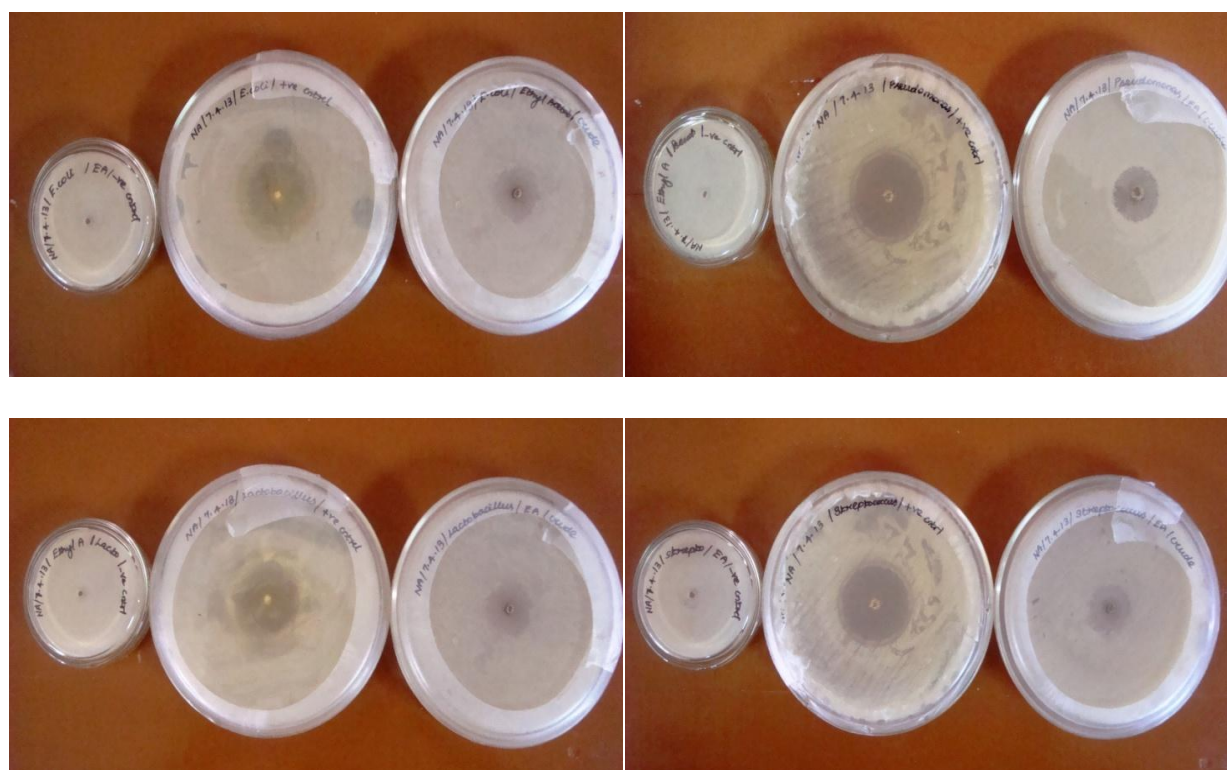


Figure1:Antibacterial activity of Ethyl acetate crude against *Escherichia coli*, *Pseudomonas putida*, *Lactobacillus acidophilus*, *Streptococcus mutans*

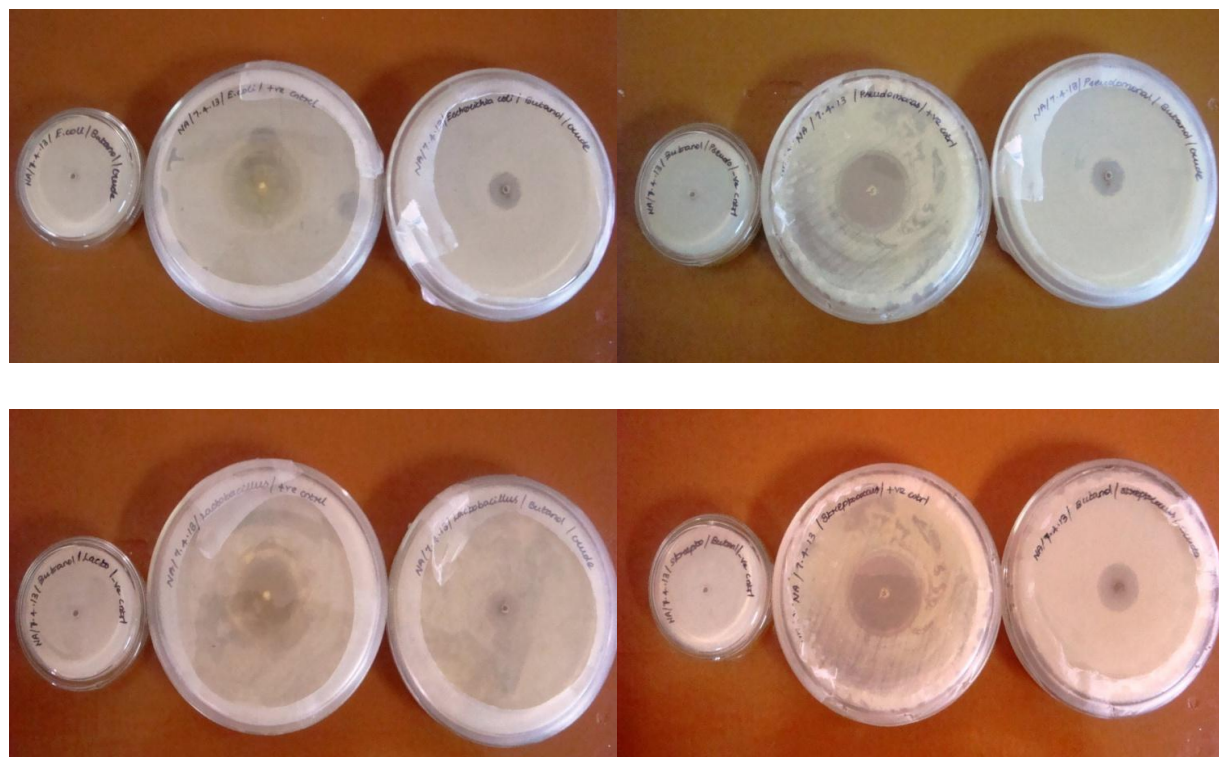


Figure 2:Antibacterial activity of Butanol crude against *Escherichia coli*, *Pseudomonas putida*, *Lactobacillus acidophilus*, *Streptococcus mutans*

Comparatively the plant extract showed the better results for both gram negative and gram positive bacteria. The Ethyl acetate crude showed 19mm of zone of clearance in both *pseudomonas putida* and *lactobacillus acidophilus* (figure 1). The Butanol extract showed highest zone of inhibition in *streptococcus mutans* with 18mm (figure2). The Methanol extract showed high zone(19.5mm) (figure 3), which is approximately equal to the commercially available synthetic antibiotic(22 mm). Thus the *A.reticulata* leaf extract showed antibacterial activity against both gram positive and gram negative bacterial strains (Table 2).

Organism	Zone of inhibition (diameter in mm)				
	Ethyl acetate extract	Butanol extract	Methanol extract	Positive control	NegativeControl
<i>Escherichia coli</i>	13	13.5	19.5	22	*
<i>Pseudomonas putida</i>	19	14	17	27	*
<i>Streptococcus mutans</i>	17	18	18	29	*
<i>Lactobacillus acidophilus</i>	19	13	10	25	*

TABLE 2: Antibacterial activity of various extracts of the *A.reticulata* leaves.

NOTE: * Indicates Zero Zone Of Inhibition

Positive control- Oxytetracycline hydrochloride

Negative control-organic solvents(Ethyl acetate, Butanol and Methanol)

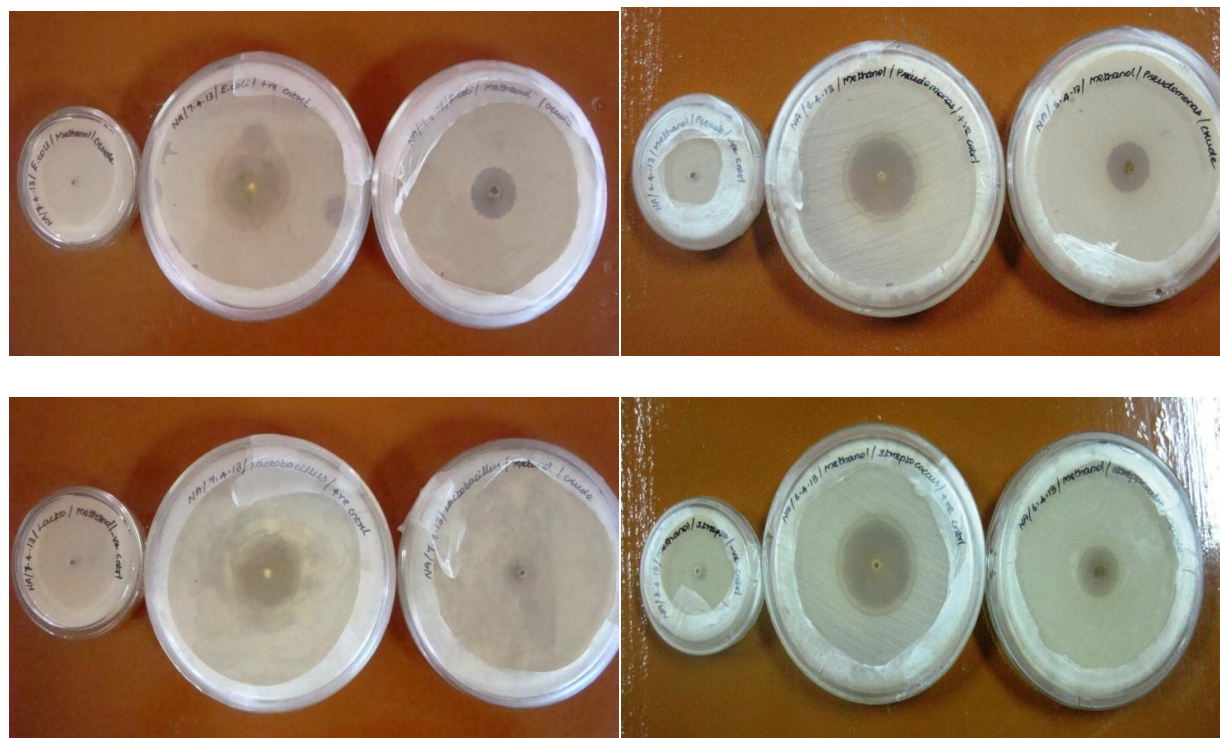


Figure 3:Antibacterial activity of Methanol crude against *Escherichia coli*, *Pseudomonas putida*, *Lactobacillus acidophilus*, *Streptococcus mutans*

FT-IR Spectroscopy:

The physico chemical properties of the leaf extract were studied by FT-IR spectroscopy. The infra red spectrum of Ethyl acetate crude revealed absorption bands as shown (figure 4). It showed a -OH stretching absorption band at 3313.48 and 3191.97 and 3145.68 cm^{-1} and the aliphatic C-H stretching between 2962.46 and 2923.88 and 2854.45 cm^{-1} . The OH stretching and the aliphatic CH stretching band are aligned and appear as a broad band from 3450 and 2850 cm^{-1} in the infrared spectrum of chitosan (saraswathy et al.,(2001)). Another peak was absorbed as 1116.71 and 1193.85 cm^{-1} which corresponds to the primary amino group -NH₂. The peak at 1668.31 cm^{-1} represents the acetyl amine group. The spectrum at 1400.22 cm^{-1} which is the standard spectrum of δ -CH spectrum, the γ -CH can be seen in the spectral standard wavelength 2923.88 cm^{-1} .

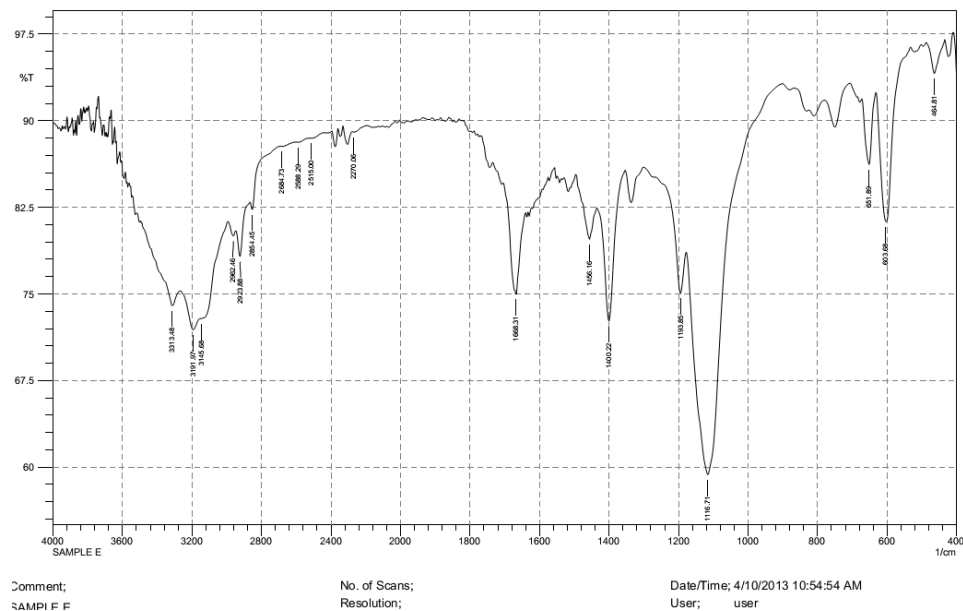


Figure 4: FT-IR spectrum of Ethyl acetate crude

The FT-IR spectrum of Butanol (figure 5) showed the major peak at 1116.71 and 1195.78 cm⁻¹ represents the CH-OH band. The spectrum at 1400.22 cm⁻¹ which is the standard spectrum of δ-CH spectrum, the γ-CH can be seen in the spectral standard wavelength 2925.81 cm⁻¹. The peak at 1666.38 cm⁻¹ represents acetyl amine group.

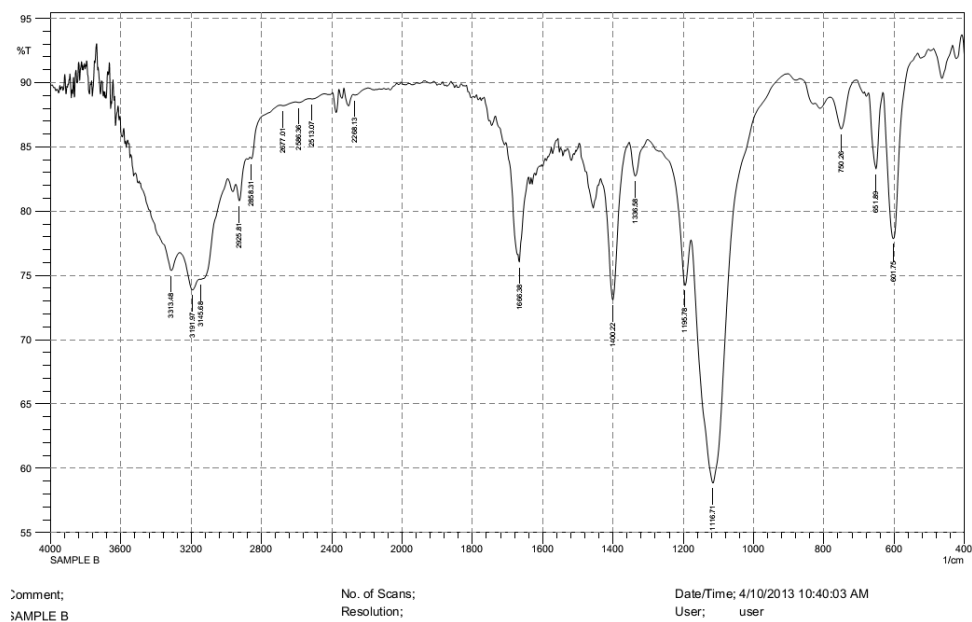


Figure 5: FT-IR spectrum of Butanol crude

In figure 6 of Methanol extract, the spectrum showed 1114.78 cm⁻¹ represents the primary amino group NH₂. The spectrum at 1400.22 cm⁻¹ which is the standard spectrum of δ-CH spectrum, the γ-CH can be seen in the spectral standard wavelength 2923.88 cm⁻¹, CH-OH spectral can be seen at 1195.78 cm⁻¹. The bands 750.26, 651.89 and 464.81 cm⁻¹ has been decreased. Thus the spectral Peak is formed at the region of CH, CH-OH, -NH₂ and -OH.

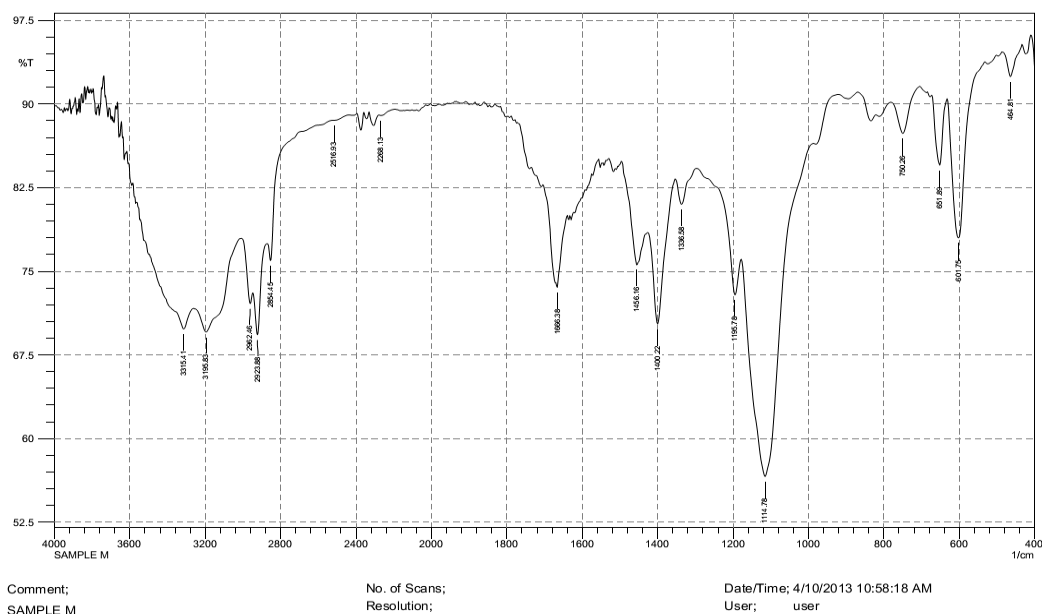


Figure 6: FT-IR spectrum of Methanol crude

The antimicrobial susceptibility of the *A. reticulata* leaf for the clinical isolates has been determined by using agar well diffusion method. The leaf extract showed the highest zone of inhibition (23mm in methanol extract) against dermatitis infection, which was usually caused by molds (figure 7). The microbes isolated from the foot cracks were also inhibited by methanol extract of *A. reticulata* leaves (Table 3).

Infections	Zone of inhibition (diameter in mm)		
	Ethyl acetate extract	Butanol extract	Methanol extract
Dermatitis	17	18.5	23
Tooth decay	8	10	15
Foot cracks	10	15	22
Blisters	8	10	13

TABLE 3: Antimicrobial activity of *A. reticulata* leaves for clinical isolates.

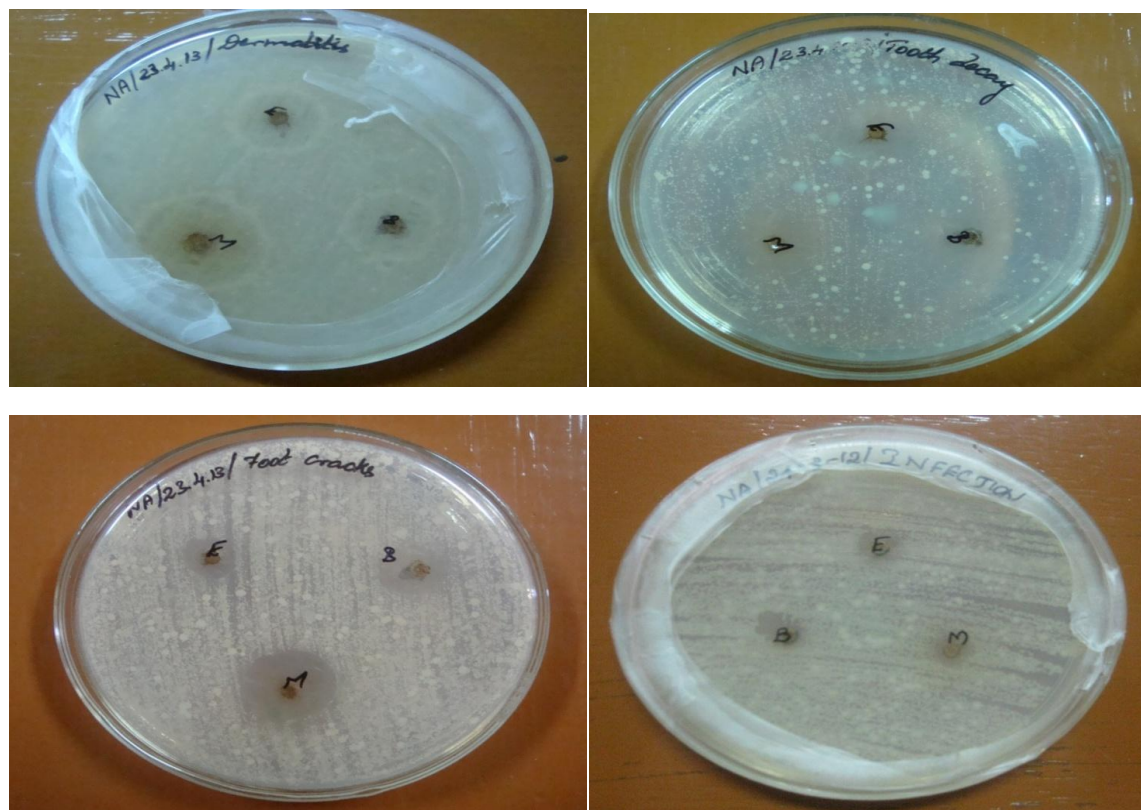


Figure 7:A.reticulata leaf extracts against Dermatitis, tooth decay, foot cracks and blisters

CONCLUSION

The leaf extracts of *Annona reticulata* contains certain amount of secondary metabolites and were extracted using different organic solvents such as Ethyl acetate, Butanol and Methonal. The leaf extracts showed better antimicrobial activity against *Escherichia coli*, *Pseudomonas putida* and *Lactobacillus acidophilus*.The clinically isolated microbes were also inhibited by the leaf extracts of *Annona reticulata*.

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