RESEARCH ARTICLE

Anticryptococcal activity of *Terminalia chebula* against clinical and environmental isolates of *Cryptococcus neoformans*

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ABSTRACT

The increasing prevalence of multidrug resistant strains of fungi and the recent appearance of strains with reduced susceptibility to antifungal agents raises the specter of untreatable fungal infections and adds urgency to the search for new infection-fighting strategies which involves the utilization of bioactive phytochemicals from plants. The ethanolic extract obtained from the fruit of ***Terminalia chebula*** was assessed for their antifungal activity against clinical and environmental isolates of ***C neoformans***. Extracts were prepared from the fruits by standard techniques and phytochemical analysis of the extract was performed.to study the bioactive compounds The compounds were identified by GC MS .analysis and the functional groups were identified by FTIR. Of the 10 isolates tested the clinical isolates were more susceptible than the environmental isolates. C5and E3 was found to be more susceptible with the zone of inhibition 25mm at a concentration of 4mg/ml.. Mass spectrometric analysis revealed the presence of phenolic compounds and tannins. FTIR analysis revealed the presence of alkanes **alkynes** esters carboxylic acids etc. as chemical groups constituting the bioactive compounds **.**

Keywords: ***Terminalia chebula,*** Cryptococcus neoformans, antifungal, pyrogallol,benzenetriol , GC MS FTIR

INTRODUCTION

AIDS has become a devastating epidemic in the world. In the recent decades, Up to 90% of all HIV patients contract fungal infections during the course of the disease, of which 10-20% die as a direct consequence of fungal infections [1,2] Cryptococcosis is the most common opportunistic fungal infection in HIV /AIDS patients and management of this infection is difficult due to growing resistance to antifungal agents, drug toxicity and high cost[3] Therefore there is a need to search for alternative therapeutic agents by the exploration of medicinal plants [4,5,6] used in local dictum and traditional medicines by the local population as important source of new, active and renewable antifungal drugs.

Terminalia chebula

Retz (family combretaceae) is a flowering evergreen tree distributed widely in India and different parts of this tree has germinated substantial compounds to cure various diseases The active compounds present in fruit bark and leaves are tannins, triterpenes, saponins and mucous substances. The fruit extracts have been reported to possess antiviral, antibacterial, anti-cancerous and antioxidant activity[7] The present work was carried out to examine the antifungal potential of crude extracts of fruits of *Terminalia chebula* against clinical and environmental isolates of *Cryptococcus neoformans*

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MATERIALS AND METHODS

Preparation of Extract:

The fruits of *T Chebula* were purchased commercially and the identification was authenticated by a botanist. .From dried fruits, seeds were separated and the remaining part was made into coarse powder .The coarse powder was then extracted with ethanol by Soxhlet apparatus . The extract was filtered using Whatmann filter paper, concentrated in vacuum and air dried.

Anti-cryptococcal activity of ethanolic fruit extract of *Terminalia chebula:*

The anticryptococcal activity of crude alkaloids was evaluated by disc diffusion method of NCCLS[8] In brief, Activated cultures of 10 isolates of *C .neoformans* in Sabouraud Dextrose broth were adjusted to 0.5 McFarland standard. 100µl of the inoculum was introduced to molten Sabouraud dextrose agar and poured into sterile petriplates

Sterile filter paper discs were impregnated with varying concentrations of ethanolic fruit extract ranging from 1mg to 4mg per disc dissolved in DMSO and dried. The discs were placed on yeast seeded plates and incubated at 37˚C for 48hrs. Disc impregnated with only 100% DMSO served as negative control Amphotericin B (100 units/disc) was used as positive control. Following incubation, antifungal activity was evaluated by measuring zones of inhibition of fungal growth. Clear zone within which fungal growth was absent were measured and recorded as the diameter (mm) of complete inhibition. Experiments were conducted in triplicates

MIC of ethanolic fruit extract of *Terminalia chebula*

The broth dilution method was used to determine the minimum inhibitory concentration of the extract against *C neoformans* Minimum inhibitory concentrations of the extract was prepared by serial doubling dilution of the extract to obtain concentrations in the range 100-1000µg/ml. Equal volume of the extract and Mueller – Hinton broth i.e. 0.5ml each were dispensed into sterilized test tubes. 0.1 ml of standardized inocula (3.3 X 10 CFU/ml) was added to each of the test tubes above. The tubes were incubated aerobically at 350C for 24 h. Tubes containing broth and plant extracts without inoculum served as positive control while tubes containing broth and inoculum served as negative control. The tubes were observed after 24 h of incubation to determine minimum inhibitory concentration, the lowest concentration that showed no evidence of growth (NCCLS, 2008)

TLC profile of ethanolic fruit extract of Terminalia chebula:

TLC of crude extract was carried out on TLC glass plates coated with 0.2mm thickness silica gel 60 .10µl of crude extract was applied on the glass plates at equal distance with the help of micropipette. The plates were kept in a chromatographic chamber after drying. Chloroform: Ethyl acetate: Formic acid (2.0: 2.0: 0.8) was used as the solvent system. The plate was removed from the chamber, when the solvent front had reached the predetermined height and the solvent front was marked precisely with pencil. Then the plate was dried and observed under UV light. The plate was sprayed with 5% ferric chloride in methanol. Rf of the band separated was noted

Phytochemical analysis of ethanolic fruit extract of *Terminalia chebula*

Phytochemical screening of *Terminalia chebula* for the presence of alkaloids flavanoids, hydrolysable tannins, coumarins, phenols, sugar etc *was* carried out as per standard procedures [9]

GC MS analysis of ethanolic fruit extract of *Terminalia chebula*

The ethanolic extract was subjected to GC MS analysis [10] using the instrument GC MS Shimadzu QP2010 with GC MS solution version 2.53 software and Elite – DB-5M column Initially oven temperature was maintained at 70˚c for 2 minutes and the temperature was gradually increased up to 300˚C at 10/35 minutes and 4µl of sample was injected for analysis. Helium gas 99.995% of purity was used as a carrier gas as well as eluent. The flow rate of helium gas was set to 1.5ml/minute ,sample injector temperature was maintained at 260˚C and the split ratio is 20 throughout the experiment period .The ionization mass spectroscopic analysis was done with 70eV.The mass spectra was recorded for the mass range 40-1000m/Z for about 30minutes. The m/z ratio obtained was calibrated from the graph obtained which was called as the mass spectrum graph which is the fingerprint of the molecule. The identification of compounds was based on the comparisons of their mass spectra with NIST library 2008, WILEY8 & FAME

FTIR analysis was done to identify chemical groups present in the bioactive compounds The samples were scanned using infrared in the range 5000-500 cm-1 The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample

RESULTS

The dried fruit pulp of *Terminalia chebula* was extracted with ethanol and the residue was recovered using a rotary evaporator

Anticryptococcal activity of ethanolic fruit extract of *Terminalia chebula:* (Table 1& 2)

The ethanolic fruit extract of Terminalia chebula was tested for its antifungal activity against 5 clinical and 5 environmental isolates of C neoformans. At the highest concentration tested 4mg/ml, of all the 5 clinical isolates maximum zone of inhibition was shown by C5(25mm±0.5)followed by C2and C3 (24mm±0.5) and C1 and C4(23±0.5) Of all the 5 environmental isolates E2 and E3 showed maximum zone of inhibition (25±0.5) followed by E4and E5 (23.5±0.5) E1 showed less zone of inhibition comparatively (19±0.62).

MIC of ethanolic fruit extract of *Terminalia chebula:*

The minimum inhibitory concentration of the extract against Cryptococcus isolates was determined by broth dilution method.The MIC for the clinical isolates C4 and C5was found to be less 600µg/ml.compared to other clinical isolates C1, C2and C3 (800µg/ml) Among the environmental isolates the minimum inhibitory concentration of the extract was found to be 600µg/ml for E2and E5, whereas it was 800µg/ml for E3and E4. The MIC value for E1 was high compared to all other isolates (900 µg/ml) The results were presented in Table3&4

TLC profile of ethanolic fruit extract of Terminalia chebula:

TLC of the ehanolic extract was performed to separate active constituents in the extract .gallic acid was used as the standard. The Rf value was found to be 0.39.

Phytochemical analysis of ethanolic fruit extract of *Terminalia chebula*

The phytochemical active compoundsof Terminalia chebula were qualitatively analysed and the results were presented in table. The ethanol extracts of Terminalia chebula fruits showed the presence of phytochemical active compounds such as tannins quinines phenols coumarines and carbohydrates etc (Table 5)

GS MS FTIR analysis of ethanolic fruit extract of *Terminalia chebula*

GC MS analysis was carried out on the ethanolic extract of *T.Chebula* and 35 different compounds were identified. The chromatogram showed 33 peaks in the retention time range 1.327min to 29.545 min. The largest peak at 3.389 min with 69.42% area was identified as Tetrahydrofuran. The second less promonent peak at 11.144 minwith 3.64%area corresponds to the compound 1, 2, 3, benzenetriol. The third prominent peak at 25.493 min with 3.46%area was due to the presence of gamma and beta sitosterol and stigmastan -3,5 diene. Other compounds identified were presented in Table 6. The IR spectral qualities of the fraction indicated the presence of ketones, aldehydes, carboxylic acids, amides, aromatic compounds alkanes alkenes alkynes and esters in their active components FTIR results were presented in Table 7.

DISCUSSION

The incidence of opportunistic fungal infections in patients treated with immunosuppressive drugs, intensive chemotherapy, suffering from AIDS and neonates is increasing at an alarming rate . Mycotic infections are very difficult to eradicate and they constitute an enormous challenge for health care providers. The fungi, like their hosts, are eukaryotic organisms, making it difficult to choose intracellular targets whose inhibition would not also be deleterious to the host cells .Although an array of

different treatments are used for fungal infections, none of them are ideal in terms of efficacy, safety or antifungal spectrum .Many of the current drugs are very toxic (Amphotericin B), lead to the development of resistance (fluconazole and 5-flucytosine) or exhibit drug-drug interactions (azoles)[11].Some Medicinal plants have therapeutic potential due to the presence of natural antioxidants functioning as reducing agents, free radical scavengers and quenchers of singlet oxygen. Majority of their antioxidant activity is due to bioactive compounds viz. phenolic and polyphenolic compound

The present investigation was undertaken to evaluate the anticryptococcal property of T.chebula fruit..Ethanolic fruit extracts were prepared and its anti cryptococcal activity was checked by disc diffusion method against clinical and environmental isolates of C .neoformans. Of all the clinical isolates C5 exhibited maximum zone of inhibition(25mm±0.5)and E3 was most susceptible(25mm±0.5) among environmental isolates at a concentration of 4mg.It was also found that clinical isolates were more susceptible than environmental isolates

Phytochemical investigation reported the presence of tannins, quinines, phenols, coumarines and carbohydrates etc The bioactive components were identified by GC- MS .analysis . Identification of components was based on comparison of their mass spectra As the compounds separated, on elution through the column, were detected in electronic signals. As individual compounds eluted from the gas chromatographic column, they entered the electron ionization detector where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments were actually charged with a certain mass. The m/z ratio obtained was calibrated from the graph obtained which was called as the mass spectrum graph which is the fingerprint of the molecule. The identification of compounds was based on the comparisons of their mass spectra with NIST library 2008, WILEY8 & FAME.

GC MS chromatogram showed 33 peaks with tetrahydrofuran showing maximum peak area 69.42%at a retention time 3.389 minutes The common properties of tetrahydrofuran is their toxicity against cell of foreign organisms. These activities have been widely studied for their potential use antiinflamatory in exhibiting anti- angionic, antibacterial, antioxidant etc. in the elimination and reduction of serious human diseases like cancer cell lines[12]The next compound is benzenetriol with peak 3.64% at retention time 11.44min is a pyrogallol with antiseptic ,antioxidant,anti-dermatic, fungicide and candidicide[13]The third prominent peak at 25.493 min with 3.46%area was due to the presence of gamma and beta sitosterol and stigmastan -3,5 diene

An aqueous extract of *T. chebula* exhibited antifungal activity against a number of dermatophytes and yeasts [14,15]. It is effective against the pathogenic yeast *Candida albicans* and dermatophytes *Epidermophyton, Floccosum*, *Microsporum gypseum* and *Trichophyton rubrum*. Its inhibitory effect on three dermatophytes (*Trichophyton* spp.) and three yeasts (*Candida* spp.) has also been documented[16].

T. chebula is one of the most versatile plants having a wide spectrum of pharmacological and medicinal activities. This versatile medicinal plant is the unique source of various types of compounds having diverse chemical structure. Though it has a number of pharmacological activities due to the presence of various types of bioactive compounds, very little work has been done on the plausible medicinal applications of this plant against the diseases particularly on fungal pathogens. Hence extensive investigation is needed to exploit their therapeutic ability to combat diseases including drug resistant infections. As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, a drug development programme should be undertaken to develop modern drugs with the compounds isolated from *T. chebula* effective against different types of diseases and also to

overcome the problem of drug resistance after extensive investigation of its bioactivity, mechanism of action, pharmacotherapeutics, toxicity and after proper standardization and clinical trials.

CONCLUSION

In the present study the ethanolic fruit extract of *Terminalia chebula* exhibited significant anticryptococcal activity against clinical isolates. The bioactive components were analysed by GC MS studies. GC MS analysis revealed that fruits of *Terminalia chebula* are rich source of tannins and polyphenolic compounds.Tetrahydrofuran and benzenetriol with typical antifungal activity were identified as predominant compounds Further analysis and fractionation of these pure compounds is needed to recommend the compounds for therapy

Table 1: Anticryptococcal activity of crude ethanolic fruit extract of *Terminalia chebula* against clinical isolates

S No	ISOLATE	Zone of inhibition in mm				Amphotericin
		1mg	2mg	3mg	4mg	100units/disc
1	C1	18±0.5	20±0.5	21±0.28	23±0.5	12±0.5
2	C2	19±0.5	21±0.28	22±0.5	24±0.5	12±0.5
3	C3	19±0.5	20±0.5	21±0.28	24±0.5	13±0.5
4	C4	21±0.28	21±0.28	22±0.5	23±0.5	12±0.5
5	C5	21±0.28	22±0.5	24±0.5	25±0.5	12±0.5

Mean values of triplicates (zone of inhibition) ± S.D

Table 2: Anticryptococcal activity of crude ethanolic fruit extract of *Terminalia chebula* against environmental isolates

S No	ISOLATE	Zone of inhibition in mm				Amphotericin
		1mg	2mg	3mg	4mg	100units/disc
1	E1	16±0.5	17±0.7	19±0.62	19±0.62	13±0.5
2	E2	21±0.28	22±0.5	24±0.5	25±0.5	12±0.5
3	E3	17±0.7	19±0.62	25±0.5	25±0.5	13±0.5
4	E4	17±0.7	17±0.7	22±0.5	23±0.5	12±0.5
5	E5	21±0.28	21±0.28	22±0.5	23±0.5	12±0.5

Mean values of triplicates (zone of inhibition) ± S . D

Table 3: Minimum inhibitory concentration of crude ethanolic fruit extract of *Terminalia chebula* for clinical isolates

S No	ISOLATE	CONCENTRATION IN µg									
		100	200	300	400	500	600	700	800	900	1000
1	C1	+	+	+	+	+	+	+	--	--	--
2	C2	+	+	+	+	+	+	+	--	--	--
3	C3	+	+	+	+	+	+	+	--	--	--
4	C4	+	+	+	+	+	--	--	--	--	--
5	C5	+	+	+	+	+	--	--	--	--	--

+ indicates growth, -- indicates no growth

Table 4: Minimum inhibitory concentration of crude ethanolic fruit extract of *Terminalia chebula* for environmental isolates

S No	ISOLATE	CONCENTRATION IN µg									
		100	200	300	400	500	600	700	800	900	1000
1	E1	+	+	+	+	+	+	+	+	--	--
2	E2	+	+	+	+	+	--	--	--	--	--
3	E3	+	+	+	+	+	+	+	--	--	--
4	E4	+	+	+	+	+	+	+	--	--	--
5	E5	+	+	+	+	+	--	--	--	--	--

+ indicates growth, -- indicates no growth

Table 5: Phytochemical analysis of Terminali chebula

S. No	Phytochemical test	Inference
1	Carbohydrate	++
2	Tannins test	++
3	Saponin test -	-
4	Flavonoid test	+
5	Alkaloid test	-
6	Quinones	++
7	Glycosides test	+
8	Cardiac glycosides test	-
9	Terpenoids test	-
10	Triterpenoid	-
11	Phenols	++
12	Coumarins	++
13	Proteins	-
14	Steroids and Phytosteroi	-
15	Phlobatannin	-
16	Anthraquinones	-

Table 6: Identification of compounds present in ethanolic leaf extract of Terminali chebula by GC – MS analysis

S no	Peak No	Ret time	Area	Area %	Name of the compound
1	1	1.327	6382584	0.21	1 Thiodiglycol , Ethanol, 2,2'-[methylenebis(thio...
2	2	1.603	7519586	0.24	Methoxymethyl 2-hydroxyethyl sul... Propanamide, 2-hydroxy- 3-hydroxy-2-thiabutane
3	3	1.719	70810819	2.29	(S)-(+)-1,2-Propanediol, Ethanethiol, Methylal
4	4	2.256	11156743	0.36	Sulfide, ethyl propyl, Ethanethiol
5	5	2.373	5970371	0.19	Disulfide, dimethyl
6	6	3.389	2147483647	69.42	Tetrahydrofuran- 2 Propane, 1,1,2-trichloro-
7	7	4.115	26218186	0.85	Dimethyl sulfone, 1,4-Cyclohexadiene, 1-methyl
8	8	6.918	7889362	0.26	Undecane
9	9	9.039	15361854	0.5	2-Furancarboxaldehyde, 5-methyl
10	10	11.144	112637154	3.64	1,2,3-Benzenetriol
11	11	20.759	63563653	2.05	Hexadecane , Heptadecane
12	12	21.035	12014231	0.39	(-)-Neoclovene-(II), dihydro- 28-Nor-17.alpha.(H)-hopane
13	13	21.267	7521333	0.24	Ergost-25-ene-3,5,6-triol, (3.be... 2 2,2,6,6-Tetramethylthiabicyclohe 2H-Inden-2-one, octahydro-3a-met
14	14	21.819	12453221	0.4	Stigmastan-3,5-diene .beta.-Sitosterol acetate , Ergosta-4,6,22-trien-3.beta.-ol
15	15	22.850	20846322	0.67	3E,5E,7E)-6-Methyl-8-(2,6,6-tri... (1R,2S,8R,8Ar)-8-acetoxy-1-(2-hy... 1-Formyl-2,2-dimethyl-3-trans-(3...
16	16	23.010	12947343	0.42	Benzamide, 2,3,4-trifluoro-N-(4-Benzenamine, 4-[2-(4-aminophenyl... Indene-1,3(2H)-dione, 2-(2-metho...
17	17	23.300	52655110	1.70	Eicosane, Tetracosane
18	18	24.520	17463703	0.56	4,10-(Methanoxymethano)-10H-cycl... Santonox 1, 3 Pyridine-3,5-dicarboxylic acid, ...
19	19	24.694	9855143	0.32	(1R,2S,8R,8Ar)-8-acetoxy-1-(2-hy... 2 28-Nor-17.alpha.(H)-hopane 3 (1R,2R,8S,8Ar)-8-hydroxy-1-(2-hy...
20	20	24.912	48118772	1.56	Eicosane, Heptadecane
21	21	25.275	41733768	1.35	1 1H-Pyrazolobis(bbn)thiolium , 2 [1,2,4]Triazolo[1,5-c]quinazolin... H-1-Benzopyran-2-one, 4,5,7-tri...
22	22	25.493	106914499	3.46	gamma.-Sitosterol , 2 .beta.-Sitosterol , 3 Stigmastan-3,5-diene
23	23	25.726	23447392	0.76	2,5-Bis(4-biphenylyl)oxazole , Terephthalic acid, hexadecyl 2-m... 3 4H-1-Benzopyran-4-one, 2-(3,4-di
24	24	26.031	34238239	1.11	28-Norolean-17-en-3-one , A'-Neogammacer-22(29)-ene , 3 Butylphosphonic acid, pentyl 4-(
25	25	26.190	19276498	0.62	Heptadecane , Octadecane , Triacontane
26	26	26.394	5759797	0.19	9,19-Cycloergost-24(28)-en-3-ol,...
27	27	26.495	6170592	0.2	5H-[1,2,4]Triazolo[3,4-b][1,3]th... .alpha.-Amyrin Urs-12-ene
28	28	27.120	47771630	1.54	1 4',6'-Bis(1,1-dimethylethyl)-3.3...
29	29	27.526	35216201	1.14	Eicosane
30	30	28.064	51373817	1.66	1 2,7-Bis-octyloxy-fluoren-9-one Cyclohexanecarboxamide, N-(2,5-d... 1-Propyne, 3,3'-[ethylidenebis(
31	31	29.080	9887139	0.32	Octadecane, 1-iodo- Heptadecane , Octadecane
32	32	29.211	19569963	0.63	N-Methyl-1-adamantaneacetamide Octasiloxane, 1,1,3,3,5,5,7,7,9,...
33	33	29.545	23329462	0.75	2-Propyn-1-one, 1-(2-thienyl)-, ...

Table 7: IR spectral qualities of fractions of ethanolic extract of *P.juliflora*

S. No	Frequency Cm^{-1}	Nature of bond	Functional Group
1	3577.05	OH stretch	alcohols and phenols
2	3096.77	C-H aromatic ring	Alkenes
3	2920.28	C-H stretch	Alkanes
4	2132.34	–C(triple bond)C– stretch	Alkynes
5	1744.64	C =O stretch	Aldehydes,ketones,carboxylic acids and esters
6	1653.02	-C=C - stretch	alkenes
7	1437.96	C-H stretch	Alkanes
8	1351.16	C-N stretch	Aromatic amines
9	1004.93	C -O stretch	Aldehydes,ketones,carboxylic acids and esters
10	789.86	C-H stretch	Alkenes

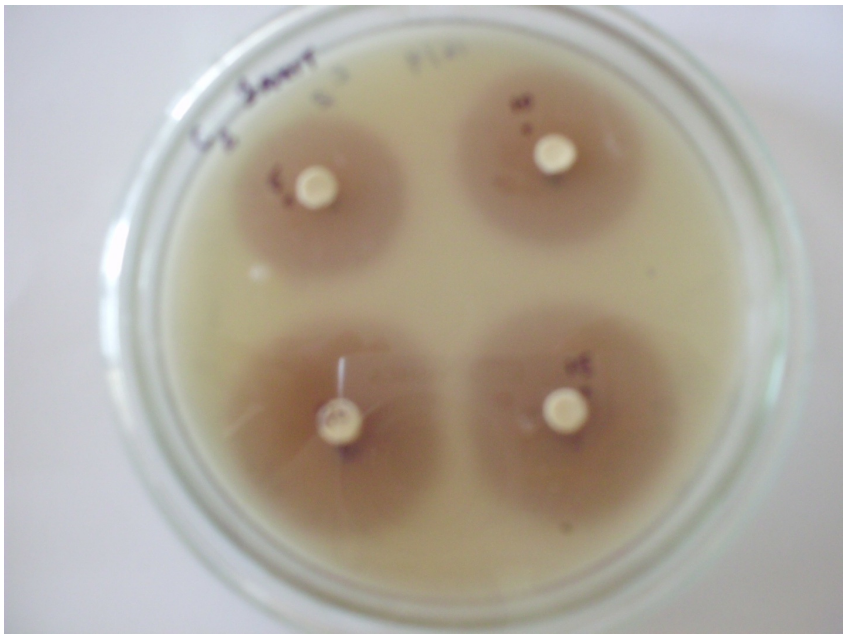

Fig 1 Disc diffusion assay of *Terminalia chebula* ethanolic extract against clinical isolates of *Cryptococcus neoformans*

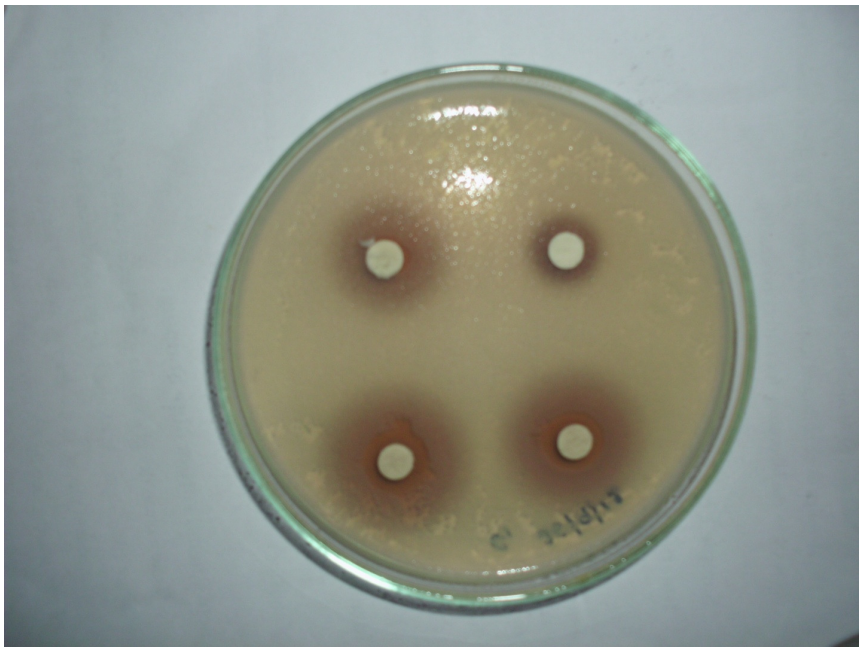

Fig 2 Disc diffusion assay of *Terminalia chebula* ethanolic extract against environmental isolates of *Cryptococcus neoformans*

Fig 3 TLC profile of ethanolic extract of ***Terminalia chebula***

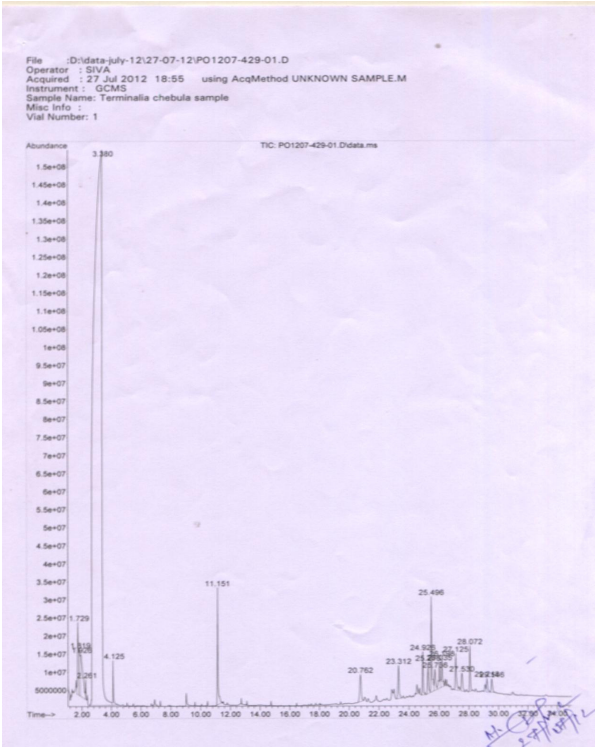

Fig 4: Gas Chromatogram of Ethanolic fruit extracts of *Terminalia chebula*

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