5.0 RESULTS

5.1 Patients manifesting clinical symptoms of Cryptococcosis

Out patients complaining of severe headache, fever and stiff neck were examined by doctors at the Stanley Medical College, Chennai. The clinical symptoms were recorded and various body fluid samples from the patients were examined for biological entities or suspected Cryptococcosis. These clinical and laboratory investigations on the patients are presented here. Patients with severe cough and headache were examined for pulmonary Cryptococcus and those with severe headache and fever were also examined for suspected meningeal Cryptococcosis. Each patient was scored using the questionnaire given in appendix I and a sample sheet is also presented in appendix II. The scores obtained were used for statistical analysis using the Student T-test (SPSS).

5.2 Clinical observation

5.2.1 Pulmonary Cryptococcosis

In this study, 38 pulmonary specimens collected from patients were examined. These included early morning sputum, induced sputum and pleural fluid. These specimens were subjected to routine microbiological growth on Staib’s agar and microscopy (including negative staining and nigrosin staining). Only specimens from which the fungus could be isolated and showed typical morphology under microscopy was considered as positive. Out of 38 samples three sputum specimens were positive for C. neoformans and all other specimens were negative. Thus the prevalence rate of pulmonary Cryptococcosis in meningitis patients was 8% (Table 6).
5.2.2 Meningeal Cryptococcosis

Patients with suspected meningeal involvement were screened. CSF samples given to us by the medical officer were then examined using culture test and microscopy. A total of 143 samples screened had a majority of patients with predisposing factors. They could be grouped as 61 patients also suffering from HIV, 79 with other predisposing factors and 3 with no known predisposing factors (Table 7).

Out of the 61 positive samples 45 were males with ages ranging from 23 to 51 with a mean age of 37 years and 19 were females with ages ranging from 12 to 48 and a mean of 30 years (Table 8).

Symptoms, incidence and patient data were compiled to perceive possible relationships. Results presented in Table 9 showed a range of symptoms among patients who had pulmonary or meningeal Cryptococcosis (n = 61 + 3). The majority reported symptoms of severe headache (92.1%), fever (73.4 %) and malaise (50%). Symptoms of stiff neck were associated with (34.3%) of cases and visual deficit with (23.4%).

Out of 143 CSF specimens, growth of *C. neoformans* in culture was seen in 61 samples. Along with microscopical staining, the prevalence of this fungus in this group of patients was 42.6 % (Table 6).
5.2.3 Laboratory observations

Pathological findings

The pathobiochemical findings collected from the laboratory records for the 64 patients at the time of diagnosis were analysed. Data presented in Table 10 indicated that CD4 cell count of the immunocompromised patient with cryptococcal meningitis infection ranged from 15 to 295 with a mean of 109.9 ± 84.4. The leucocyte counts in the CSF ranged from 1 to 68 with a mean of 14.7 ± 19.6. Glucose levels ranged from 15 to 110 mg/dl (mean of 58.4 ± 27.5) and protein levels ranged from 26 to 280 mg/dl with a mean of 109.2 ± 71.2. These elevated levels are in accordance with the published reports (Jones, 2004).

5.3 Laboratory growth, identification and characterization of Cryptococcus neoformans spp. complex

Diagnosis and correct identification of the yeast in clinical specimens or environment samples include culture on various media, culture at 25°C and 37°C, Microscopy, Biochemical tests such as Phenoloxidase production, Creatinine utilization, Glycine utilization, Proline utilization, Urease production, Nitrate reductase activity, serological detection using Latex agglutination test and confirmatory tests using PCR of specific genes of the pathogen.

Specific tests include growth response on specific media that are used for distinguishing the variants of Cryptococcus neoformans viz., serovar grubii (A); neoformans (D, A/D), and gattii (B, C). The two species C. neoformans and C. gattii are easily identifiable based on their response on differential media.

Isolates obtained in this study were used for identification and confirmation of identification using laboratory tests described below.
5.3.1 Growth of isolates from clinical specimens:

*C. neoformans* was isolated from pulmonary specimens (8%), CSF specimens (43%) and extraneural sites (blood culture 32.4%) as indicated in Table 6. Their ability to be cultured on three different, predictive identification media (*vide*, 4.8) *viz.*, NSDA, Staib’s agar with biphenyl (0.01%; SAB) and sunflower seed agar (SSA) is presented in Table 11. Only 3 isolates from the pulmonary specimens could be grown on the selective SAB medium. One of these also grew on NSDA, while two others could be grown on sunflower seed agar.

Among the CSF specimens 61 isolates could be grown on SAB medium while only 45 or 52 numbers were culturable on NSDA and SSA medium respectively. Among the specimens from extraneural sites, 12 isolates could be grown on SAB medium while only five or eight isolates were culturable on NSDA and SSA medium respectively.

5.3.2 Occurrence of *Cryptococcus* from Environmental sources

Pigeon droppings from residential pigeon pens and dried plant material (1 g) from beneath Eucalyptus trees were collected in 9ml of saline. In the laboratory, they were cultured on neutral Sabaroud’s Dextrose agar (NSDA), Staibs agar (SAB) medium and SSA medium (*vide*, 4.8).

Out of 42 specimens of pigeon droppings collected from 6 different locations in North Chennai (Tamilnadu), *C. neoformans* was isolated from 9 specimens (21.4%). Out of 25 specimens of dried plant material of *Eucalyptus* tree, four samples were positive for *C. neoformans* (16%; Table 12).

Growth characteristics on different media and populations in the 13 samples that were positive for *C. neoformans* were then estimated. As seen from
Table 13, on SAB medium 13/67 samples were positive with a population of $7 \times 10^2$ to $3.3 \times 10^5$ CFU/gm recovered.

5.3.3 Microscopic Examination using negative staining:

The principle of this staining method is to detect the polysaccharide capsule around this yeast. Capsule is an important virulent factor and hence its detection in samples either by simple microscopy or serology would be a very definitive identification factor.

Negative staining (for capsule detection) was done with pulmonary, CSF specimens and isolates obtained from the environment using Indian ink and Nigrosin wet mount. A drop of Indian ink or nigrosin solution (aqueous solution, 1% nigrosin powder, Himedia) and processed specimen were mixed on a clean glass slides and mounted with a coverslip.

The wetmount was examined under 10x and then 40x objective (Nikon, USA) for the presence of encapsulated yeast. Cells with a clear halo against the background were scored as positive (Fig. 3).

Microscopy of the specimens using Indian ink and nigrosin staining showed that one out of 3 culture positive specimens were positive when pulmonary samples were tested. Among 143 CSF samples, negative staining revealed capsulated yeast in 40 samples only although 61 were culture positive (Table 14). Cultures recovered from the environment also showed the clear presence of the encapsulated yeasts.

5.3.4 Culture on various media:

Definitive tests for identification of this encapsulated yeast are their growth on selective media. Isolation of the yeast from specimens on BHIA medium
containing chloramphenicol could vary from 2 days – 3 weeks. Each of the isolates were numbered and used independently for their characterization. Pigmentation, growth characteristics and colony morphology at 27°C being an important virulence character was studied on the following media.

5.3.4.1 Presumptive identification on media inducing pigmentation:

One important medium used for identification of *Cryptococcus neoformans* species is the Staib agar (1962) in which the fungus produces characteristic dark brown pigmentation. Media containing seed extracts of *Guizotia abyssinica* (Staib, 1962) Sunflower seeds (Pal and Baxter, 1985) or mustard seeds (Nandhakumar *et al.*, 2006) were used to show that *C. neoformans* spp. selectively produced a brown pigment unlike other yeasts like *Candida* spp. Media containing polyphenolics or polyaminobenzene compounds induce the phenoloxidase in this fungus resulting in the brown pigmentation. The Staib’s Agar amended with biphenyl (SAB) was used to control hyphomycetes.

**Results of CDC isolates:** On SAB, NSDA and SSA media, all the four isolates grew well. On SAB media (Fig.4a) and SSA media (Fig 4b), all four serotypes grew well and were deeply brown pigmented (melanin). On NSDA media, serotype A only was darkly pigmented while serotype D, B and C was mildly pigment (Fig.4c).

**Results for collected samples:** The NSDA plates were contaminated predominantly with the species of *Candida* and *Aspergillus*. The colonies on Staibs agar observed were well grown and deeply melanin pigmented, but the colonies on sunflower seed agar were comparatively less pigmented, few and smaller in size (Fig. 4 b, c).
The data obtained on growth of cultures on the three media and microscopy for the clinical specimens were subjected to statistical predictive SPSS analysis (Table 14). Only the Staibs agar test showed 100% specificity for cultures obtained from all specimens (n = 3; n = 61; n = 12). Negative staining was 66% sensitive for CSF samples but 33% for pulmonary samples. The NSDA culture test was also variable in sensitivity, being 73% for CSF samples, 42% for extraneural sites and 33% for pulmonary samples. The sunflower seed agar showed a better sensitivity for culture of this yeast and was 67% for both pulmonary and extraneural site and 85% with CSF specimens. Here we concluded that staining gave a negative predictive value of 80% for pulmonary and 95% for CSF samples. Growth on SAB medium gave 100% positive and negative predictive values for all types of specimens. However positive culture showed 100% specificity for all the four tests employed. Thus, the growth on SAB medium may be considered the most selective /definitive method for diagnosis of this pathogen from our studies.

5.3.4.2 Presumptive biochemical Tests for Cryptococcus species using growth on specific media

Type isolates (CDC), isolates obtained from the environment and isolates from patients were subjected to presumptive biochemical tests. These three groups of isolates were tested for urease hydrolysis, nitrate utilization, and proline assimilation.

Four CDC isolates, 13 isolates from the environment and 64 isolates of patient were cultured on the respective media (vide, 4.9). On Christensen’s Urease agar (Fig. 5) the colour of the medium changed from yellow to pink in cultures of Cryptococci while C. albicans did not utilize urea. Nitrate utilization tested showed no change in colour for all isolates tested (Fig 6) differing from other
yeasts in this phenotype. Proline assimilation was detected by an overgrowth surrounding the proline containing disc placed on a culture plate. The results are presented in Table 15 and Fig. 7.

5.3.5 Biochemical Serogrouping of isolates:

Fromtling *et al.* (1982) and Shadomy *et al.* (1987) first utilized the differences in the growth abilities of *Cryptococcus neoformans* serovars in the presence of glycine or L-canavanine as a sole carbon source and resistance of these yeasts to Cycloheximide. These tests were typical to Cryptococci and none of the *Candida* species were known to grow on these media.

5.3.5.1 Standard (CDC) isolates

Standard (CDC) isolates of *C. neoformans* var. *grubii* serotype A, *C. neoformans* var. *neoformans* D and *C. gattii* serotype B and serotype C were cultured on CGB medium (L-Canavanine-glycine bromothymol blue) and GCP medium (Glycine-cycloheximide-phenol red medium). Pigmentation of the medium were observed at the end of the experiment and tabulated.

The colour changes seen on CGB media (*vide*, 4.10) for the four type isolates are presented in Fig 8. On this medium type isolates of *Cryptococcus gattii* (serotype B and Serotype C) showed changes in pH of the medium resulting in blue or green colour. The strains of *Cryptococcus neoformans* var. *grubii* A and *Cryptococcus neoformans* var. *neoformans* D did not or showed marginal colour changes on this medium.

Growth on GCP medium again showed typical change in colour from yellow to pink or red (Fig.9) only in cultures of *Cryptococcus gattii* (serotype B
and C). *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) did not or showed marginal colour change on this medium.

5.3.5.2 Biochemical serogrouping of Cryptococcus from Clinical samples

CSF samples from patients were examined by culture on SAB medium (*vide*, 4.8.2) with 64 samples being positive (Table 6). Their growth response on differential agar media like CGB and GCP was used to determine the serotypes.

Result presented in Table 16 and Fig 10 indicated the differential response of the 64 isolates and a putative classification was made. Of the 64 positive isolates 53 did not produce any colour change on CGB or GCP agar. These were grouped as *C. neoformans* var. *grubii* (serotype A) and eight of the isolates produce a mild change in colour in the media (CGB and GCP) from light yellow to orange. These were grouped as *C. neoformans* var. *grubii* (serotype D). Three isolates produced intense blue colouration and deep red colouration on the two media (CGB and GCP respectively) and were hence grouped under *Cryptococcus gattii* serotype B/C. Thus, in accordance with the literature *C. neoformans* var. *grubii* (serotype A or D) showed 82.80% preponderance in the patient samples.

5.3.5.3 Biochemical serogrouping of Cryptococcus from environmental samples

Pigeon droppings (42 samples) and Eucalyptus tree debris (25 samples) were processed (*vide*, 4.8) and plated on NSDA, SAB medium and SSA medium. The 13 samples that were positive for *Cryptococcus* spp (*vide*, Table 13) were then tested for a colour reaction on CGB and GCP agar.
The result presented in Table 17 and Fig 11 showed that on CGB agar of the 13 positive isolates 11 produced the corresponding change in colour from yellow to cobalt blue. On GCP agar medium colour changed from yellow to deep red for 11 of these isolates. This indicated the presence of putative *Cryptococcus gattii* serotype B or C isolates in the environment. Two isolates may be *Cryptococcus neoformans var grubii* serotype A/D as they did not show change in colour on these two media (Fig 11).

### 5.4 Latex Agglutination test

All 143 CSF samples from patients with suspected meningitis were tested with the commercially available Latex agglutination test kit (Pastorex @Cryptococcus, Sanofi Diagnostics Pasteur, France) for confirmation. Of these samples from patients 38% of them were positive. However, subsequently, when we cultured these samples on SAB medium to recover the organism, in 61 of the CSF samples (43%) the yeast was present.

The results of the agglutination test and the predictive value for the test is presented in Table 18; Fig.12. It is obvious that this test failed only in 6 of the CSF samples and was positive in 54 (88%) of the 61 culture positive samples. This test was however not serotype specific.
5.5 MIC of *Cryptococcus* spp isolated from Clinical specimens

The treatment for Cryptococcal meningitis generally involves administration of Amphotericin B. Under chronic conditions Fluconazole and 5-Flucytosine are also prescribed. Hence it was necessary to determine if the clinical isolates obtained from our studies were inhibited by the three most commonly used antibiotics.

The clinical isolates (n = 64; *vide*, Table 6) were grown in RPMI carrying increasing dilutions of Amphotericin B 1µg/ml or Fluconazole (16 µg/ml) or 5-Flucytosine (16 µg/ml). The MIC$_{50}$ values for 64 isolates in Amphotericin B was 0.5 µg/ml; 2 µg/ml for 5-Flucytosine and (4 µg/ml) for Fluconazole. The results are presented in Table 19. The MIC 90 values determined for each of the 64 isolates was 1 µg/ml for Amphotericin B; 6 µg/ml for 5-Flucytosine and 13 µg/ml for Fluconazole.

These isolates did not exhibit any unusual resistance to Amphotericin B.
5.6 Immunoblotting using serum of patients with Cryptococcosis

5.6.1 Profiles by SDS PAGE of yeast total proteins

Blood samples of five AIDS patient were cultured in Staib agar (SAB) for presence of yeasts. The yeast strains identified as *C. neoformans* var *grubii* (sero type A), *C. neoformans* var *neoformans* (sero type D; vide, Table 16) and one strain of *Candida albicans* were cultured in liquid medium. Total cellular proteins from these identified strains of the yeast were estimated and used to separate on SDS gel to obtain their protein profiles (vide, 4.13). As seen in Fig 13 silver staining of gel, twelve different peptides which ranged from 75 kD to 15 kD were apparent in *C. neoformans* var *grubii* (sero type A), *C. neoformans* var *neoformans* (sero type D) and one strain of *Candida albicans*. Most of the proteins seen here were probably identical in both yeast species.

5.6.2 Immunoblotting with patient’s serum

The proteins separated as above were transferred to Nitrocellulose membrane (vide 4.14) and probed with serum of AIDS patients (a selected five) was diagnosed with Cryptococcal meningitis (the yeast was isolated from these patient's). A representative blot probed with patient sera is presented in Fig.14. These immunoblots were detected using chromogenic substrate 3,3-diaminobenzidine tetra hydrochloride (DAM).

Table 20 showed that one protein was shared with unrelated species, *C. albicans*. Four proteins in sera of patients reacted with both A and D cellular antigens indicating no specificity.
5.7 PCR

Diagnosis of Cryptococcal infections in neat CSF samples and clinical samples (like blood) is required to begin appropriate therapy. PCR diagnosis is a test of choice as early results determines the clinical treatment procedures.

Published protocols on using PCR for clinical samples indicated the need for optimization in individual laboratories. This test was therefore optimized for our samples for the first time in Tamil Nadu and is presented below.

5.7.1 DNA isolation and Quantification

Organisms cultured on SAB agar and confirmed as Cryptococcus neoformans serovars were selected for isolation of total DNA. These were obtained from CSF of clinically sick patients (n = 61; vide, Table 6). The extraction of genomic DNA was performed with a slightly modification of the procedure of Sandhu et al., (1995). The isolated DNA was checked in a 0.8% agarose gel. The results are presented in Fig. 15 and showed genomic DNA.

The DNA was quantified by using UV spectrophotometer at 260/280 nm. This DNA was diluted suitably for PCR analysis. The results of this quantification are tabulated in Table 21. It was possible to prepare a fairly good quality of DNA in 12% of the samples while majority of preparations yielded total DNA containing protein and other contaminants. The yields of the DNA ranged from 20.15 to 155 µg.

5.7.2 PCR using fungal specific primers to rRNA

Primers flanking the internal ribosomal sites of ‘yeasts’ were used to optimise PCR conditions. Template DNA for each isolate was prepared in two ways. CSF samples that were culture positive (n = 61; Table 6) were either used
directly as template DNA or DNA was prepared from cultures obtained from these CSF (vide, M & M 4.15). The DNA from these two preparations were used to amplify using four sets of primers (vide, M & M 4.15.3). The diagrammatic representations of the relative positions of the primers are given in Fig. 16a (Rappelli, et al., 1998).

5.7.2.1 PCR analysis using ITS1 and ITS4

When CSF samples were directly used in PCR with these set of primers only 38 / 61 samples showed a 600 bp product (62.2%). Some of PCR results are seen in Fig. 16 b, c and in Table 22. When template DNA was prepared from the isolated cultures all the samples gave a 600 bp fragment (100%). It is possible that PCR inhibitory factors present in the clinical CSF samples used as template; interfered resulting in absence of a product. The marker DNA (lane M) used to compare amplicon sizes was 100 bp ladder.

5.7.2.2 PCR analysis using primers CN4 and CN5

Template DNA from cultured yeast cells or clinical, CSF samples were amplified using CN4 and CN5 primers which were considered more specific to C. neoformans species.

A 136 bp fragment expected from Cryptococcus genome was seen in all 61 samples that were cultured (Fig 17a, b).

Again when amplification of CSF was attempted only 45 / 61 samples showed the 136 bp fragment (74%). This was marginally better than the earlier primer pair (ITS1 and ITS4). The results are presented in Table 23 and Fig 17 (b and c). The DNA marker run for comparison was 100 bp.
5.7.2.3 PCR using ITS1 and CN4 primers

A change of primer pairs for detection (using two DNA templates) was attempted with a combination of generic and species specific primers. Again DNA obtained from the cultures showed amplification of a 415 bp fragment in all 61 isolates, when ITS1 and CN4 were used as a primer.

From CSF, amplification was obtained in 57 of the 61 samples. This showed a remarkable improvement in detection of 93.4 % without the need to isolate yeasts or culture them. DNA templates from all the cultured isolates were positive (100%). The DNA marker run for comparison was 100 bp. The results are presented in Table 24 and Fig 18 (b and c).

5.7.2.4 PCR using internal primers CN5 and CN6

In the earlier experiment with species specific primers, the primers could not amplify the Cryptococcus gene from all the samples of CSF. One of the reasons for such a lack of amplification could be the rare amount of template DNA. Hence in this experiment, a double PCR was attempted.

PCR product (415 bp) obtained earlier (vide, 5.7.2.3) was used as a template (2 µl from the remaining 10 µl product) for a second round of PCR using internal primers CN5 and CN6. Here again, all templates from cultured yeasts were positive (116bp) while only 93.4% of CSF samples were positive (Table 25; Fig 19 b, c). This suggests that template DNA is not limiting but PCR interfering substances in CSF samples could play a role in 3 of the samples.
5.8 Fourier Transform Infrared Spectroscopy (FTIR) characterization of CSF samples

CSF samples from patients were withdrawn (vide, 4.3.2) and stored appropriately. These samples were then subjected to microscopy and culture to confirm the presence of the pathogenic yeast. Fifteen samples from which the yeast was recovered (vide, 4.8.2) were selected. Of these, 8 CSF samples were from patients also suffering from AIDS (patient 1-8); four samples were from TB infected (patient 9-12) and three others had other disorders (patient 13-15).

Three CSF samples drawn from patients with suspected symptoms but from which no *Cryptococcus* or other infectious agents could be isolated were taken as controls (control 1-3). Spectral images of all samples were performed over a wave number range of 700-4000 cm\(^{-1}\) in the mid-IR region with a Spectral resolution of 4 cm\(^{-1}\). The number of co-added scans was increased to 128 to achieve a high signal to noise ratio.

5.8.1 FTIR spectra of controls vs patient CSF samples

FTIR spectra of CSF samples obtained from three controls are shown in the Figures 20a & b. All three FTIR spectra of CSF samples from controls were very similar in both lower (Fig. 20a) and higher (Fig. 20b) wave number regions. The Standard Deviation calculated for three spectra of CSF samples from controls was found to be 0.013 and 0.019 for lower and higher wave number regions, respectively. Since the variation among controls was low, which is evident from the SD values, the average spectra of the three controls is used for a comparison with test samples in the rest of the Figures. FTIR spectra of control CSF samples exhibited diagnostics IR bands at 990, 1066, 1328 and 1648 cm\(^{-1}\) (Table 26a, Fig. 22a, b).
The average spectra of controls here in after called as average controls depicted as a red thick spectrum was compared with spectra of samples from patients 15nos; (Cryptococcus positive). The results are presented in Fig. 21a for low range and high range (Fig. 21b) wave numbers. Significant IR spectral changes (Fig. 21 a & b) were observed with CSF samples obtained from cryptococcal infected patients. Spectral shifts were observed with symmetric stretching vibrations of phosphate group such as 1014 and 1046 cm⁻¹ which appears at 990 and 1066 cm⁻¹ in the CSF samples (Table 26b). The IR asymmetric stretching vibrations of phosphate group appeared prominently at 1124 cm⁻¹ which was absent in the case of control samples. IR band at 1746 cm⁻¹ was a new one observed with patient samples which was completely absent with control CSF samples. Various IR bands for control and patient CSF samples can be compared from Table 27a and 27b.

From the spectral overlaps seen in Figures 21 and the table 26a and b, it is obvious that significant spectral shifts corresponding to particularly phosphate groups and asymmetric stretching of aminoacid side chains have occurred in patient samples while a new peak was also observed at 1746 wavenumber cm⁻¹.

5.8.2 FTIR spectra of CSF samples from AIDS patients

Cryptococcal meningitis being opportunistic is known to infect persons with other immunocompromised conditions. Hence comparisons of spectra among these compromising conditions would help ascertain if the differences are unique to Cryptococcal infections.

CSF samples from eight patients (patient 1,2,3,4,5,6,7 and 15) were culture positive and diagnosed with AIDS. The individual spectrum was compared with
average control in the region between 800-1800 cm\(^{-1}\) and between 2500-4000 cm\(^{-1}\) (Figs 22a to 22p).

IR absorbance in the region 950-1200 cm\(^{-1}\) was observed to be lower for patient subjects 4-8 (Fig 23g, I, k, m & o) compared to average control subjects. Three patients (1-3) CSF samples (Fig 23a, c & e) showed higher IR absorbance than the average control subjects CSF sample. In particular, in two patients CSF samples (2-3), the IR absorbance was almost twice that of average control (Fig. 23c & e). The IR region between 950-1200 cm\(^{-1}\) arises due to symmetric and asymmetric stretching vibrations of PO4\(^{3-}\) and hence phosphate content variations are significantly observed with control and patient CSF samples.

Regions between 1200 -1410 and 1500-1700 cm\(^{-1}\) showed higher IR absorbance for average control of CSF samples compared to other AIDS patients except in the case of patient No. 1. In this sample, there were significant spectral shifts observed with amide band I at 1650 cm\(^{-1}\). Both average of controls and patient No: 1 showed amide band I at 1652 cm\(^{-1}\) (Fig. 22a). But other 7 AIDS patients showed spectral shift of 14 -36 cm\(^{-1}\). It may be due to the changes with secondary structures of proteins present in CSF samples.

In addition to spectral shifts with respect to amide I band, pattern changes were also observed around the region of amide I band. There was a small IR band at 1648 cm\(^{-1}\)(could be seen on expansion of IR spectral region) in the case of patient 2, 3 (Fig. 22c & e) and it was absent in all other patients and the controls.

IR bands around 1740 cm\(^{-1}\) was only observed with patient 2-7 (Fig. 22 c, e, g, I, k, m, & o) and it was completely absent in the case of patient No:1 and average control samples.
IR absorbance for 2886 cm\(^{-1}\) for CSF samples obtained from patient subjects CSF (1-3; Fig. 23b, d & f) was higher compared to average control. Other patient subjects CSF (4-8; Fig. 22 h, j, l, n & p) showed lower values compared to average control. This trend matched with 950-1200 cm\(^{-1}\) region where IR absorbance was observed to be higher with patient subjects 1-3 and lower for patient subjects 4-8 compared to the average control.

But the absorbance value for IR band at 2816 cm\(^{-1}\) were higher for patient subjects 1-3, 5, 7 & 8 and lower with patient subjects 4, 6 compared to average control subjects. Asymmetric stretching vibrations of CH\(_2\), CH\(_3\) groups present in lipids, amino acid side chains, nucleic acids account for IR bands in the region between 2800-3000 cm\(^{-1}\).

Comparing the wave number region 950-1200 cm\(^{-1}\) and amide band I with higher wave number region suggests that the absorbance variations among various AIDS patient subjects would be attributed to changes in nucleic acids or lipid content in the CSF sample obtained from the patient subjects.

A consolidation of the individual spectra in the two regions was compared with average control to bring out the changes seen between healthy controls and infected CSF samples (Fig. 23a & b).
5.8.3 FTIR spectra of CSF samples from TB patients

Four patients whose CSF was positive for Cryptococcus and also were affected by TB were selected (patient 9, 10, 11, 12). Spectra of CSF from each of these individual TB patient subjects are shown in Fig. 24 (a, c, e) in the region of 800 - 1800 wave number cm⁻¹. In the region 2500 – 4000 wave number cm⁻¹ the spectra are presented in Fig 24 (b, d, f, h). Each of the spectra was compared with average control.

Absorbance of IR band at 2890 cm⁻¹ was higher for average control compared to all four TB patient subjects. But in the case of IR band at 2818 cm⁻¹ the absorbance was higher for control compared to patient subjects (9 & 10; Fig 24 b, d) and lower to other 2 patient subjects (11 &12; Fig 24 f, h). Analysis of higher wave number region suggested changes in the contents of various biomolecules such as nucleic acids, proteins and lipids. This is substantiated by higher absorbance of average control subjects in the entire region of 900-1800 cm⁻¹ compared to the TB patient subjects.
5.8.4 FTIR spectra of CSF samples obtained from other disorders

FTIR spectrum of CSF samples that were positive for the Cryptococcus and obtained from Lymphoma, Chronic alcoholism and renal transplant patients are shown in Fig 25. Three Patients (patient 13, 14 and 15) were selected and their spectrum for both regions viz., 900-1800 and 2500-4000 wavenumber cm\(^{-1}\) were compared to average controls.

IR absorbance was much higher with average control compared to the patients suffering due to other disorders such as lymphoma, chronic alcoholism and renal transplant, in the region between 980-1680 cm\(^{-1}\). In addition, an IR band at 1740 cm\(^{-1}\) was observed with CSF obtained from the patients belonging to other disorders and it was absent in the case of average control sample. In the higher wave number region, IR band at 2888 cm\(^{-1}\) showed higher absorbance for average control compared to the patients. IR band at 2814 cm\(^{-1}\) was much lower in the case of Lymphoma patient where as other two patients showed only moderate decrease compared to the average control.

5.8.5 Cluster analysis of FTIR spectra of CSF samples

5.8.5.1 Comparison of Cryptococcus infected samples with healthy controls

Cluster analysis of the spectra was performed on four regions of FTIR spectra (950 -1150, 1200-1500, 1500-1800 and 2750-2950 cm\(^{-1}\)). CSF samples obtained from all the 15 patients and three controls (vide, materials and methods 4.16) were subjected to FTIR analysis (results presented in 5.8.1). Figures 26 show the dendograms generated showing the cluster elements in the X-axis and level of heterogenicity between the various elements in the Y-axis.

Best classification was observed with the spectral region 1200-1500 cm\(^{-1}\) having only two false negative cluster elements. Cluster analysis of other spectral
regions resulted in higher number of (more than eight) false negative cluster elements.

5.8.5.2 Comparison of Cryptococcus infected samples and with AIDS with healthy controls

FTIR spectra of cluster analysis of CSF samples from AIDS patients along with three controls are shown in the Figure 27. Results indicated that the best classification was obtained from region 1200-1500 cm\(^{-1}\) having two false positive and no false negative elements. In the major cluster (1200-1500 cm\(^{-1}\)) having 10 elements, two controls were grouped into a minor cluster and six patients were grouped separately into a different sub-cluster. Other spectral regions (950-1150, 1500-1800, 2750-2950 cm\(^{-1}\)) showed more false negative elements compared to 1200-1500 cm\(^{-1}\) region. Hence, other spectral region showed poor classification between patients and controls.

5.8.5.3 Comparison of Cryptococcus infected samples and with TB infection with healthy controls

Regions of cluster analysis for TB patients are shown in Figure 28. As in the case of AIDS patients, 1200-1500 cm\(^{-1}\) region provided best classified between patient and controls having one false negative element. Other spectral region (1150-950, 1500-1800, 2750-2950 cm\(^{-1}\)) showed 2-3 false negative elements providing poor classification between TB patients and controls.
5.8.6 Statistical analysis of FTIR collected on CSF samples obtained from *Cryptococcus neoformans* infected patients and healthy controls subjects

**Regression Analysis**

Regression was performed for the two different regions (700- 1800 and 2500-4000 cm\(^{-1}\)) of FTIR spectra of CSF samples obtained from *Cryptococcus neoformans* infected patients and control subjects.

The results of regression analysis are shown in the table 27a and b. Results indicated that unstandardized coefficient value obtained was \((3.2\times10^{-4})\) for controls and \((0.8\times10^{-4})\) for patient subjects in the 700-1800 cm\(^{-1}\), which was significantly higher for controls subjects compared to patient subjects. However unstandardized coefficients value did not differ significantly for 2500-4000 cm\(^{-1}\)region between controls \((-1.9\times10^{-4})\) and \((-1.5\times10^{-4})\) for patient subjects. This indicates that for every single wave number change, the IR intensity was four times lesser with patient subjects compared to the control subjects.

5.8.7 STUDENT’S T- test

Unpaired student’s T – test was also performed treating both the populations (control and patient subjects) as independent groups (Table 28a and 28b).

Results showed very low probability \((P<0.0001)\) for both the IR spectral regions (700- 1800 and 2500-4000 cm\(^{-1}\)) rejecting the null hypothesis. The student’s T –test results indicated that two different groups differ in their mean FTIR values.
5.9 Bioactivity of Extracts from Mahagony

5.9.1 TLC analysis of crude leaf extracts of *Swietenia mahagoni*

Dried leaf powders of *S. mahagoni* were extracted in methanol and this fraction (SM 12) was used for separation by TLC. Further fractions SM12- E1 and SM12 – E2 were also included for this separation (*vide*, 4.17.3). Results are presented in Fig 29 and Table 29. Crude extracts of methanol gave the fractions from 0.2, 0.5 and 0.6 $R_f$s indicating that this extracts had all the relevant compounds. Brown, non- diffusible substances remained at the origin.

SM12-E1 and SM12-E2 were prepared from the crude leaf powder representing the chloroform soluble or alkaline chloroform fractions respectively. TLC’s eluted in Ethyl acetate: chloroform (1:3) were sprayed with 5% Methanolic sulphuric acid and $R_f$s noted. While SM12 –E2 fractions ranged from 0.05 – 0.38 $R_f$s, those from SM12 –E1 spanned $R_f$s 0.12 – 0.53.

These TLCs confirmed the presence of several compounds in crude extracts which may or may not be bioactive against *Cryptococcus* species.
5.9.2 TLC Bioautography

The scheme for crude leaf extracts is outlined in Fig 2 (vide, 4.17.4). Methanolic extracts of dried leaf powder of SM12 and further fractions E1 and E2 were used for this assay. The sample were eluted on TLC and then incubated with *Cryptococcus neoformans* culture to detect bioactivity.

Growth inhibition was seen as clear white surfaces against the rest of TLC that was pink (TTC) due to growth of the yeast cells. Results are presented in Fig 30 and Table 30.

The methonolic extracts of E1 and E2 fractions of SM12 leaf powder showed good inhibition against *Cryptococcus*. The E1 extract showed 4 zones though they are all small in diameter - 3-4mm. The E2 extract showed the highest activity with a width of 13 (R$_f$ 0.40) and 14mm (R$_f$ 0.10) in diameter (Table 30). The inhibition with Amphotericin -B is shown in spot1.

Crude leaf powder extracts showed a major inhibitory fraction at R$_f$ 0.59. Four zones were obtained in polar fractions of E1 (Chloroform soluble) and two (0.41 and 0.50 R$_f$) corresponded to the R$_f$ seen in (SM12) extracts. Chloroform insoluble fraction, E2 showed good inhibition at 0.10 and 1.40 R$_f$’s.

These compounds should be further purified and compared with already reported molecules for antifungal activity.
5.9.3 Column separation of crude leaf extracts

SM12 leaf powder obtained as extracts E1 (1g) and E2 (1g) (*vide*, 4.17.2) were separated using a silica gel column of 50 cm. Fractions of 5ml were collected, pooled and separated on TLC. TLC was sprayed with reagent or inspected under UV. The results are presented in Fig. 31, 32 and 33).

The compounds could be separated into three distinct pools for SM12-E1. Compounds with larger $R_f > 0.50$ emerged in the first fractions pooled as 15-35. Compounds with $R_f$ of 0.35 fractioned from tubes 40-50 and those with $R_f$ of 0.12 pooled as 50-65 (Fig. 31, panels A - E).

Sample of SM12-E2 (Fig. 32, panels F - I) could be column separated into three distinct pools of $R_f$ 0.35 into tubes 15-25, $R_f$ 0.15 into tubes 25-40 and $R_f$ 0.05 in to tubes 45-55. These pooled samples were used for testing against *Cryptococcus*.

The results of these TLC’s under UV for the extract SM12- E1 (Fig.32) and for extracts SM12- E2 (Fig. 33) are also presented here.
5.9.4 Agar diffusion Assay

Antimicrobial assay was performed by Agar well diffusion method. Inoculum (0.1ml) of test organism was spread on LB agar medium (1.5%). Antimicrobial activity was evaluated by measuring the zone of inhibition (diameter in mm) against the test organism. Antimicrobial activity against *Cryptococcus* was observed with different crude fractions of *S. mahagoni* leaf extracts (SM12, SM12- E1 and SM12 E2).

Crude SM12 and SM12-E1 (Methanol) extract showed moderate inhibition zones of 3-4 mm in diameter. The best results were obtained with the extracts of *S. mahagoni* SM12-E2 (Methanol) that showed inhibition zones of 8 mm in diameter. No zones were produced by solvent control (methanol). The antifungal drug Amphotericin-B (10ppm) that was used as a positive control showed 10 mm of inhibition. The results are shown in (Table 31 and Fig. 34).
5.9.5 Assay of column fractionated samples

In the bioautography assay (vide, 4.17.5) good inhibition was seen with SM12-E2 extract. Hence column separation of this E2 sample was done and pooled 5ml samples based on the sprayed plates were tested using agar diffusion assay. The column purified fractions (20-25, 30-35, 45-50) of SM12-E2 (methanol) extracts were tested against Cryptococcus. The results are shown in Table 32 & Fig 35. Fractions 20-25 showed a better inhibition zone of 6 mm in diameter. Fraction 30-35 and 45-50 did not show inhibition against test organism, but showed no growth inside the well. This shows that their concentration may be too less for bioactivity, and further needs to be confirmed.
5.9.6 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the plant extract against *Cryptococcus* was tested by adding various concentrations 0.5µg to 5µg of the *S. mahagoni* crude leaf extracts in to the culture tube containing 0.5 O.D (Mac Farland turbidity constant). After 24 hours a loop full of each tube is streaked on a LB agar plate.

The tubes containing 1.5µg to 5µg of the SM12-E2 extract showed good inhibition of growth to the test organism in the tubes. The SM12-E1 extracts showed good inhibition of the test organism at 2µg to 5µg. The solvent control showed good growth. The positive control antifungal drug Amphotericin-B (10ppm) and the blank showed no growth in their respective tubes. The results are shown in the Table 33 and Fig. 36.

The plates streaked for re-growth of *Cryptococcus* were examined. Re-growth of the *Cryptococcus* organism was seen in 2µg/ml of SM12-E1 and in SM12-E2 extract 1µg/ml shown few colonies in NSDA plates.

There is no re-growth of the organism *Cryptococcus* seen in 1.5µg/ml of SM12 -E2 and 2µg/ml of SM12-E1. The results are shown in Table 34 and Fig 37. The MIC values for those crude extracts appeared high as the relative concentrations of individual components would be low. Further purification of these bioactive fractions would give us a better insight into this class of compounds.
To summarize these results:-

The separation of compounds (SM12-E1& E2) from leaf extracts of *Swietenia* indicated presence of at least 6 compounds in the methanolic extracts. These compounds showed reactivity to UV and spray reagents (Table.30).

TLC Bioautography using *Cryptococcus neoformans* showed inhibition of the human pathogen at about *R*$_f$’s 0.05, 0.25, 0.41 & 0.50 in E1. *R*$_f$ 0.10 & 0.41 in E2. However, these inhibitory zones did not match with Rf of TLC separated visible (reagent sprayed) compounds nor UV reactive compounds.