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A novel modification of culture media for cultivation of *Cryptococcus neoformans* by using extracts of different plants from Solan area of Himachal Pradesh (India)

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ABSTRACT

Cryptococcus is an encapsulated yeast, ubiquitous in nature. The concept of using differential media for isolating specific fungal pathogens is not new. The present study reveals that all plant based media are found to be supporting the confluent growth of *Cryptococcus neoformans, Lowsonia inermis* (Henna) leaf based media, *Brassica campestris* (Mustard) seed based media and *Rhus cotinus* (Smoke tree) leaf based media exhibits appreciable brown color effect (BCE) which makes them a good option as selective media for *Cryptococcus neoformans.* Not only plant based agar media, the plant based broths also supported confluent growth of this organism. This is perhaps the first report. Thus, these plant based media are selective, simple to prepare and economical to use and offers a novel alternative for currently available synthetic media.

Introduction

Cryptococcus neoformans, a capsulated yeast was first reported in peach juice. It is ubiquitous in nature and belongs to the family basidomycetes. The life cycle of *Cryptococcus neoformans* involves asexual and sexual forms. The asexual form exists as yeast and reproduces by budding.

It causes infections mainly in immunocompromised patients and is thus, gaining significance as one of the most life-threatening pathogen in immunocompromised patients. *Cryptococcus* (Cryptococcal) infections are believed to be acquired by inhalation of airborne yeast or basidiospores from environmental source, which get deposited in the alveoli in the lungs of host (Sanfelice1884).

Virulence of *Cryptococcus neoformans* may be attributed to several factors like capsular polysaccharide, urease production, phospholipase, melanin synthesis and mating type. Some of the virulence factors of *Cryptococcus* are: the presence of polysaccharide capsule, phenol oxidase production and the ability to survive at 37^0C. *Cryptococcus* infection has been on the rise during past two decades (Donald L. *et al.*, 1985). There is an urgent need for, timely and proper diagnosis of *Cryptococcus*. States of Himachal Pradesh, Punjab, Haryana, Uttar Pradesh, Tamil Nadu, Delhi and Union Territory (Chandigarh) have a wide prevalence of *Cryptococcus neoformans* and *C. gattii.* Conventional culture media available nowa days are poorly sensitive and cost more (Nandhakumar *et.al.*, 2007). The concept of using differential media for isolating specific fungal pathogens is not new, but problems encountered with these media for identification of *Cryptococcus neoformans* are: elevated cost of media and a complex medium preparation procedure. *Eucalyptus camaldulensis, Syzgum cumini, Ficus religiosa, Butea monosperma, Tamarindus indica, Polyalthya longifolia, Mimusops elengi* and *Manilkara hexandra* have been studied for isolation of *Cryptococcus* spp. Both the species, *Cryptococcus neoformans* and *C. gattii* have been successfully isolated from decaying wood in trunk hollows, bark and soil near the base of all these trees. Himachal Pradesh has rich plant diversity due to varying degree of agro-climatic zonation from subtropical to extreme cold. The state is a bucket of large variety of medicinal herbs. In the present study, some plants specifically the *Rhus cotinus* (Smoke tree) and *Pinus roxberghii* (Pine) of this region were tested to prepare natural media for supporting growth of *Cryptococcus neoformans*.

Materials and Methods

Plant materials

Bark of *Pinus roxberghii* (Pine), Leaves of *Lowsonia inermis* (Henna), *Rhus cotinus* (Smoke tree) and Seeds of *Brassica campestris* (Mustard) were utilized for preparation of broths and plant agar medium.

Preparation of inoculum of *Cryptococcus neoformans*

The fungal cells were counted using a hemocytometer as per standard protocol followed for counting cells such as leucocytes. Inoculum containing 10^6 cfu/ml was prepared and inoculated in 100µl vol to respective plant broth incubated at 30ºC for 24h, 48h and 72 h in a shaking incubator. The optical densities of inoculated broths were measured at 600 nm at the mentioned time intervals.

Preparation of broths

Filtrates of plant extracts were sterilized in autoclave at 121ºC for 15 minutes at 15 lb pressure and 15-20ml volume poured into sterile Test-tubes in a volume of 15-20ml of1%,2% and 5% concentration (Fig-1a).

Preparation of agar based media

Composition of plant Agar media was Pine bark powder /Henna leaf powder/Mustard seed powder/ *Rhus cotinus* leaf powder 1gm, 2gm, 5gm/100ml, Agar 2.5gm, Distilled water 100 ml and pH 5.6-5.8. For the preparation of these media, the

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weighed amounts of dry powder of plant material were added to 100ml.of sterile distilled water in a conical flask and boiled for 20 minutes with occasional shaking. The extract was then filtered through a muslin cloth for coarse residue and finally filtered through *Whatman* No.1 and the volume adjusted to 100 ml of each respective concentration. The Ingredients were heated to dissolve and sterilized in autoclave at 121ºC for 15 minutes at 15 lb pressure. About 15-20 ml of media was poured into sterile Petri-dishes to a thickness of 4mm (Nandhakumar *et al.*, 2007).

Spread plate method for inoculation of *Cryptococcus neoformans* on plant agar

Inoculum containg 97 cells of *Cryptococcus neoformans* in 20 µl vol. was seeded over pine bark agar, Henna agar, Mutard agar and *Rhus cotinus* agar separately and spread with sterile L-shaped spreader. The plates were incubated at 30°C for 72 hrs. The growth and characteristics of fungal colonies in the plates were recorded, and colonies were counted in a colony counter.

Results and discussion

The present study aimed at the evaluation of plant extract broths of *Rhus cotinus* (Smoke tree), leaf based broth, *Brassica campestris* (Mustard) seed based broths, *Pinus roxberghii* (Pine) bark based broth and *Lowsonia inermis* (Henna) leaf based broth for supporting the growth of *Cryptococcus neoformans.* The plant broths were inoculated with *Cryptococcus neoformans* and their O.D *values* recorded for *Rhus cotinus* (Smoke tree) Fig-2b Table-1, *Brassica campestris* (Mustard), *Pinus roxberghii* (Pine) and for *Lowsonia inermis* (Henna) Fig-2a. Our study reveals that all the plant extract broths in all concentrations 1%, 2% and 5% (w/v) support the growth of this organism progressively during 72 hrs of incubation of with the exception of Henna plant broth where inhibition of growth was seen at higher concentrations as reflected by the decrease in the optical densities on incubation (Table-1).

Fig-1 (a) Plant broth (b) Mustard Seed powder

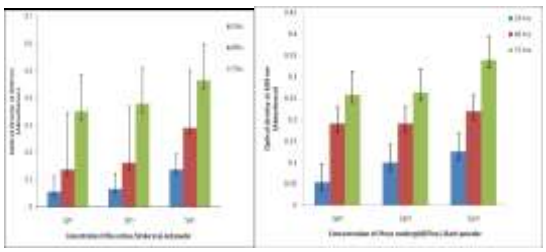
The mean of optical densities in case of *Rus cotinus* (smoke tree) increased progressively with incubation time and concentration of plant material in the broth.

Fig-2 (a) Henna powder (b) *Rhus cotinus* (Smoke Tree)

At 72h, the O.D values were 0.351, 0.378 and 0.464 with 1%,2% and 5% concentration respectively (Table-1). Similar trend was observed in case of *Brassica compestris* (Mustard) and *Pinus roxberghii* (Pine). The O.D values after 72h of incubation were; 0.291, 0.396 and 0.482 in case of *Brassica compestris* (Mustard) and 0.258,2.62 and 0.339 in case of *Pinus roxberghii* (Pine). In case of lowsonia innermis however, the maximum O.D values were recorded after 24h of incubation and 1% concentration; the values decreased with increase in conc. and time of incubation in this case (Table-3 and Table-4). All these observations point towards the fact that all the broths under study supported the growth of *Cryptococcus neoformans.* In fact, these are secondary reports of this nature with regards to fungi in general. Moreover, this is an important observation in the direction of using such media as a replacement for synthetic media like Sabouraud dextrose broth (SDB), Brain-Heart infusion (BHI), Buffered yeast nitrogen base (BYNB), RPMI160), which are complex and expensive (Koneman, 1985; McGinnis 1980; Roberts 1985).

Fig-3 (a) Light brown colored colonies of *Cryptococcus neoformans* in *Brassica campestris* (Mustard) agar medium after 72 hrs. of incubation. (b) Brown color colonies of *Cryptococcus neoformans* on *lowsonia inermis* (Henna) agar medium after 72 hrs. of incubation.

Recovery of *Cryptococcus neoformans* in colony forming unit (cfu) at 72 hrs of incubation are presented in Table-3. The organism was recovered with all the measured amounts of the plant material but the best recovery was observed in Henna agar at 1gm (381.8cfu/ml) than at 2gm (172.4 cfu/ml) and 5gm (14.8) concentration (Table-5). The O.D values were taken for assessment of fungal growth in respective media. The plant extract agar material from these plants have been used as differential medium by various workers (Hopfer and Blank, 1975; Chaskes, 1978; Denning *et al*., 1990; Khan et al., 2004; Nandhakumar *et. al.*, 2006), but similar studies regarding the plant based broth media supporting the growth of *Cryptococcus neoformans* have not been conducted. This is perhaps the first study which reports the use of plant based broth media supporting confluent growth of *Cryptococcus neoformans*. Various culture media including both synthetic and natural have been tested as differential and selective media for the isolation and presumptive identification of *Cryptococcus neoformans* from environmental and clinical samples (Fleming, 1976). Sabourauds dextrose agar (SAB) which is standard medium for maintenance of fungi is supplemented with antibiotics and trypan



Graph-1 shows the optical densities of plant *Rhus continus* broth at 1%, 2% and 5% (w/v) at 24h, 48h and 72h post inoculation at 600 nm. The values are expressed as mean and ± S.D. (n=8). Graph-2 The figure shows the optical density values of plant *Pinus roxberghii* broth at 1%, 2% and 5% (w/v) at 24, 48 and 72 hrs post incubation at 600 nm. The values are expressed as mean and ± S.D (n=8).

cutaneum and *Rhodotorula* species also forms dark blue colonies in it (Fleming, 1976). Various modifications of Litman oxgall medium have also been done including the incorporation of caffeic acid, Ferric citrate etc. This medium was found to be a good differential medium for identification of *Cryptococcus neoformans* as it produces brown pigmentation on it (Botard, 1968; Hopfer, 1975). Also agar supplemented with Inositol and urea proved to be highly selective against genera other than *Cryptococcus* which showed heavy growth and light brown pigmentation on it (Mark, 1977).

Melanin production by phenol oxidase activity is a distinctive and characteristic property of *Cryptococcus neoformans* isolates which gives rise to brown color effect (BCE) of *Cryptococcus neoformans* (Nandhakumar *et.al.*, 2007). Therefore, melanin production has also been tested on plant based media which contains precursors of melanin like Sunflower seed agar, Tobacco agar, Birdseed agar, Mustard seed agar, Henna agar etc by various workers (Hopfer and Blank, 1975; Chaskes, 1978; Denning *et. al*., 1990; Khan *et al*., 2004; Nandhakumar *et.al*., 2006).

In our study, four plant based media were tested for *Cryptococcus neoformans.* Plant decoctions 1%, 2% and 5% (w/v) made by boiling the powder in distilled water were used to prepare agar plates.

Rhus cotinus (Smoke tree) leaf based medium was light brown in color and showed heavy growth. Brown color effect (BCE) of *Cryptococcus neoformans* was observed after 72 hrs at all the three concentrations. The effect was however, found to increase with increasing concentration. Therefore, this media could be considered as selective for *Cryptococcus neoformans* as it exhibits brown color effect (BCE) which is also exhibited in other plant based media and is characteristic of differential media (Hopfer and Blank, 1975; Chaskes, 1978; Denning *et.al*., 1990; Khan *et.al*., 2004; Nandhakumar *et.al.*, 2006).

Brassica campestris (Mustard) seed based medium was translucent and grayish in color and showed good and countable growth (fig-3a). It also exhibits brown color effect (BCE) after 72 hrs at all the three concentrations used and the effect was found to increase with increasing concentration. So, this media also seems to be selective for *Cryptococcus neoformans* as other plant based media (Khan *et.al*., 2004; Nandhakumar *et. al.*, 2006).

Pinus roxberghii (Pine) bark based medium was dark brown in color and showed heavy growth of *Cryptococcus neoformans.* There was no brown color effect (BCE) after 72 hrs at all the three concentrations. The colonies were grayish white in color and very small in size (Fig-3a and 3b).

Lowsonia inermis (Henna) leaf based medium was dark brown in color. Colonies on this media showed light brown color after 72 hrs which intensified on further incubation for four more days (Fig-3b). Also this medium seems to be inhibitory at higher concentration as the best growth in terms of optical density was observed at 1gm conc. This was further supported by the fact that and the cfu/ml count was found to be greater at this concentration than with 2gm and 5gm concentrations. The cfu/ml count was in agreement with the results obtained in case of broth i.e. best count was found at 1gm (381.8) which and the count decreased with increasing concentrations i.e. at 2gm (172.4) and at 5gm (14.08). This medium can be said as the best plant based medium of this study as it shows brown color effect (BCE) as well as supports the growth of *Cryptococcus neoformans* appreciably at low concentration. Further studies are required to be done in this regard it can be used as replacement to synthetic media. All these media were also tested for the growth of environmental contaminants but none of the media showed any contamination as the uninoculated petri plates were kept as control in the safety cabinet and incubated along with the seeded plates. The present study thus, supports the fact that the plant based media have better potential to be used as alternative to synthetic media.

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Table -1 The optical density values of plant *Rhus cotinus* broth inoculated with *Cryptococcus neoformans* at con. of 1%, 2% and 5%(w/v) after 24, 48 and 72 hrs. respectively at 600nm.

Rhus cotinus (Smoke tree) – Leaf										
Conc.	Sample Tube no.								Mean	SD
1gm	I	II	I	II	III	I	II	III		
	0.026	0.089	0.05	0.061	0.05	0.053	0.059	0.059	0.05588	0.01737
	0.137	0.134	0.161	0.158	0.167	0.109	0.115	0.111	0.1365	0.02351
	0.39	0.392	0.343	0.358	0.369	0.298	0.314	0.343	0.35088	0.03355
2gm	I	II	I	II	III	I	II	III		
	0.094	0.127	0.047	0.051	0.061	0.036	0.057	0.052	0.06563	0.02998
	0.13	0.127	0.168	0.198	0.158	0.159	0.18	0.167	0.16088	0.02374
	0.41	0.431	0.383	0.393	0.343	0.371	0.381	0.309	0.37763	0.03803
5gm	I	II	I	II	III	I	II	III		
	0.172	0.264	0.101	0.111	0.106	0.114	0.121	0.106	0.13688	0.0561
	0.533	0.705	0.168	0.172	0.189	0.181	0.178	0.184	0.28875	0.20906
	0.61	0.735	0.386	0.391	0.401	0.392	0.396	0.402	0.46413	0.13298

(w/v) after 24, 48 and 72 hrs. respectively at 600nm.

Table-2 The optical density values of plant *Lowsonia inermis* broth inoculated with *Cryptococcus neoformans* at con. of 1%, 2% and 5% (w/v) after 24, 48 and 72 hrs. respectively at 600nm.

Lowsonia inermis (Henna) – Leaf										
CON.	Sample Tube no.								Mean	STDEV
1gm	I	II	I	II	III	I	II	III		
	0.152	0.145	0.139	0.0.149	0.152	0.161	0.159	0.167	0.15186	0.00914
	0.442	0.549	0.21	0.261	0.232	0.196	0.237	0.291	0.30225	0.1206
	0.771	0.786	0.61	0.67	0.596	0.396	0.46	0.53	0.60238	0.13883
2gm	I	II	I	II	III	I	II	III		
	0.121	0.129	0.131	0.127	0.120	0.129	0.131	0.142	0.13017	0.00688
	0.276	0.284	0.268	0.289	0.286	0.252	0.242	0.269	0.2715	0.01533
	0.73	0.728	0.591	0.618	0.644	0.388	0.391	0.406	0.58113	0.17793
5gm	I	II	I	II	III	I	II	III		
	0.12	0.1	0.172	0.147	0.168	0.152	0.164	0.153	0.147	0.02494
	0.230	0.248	0.232	0.241	0.238	0.262	0.257	0.248	0.2445	0.01139
	0.652	0.626	0.637	0.589	0.668	0.433	0.441	0.487	0.56663	0.09748

Table -3 The optical density of plant *Brassica campestris* broth incubated with *Cryptococcus neoformans* at con. of 1%, 2% and 5% (w/v) after 24, 48 and 72 hrs. respectively at 600nm.

Brassica campestris (Mustard) – Seed										
Conc	Sample Tube no.								Mean	SD
1gm	I	II	I	II	III	I	II	III		
	0.024	0.08	0.028	0.021	0.035	0.053	0.056	0.052	0.04363	0.02018
	0.206	0.186	0.106	0.115	0.138	0.116	0.109	0.121	0.13713	0.03796
	0.319	0.351	0.286	0.315	0.292	0.216	0.269	0.284	0.2915	0.0399
2gm	I	II	I	II	III	I	II	III		
	0.09	0.095	0.037	0.055	0.041	0.096	0.092	0.128	0.07925	0.03167
	0.315	0.288	0.215	0.268	0.286	0.284	0.234	0.268	0.26975	0.03191
	0.576	0.65	0.33	0.36	0.383	0.291	0.293	0.286	0.39613	0.13963
5gm	I	II	I	II	III	I	II	III		
	0.092	0.122	0.038	0.049	0.089	0.098	0.102	0.134	0.0905	0.03285
	0.144	0.138	0.186	0.219	0.315	0.271	0.32	0.279	0.234	0.0729

Table -4 The optical density of plant *Pinus roxberghii* broth incubated with *Cryptococcus neoformans* at 1%, 2% and 5% (w/v) after 24, 48 and 72 hrs. respectively at 600nm.

Pinus roxberghii (Pine) – Bark										
CON.	Sample Tube no.								Mean	STDEV
1gm	I	II	I	II	III	I	II	III		
	0.07	0.09	0.026	0.039	0.059	0.047	0.048	0.051	0.05375	0.01956
	0.221	0.26	0.15	0.159	0.174	0.182	0.186	0.192	0.1905	0.03538
	0.281	0.277	0.261	0.283	0.268	0.226	0.238	0.228	0.25775	0.02375
2gm	I	II	I	II	III	I	II	III		
	0.055	0.081	0.17	0.114	0.147	0.045	0.096	0.084	0.099	0.04306
	0.194	0.215	0.185	0.145	0.194	0.198	0.201	0.196	0.191	0.02042
	0.228	0.241	0.279	0.272	0.276	0.268	0.261	0.278	0.26288	0.01878
5gm	I	II	I	II	III	I	II	III		
	0.1	0.169	0.186	0.105	0.159	0.083	0.101	0.097	0.125	0.03957
	0.279	0.278	0.217	0.189	0.172	0.21	0.198	0.206	0.21863	0.03943
	0.293	0.393	0.281	0.293	0.284	0.378	0.389	0.401	0.339	0.05529