



Novel Preparations of Culture Media for Cultivation of *Cryptococcus neoformans* with Extracts of Different Plant Products

Naveen Minhas, Bharti, Sandip Patil, P.C. Sharma

Department of Microbiology, School of Biotechnology, Shoolini University, Solan, India.

*Corresponding author's E-mail: dr.sharmapc@gmail.com

Accepted on: 15-10-2013; Finalized on: 31-12-2013.

ABSTRACT

Cryptococcus neoformans is a fungal human pathogen which causes cryptococcosis. The present study reveals that *Lycopersicon esculentum* (tomato) agar media, *Spondias dulcis* (golden apple) agar media, *Daucus corota* (carrot) agar media and *Beta vulgaris* (sugar beet) agar media support confluent growth of this organism and produce appreciable Brown Color Effect in colonies as compared to standard media like Sabouraud Dextrose Agar and Potato Dextrose Agar. Plant broths prepared from these extracts also promoted growth of *C. neoformans*. The plant based agar media are prepared from inexpensive and readily available materials. Phytochemical analysis of extracts of these plants revealed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, reducing sugars, coumarins, saponins and tannins. In HPLC, the peaks corresponding to lycopene were obtained in the samples of tomato, apple and carrot. It may be inferred from the present study that tomato, apple, carrot and sugarbeet agar media offer a novel alternative for currently available synthetic media for the cultivation of *Cryptococcus neoformans*.

Keywords: Brown Color Effect, *Cryptococcus neoformans*, Cryptococcosis, HPLC, Plant media.

INTRODUCTION

Cryptococcus neoformans is a cosmopolitan fungal pathogen which causes life-threatening meningoencephalitis in immunocompromised people including patients of acquired immunodeficiency syndrome (AIDS). This pathogen belongs to the family *Basidiomycetes* and is the most common species in temperate climates throughout the world. *C. neoformans* is found in aged pigeon droppings and its life cycle involves both asexual and sexual forms. In the asexual form, this organism exists as yeast (unicellular) which reproduces by budding whereas the sexual form involves mating of hyphae and production of basidiospores. The most serious cryptococcal infections develop in patients with defective cell-mediated immunity usually in patients with AIDS or reticuloendothelial malignancy, organ transplantation and corticosteroid treatment.¹ *Cryptococcus* infections are generally acquired by inhalation of airborne infectious propagules of basidiospores which are highly pathogenic for the following reasons: they are resistant to desiccation, easily aerosolized, and have an ideal size to penetrate and lodge in the alveoli of the lungs. *C. neoformans* normally grows as budding yeast in nutrient-rich medium or host tissue. The virulence factors include: the ability to grow at 37°C, the production of a polysaccharide capsule (composed of glucuronoxylomannan, galactoxylomannan and mannoproteins), and enzyme phenol oxidase which acts on certain substrates (dihydroxyphenylalanine, caffeic acid) to produce melanin. The conventional media for cultivation of *C. neoformans* are less sensitive and costly.² The preparation of media alone is time consuming process. The present study has been planned to formulate media from our natural resources (plant materials) for cultivation of *Cryptococcus neoformans*.

The phytochemical analyses and HPLC of such media might reflect the growth promoting constituents. Plant based agar media have advantage of being prepared easily from readily available and inexpensive plant materials. *Lycopersicon esculentum* (tomato), *Spondias dulcis* (golden apple), *Beta vulgaris* (sugar beet) and *Daucus corota* (carrot) have been selected in the present study for cultivation of *Cryptococcus neoformans*. This study might prove helpful in formulating new natural media to promote the growth of *Cryptococcus neoformans* as an alternative to the costly and complex conventional media.

MATERIALS AND METHODS

Isolate

Cryptococcus neoformans strain ATCC 3542 was obtained from National Chemical Laboratory, Pune (Maharashtra). This strain was maintained on slants of Sabouraud's Dextrose Agar (Himedia, Mumbai) and preserved in 10% glycerol at -20°C and was subcultured on regular intervals so as to maintain fresh cultures.

Plant Materials

Fruit of *Lycopersicon esculentum* (tomato), Fruit of *Spondias dulcis* (golden apple), Root of *Daucus corota* (carrot) and Root of *Beta vulgaris* (sugar beet) were utilized for preparation of plant agar medium and plant broths.

Preparation of *Cryptococcus neoformans* Inoculum for Agar Media

The fungal cells were counted using a hemocytometer. Inoculum (10µl) containing 10⁴ cfu was seeded over respective plant agar media and incubated at 30°C for 24h, 48h and 72 h respectively.



Preparation of *Cryptococcus neoformans* Inoculum for Broth

Inoculum containing 10^6 cfu/ml of *Cryptococcus neoformans* was prepared by using 0.5 McFarland standard. Inoculum in a volume of 100 μ l was added to the broth, incubated at 25°C for 24h, 48h and 72 h respectively and optical densities measured at 600 nm at the specified time intervals.

Preparation of Broths

Plant extracts were sterilized in autoclave at 121°C for 15 minutes at 15lb pressure. Each extract in 20ml volume was poured into sterile test tubes.

Preparation of agar based media

For the preparation of these media, known amounts (1g, 2g and 5g) of powder of each plant material were added to 100ml of sterile distilled water in separate conical flasks and boiled for 10minutes with occasional shaking. The extract was then filtered through muslin cloth for coarse residue and finally filtered through Whatman No.1. The final volume adjusted to 100ml. Agar-Agar powder (3g) was then added to it as solidifying agent and sterilized by autoclaving at 121°C for 15 minutes at 15lb pressure. The media (15-20ml) was poured into sterile Petri dishes to a thickness of 4mm.

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

Inoculum containing 90 cells of *Cryptococcus neoformans* was seeded over Tomato agar, Apple agar, Carrot agar and Sugar beet agar in petri plates separately and spread with sterile L- shaped spreader. The plates were incubated at 30°C for 72 hours. The growth of the fungal cells and characteristics of fungal colonies developed in the plates were recorded and counted in a colony counter.

Test for Phytochemical Analyses

The plant extracts were analyzed for the presence of alkaloids, terpenoids, reducing sugars, saponins, tannins, flavonoids, steroids, proteins, coumarins, glycosides and phenol³⁻⁴ (Table 1).

Test for Alkaloids

One ml of each plant extract was taken in a test tube and a few drops of Mayer's Reagent were added to it. The development of white-yellow precipitate indicated the presence of alkaloids in the extract.

Test for glycosides

To plant extract in glacial acetic acid, a few drops of ferric chloride and concentrated H₂SO₄ were added. The development of a reddish brown colour at the junction of two layers and the bluish green color in the upper layer indicated of the presence of glycosides.

Test for Terpenoids

Plant extract (0.5g) was added to 2 ml of chloroform in a test tube, H₂SO₄ was slowly layered on it. The appearance of reddish brown color at the interface showed the presence of terpenoids in the extract.

Test for Steroids

Plant extract (0.5g) was added to 3 ml of chloroform in a test tube, filtered and concentrated H₂SO₄ added to it carefully so as to form a layer. The development of reddish brown color at the interface was indicative of presence of steroids in the extract.

Test for reducing sugars

To 0.5 ml of plant extract, one ml of distilled water was added followed by 5-8 drops each of Fehling A and B. Brick red precipitate indicated the presence of reducing sugars.

Test for Saponins

To one ml each of plant extract equal volume of distilled water was mixed and shaken vigorously. Froth formation indicated the presence of saponins.

Test for Tannins

Few drops of 10% lead acetate were added to one ml of the plant extract. The precipitation indicated the presence of tannins in the extract.

Test for Flavonoids

Plant extract (0.2g) was dissolved in dil. NaOH and dil. Hydrochloric acid was then added to it. The color changes from yellow to colorless indicate the presence of flavonoids.

Test for Coumarins

To one ml of plant extract equal volume of ethanol – KOH (1:1) solution was added. The appearance of yellow precipitates indicates the presence of coumarins.

Test for Phenols

One ml of plant extract was added to 5 ml distilled water in a test tube. A few drops of neutral ferric chloride were then added to it. The development of dark green color was suggestive of presence of phenols.

Test for Proteins (Biuret test)

To 3 ml of plant extract 4% NaOH and few drops of 1% CuSO₄ solution were added. The appearance of violet or pink color indicated the presence of proteins.

HPLC

The samples were processed for analysis by reverse phase HPLC using mixed phosphate buffer as solvent.

RESULTS AND DISCUSSION

Selected plant extracts in the form of plant based media were evaluated for the cultivation of *Cryptococcus neoformans*. All natural media support the growth of



Cryptococcus neoformans, but the best recovery rates in terms of colony forming unit (cfu) were obtained in *Lycopersicon esculentum* (tomato) agar media (Figure 1a), *Spondias dulcis* (apple) agar media (Figure 1b), and *Beta vulgaris* (sugar beet) agar media (Figure 1c) and in terms of colony size, it was observed greater in case of *Daucus corota* (carrot) agar media (Figure 1d). Also the growth in each plant broth was compared with that on standard broths such as PDB (Potato dextrose broth) and YPD (Yeast extract peptone dextrose). All the concentrations of broth used i.e. 1%, 2% and 5% (w/v) in the study supported the growth of *C. neoformans* during 72 hrs of incubation progressively Figure 2(a-d) but maximum increase in optical density values was observed for *Lycopersicon esculentum* (tomato) (Figure 2a), *Spondias dulcis* (apple) (Figure 2b), and *Daucus corota* (carrot) (Figure 2d). Growths in the plant based broths were comparable with the standard media. All these findings point towards the fact that all these media can replace synthetic media which are being used routinely such as Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA), Brain-Heart Infusion (BHI), Buffered Yeast Nitrogen Base (BYNB), RPMI160 for growing *C. neoformans*. The synthetic media are complex and expensive.⁵⁻⁷

Recovery of *Cryptococcus neoformans* in colony forming unit (cfu) at 72 hrs of incubation are presented in Table 2. The recovery of the organism is seen with all the amounts (1%, 2% and 5%) of the plant material and the recovery

was compared with standard media used (SDA, PDA) but the best recovery was observed in Tomato agar, Apple agar and Sugar beet agar at 1 gm. The cfu/ml in Tomato agar at 1 g was 671 followed by 454 and 443 at 2g and 5g respectively. These values for Sugarbeet agar were 613, 471 and 488 at 1, 2 and 5 g amounts respectively. The cfu/ml in Apple agar was 570, 419 and 455 at 1, 2 and 5g amounts respectively. It can be seen that cfu/ml decreased with increasing concentration of plant product so these media can be more efficient and cost effective than synthetic media, since these support the growth of *Cryptococcus neoformans* appreciably at low concentration, i.e. 1g.

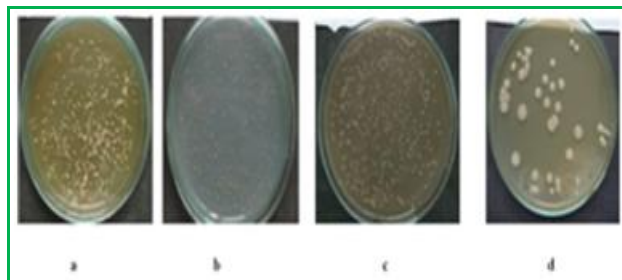


Figure 1: (a-d) Brown colonies of *Cryptococcus neoformans* on agar medium of (a) *Lycopersicon esculentum* (tomato) (b) *Spondias dulcis* (apple) (c) *Beta vulgaris* (sugar beet) and on (d) *Daucus corota* (carrot) after 72 hrs of incubation.

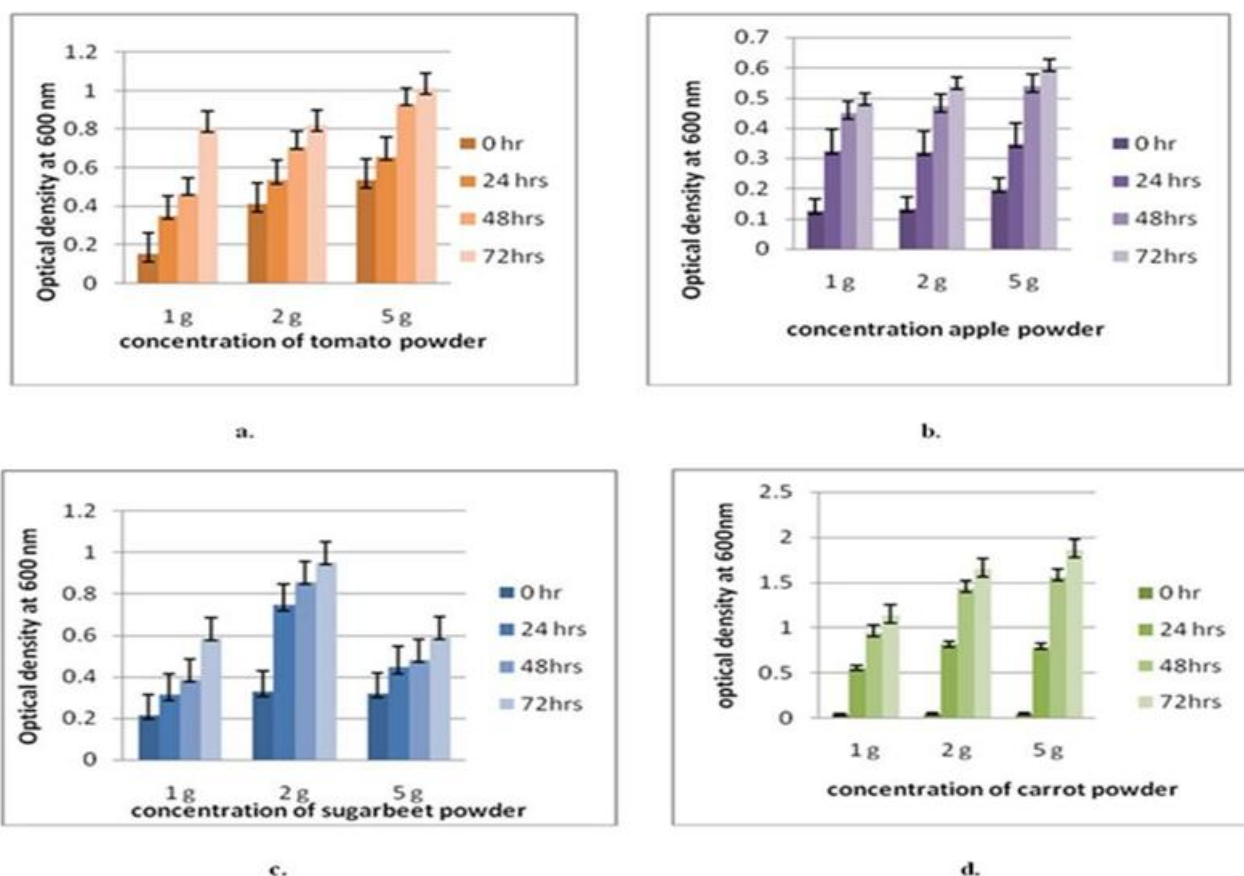


Figure 2: The optical density values of (a) *Lycopersicon esculentum* (tomato) (b.) *Spondias dulcis* (apple) (c) *Beta vulgaris* (sugar beet) and (d) *Daucus corota* (carrot) broth at conc. of 1%, 2% and 5% (w/v) after 24, 48 and 72 hrs of incubation at 600 nm. The values are expressed as mean and \pm S.D (n=3).

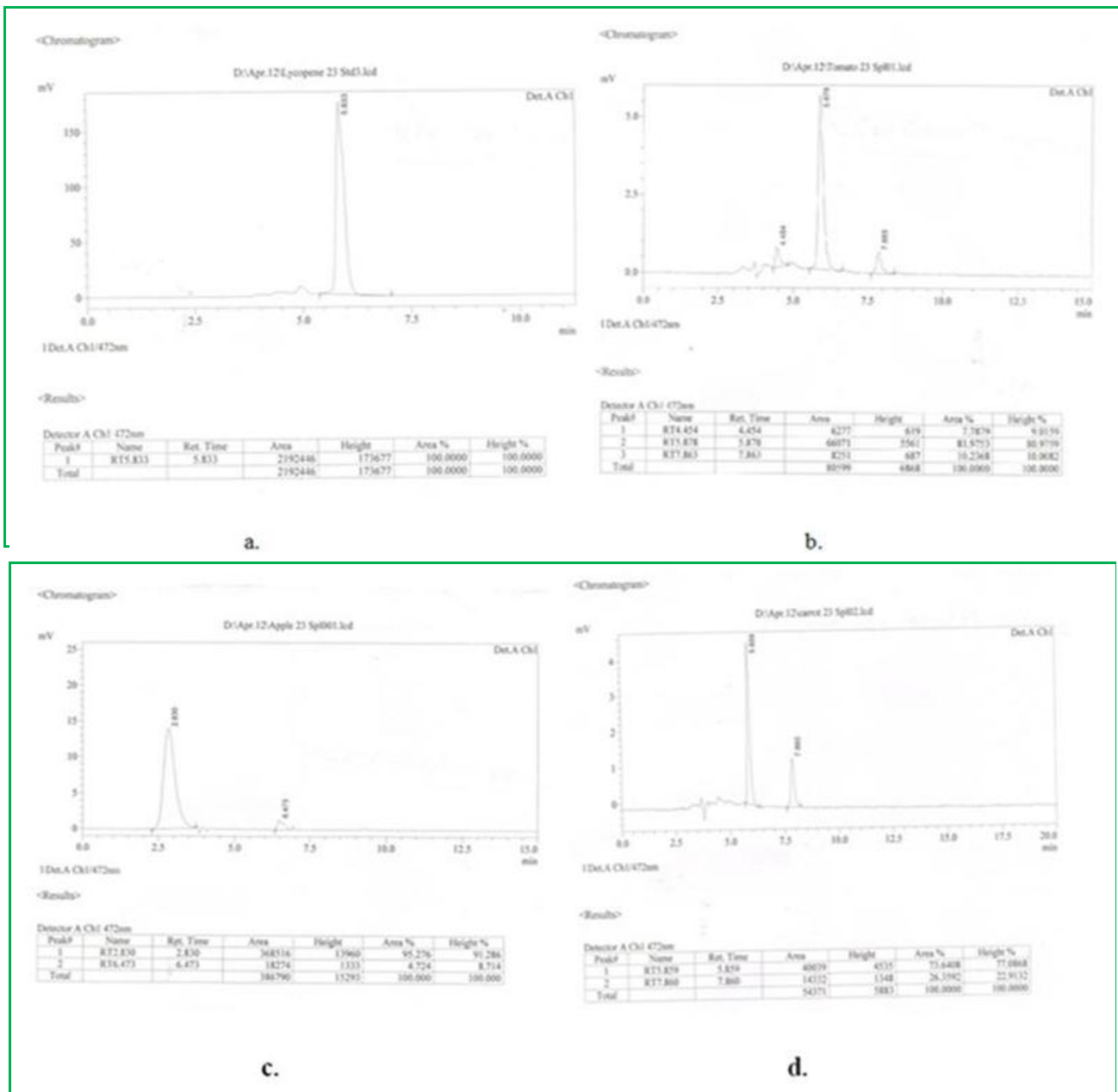


Figure 3: HPLC analysis of (a) Lycopene (standard) (b) Tomato (c) Apple (d) carrot (peak corresponding to the standard Lycopene is visible only in b and c).

The plant extract agar media have been used as differential media by various workers,⁸⁻¹³ but similar studies regarding above plant based solid media and plant based broth supporting the growth of *Cryptococcus neoformans* have been scanty. This is perhaps the first study which reports the use of such plant materials for supporting confluent growth of *Cryptococcus neoformans*. Wide range of 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 environment and clinical cases.¹⁴ Plant based media such as Tomato agar media, Apple agar media, Sugar beet agar media, and Carrot agar media have been found to be supporting the growth of *Cryptococcus neoformans* at low concentration appreciably, which makes them a good option for being a more efficient, economically affordable and selective

media for *Cryptococcus neoformans*. Sabouraud Dextrose Agar (SDA) which is standard medium for maintenance of fungi has been supplemented with antibiotics and trypan blue dye. *Cryptococcus neoformans* forms dark blue colonies in it but contaminants such as *Tricosporum cutaneum* and *Rhodotorula* species also form dark blue colonies in it.¹⁴ Various modifications of Littman oxgall medium have been made including the incorporation of Caffeic acid, ferric citrate etc. and the preparation was found to be a good differential media for identification of *Cryptococcus neoformans* as it forms brown pigmented colonies on it.^{15,8} Agar supplemented with Inositol and urea was also used and this medium proves to be highly selective against genera other than *Cryptococcus* which showed heavy growth and light brown pigmentation on it.

Phenol oxidase activity is the distinctive and characteristic property of *Cryptococcus neoformans* to produce

melanin, a brown coloured pigment.² The production of melanin 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, Pine agar etc.⁸⁻¹³

Table 1: Phytochemical Analysis of Plant Extracts

Components	Tomato	Apple	Sugarbeet	Carrot
Proteins	–	–	–	–
Alkaloids	–	+	–	–
Glycosides	+	+	+	+
Terpenoids	+	+	+	+
Steroids	+	+	+	+
Flavonoids	+	+	+	–
Saponins	+	–	+	+
Reducing sugars	–	+	–	–
Coumarins	–	+	+	+
Phenols	–	–	–	–
Tannins	+	+	+	+

- indicates absence of compound; + indicates presence of compound.

Table 2: Colony forming units (cfu) recovery of *Cryptococcus neoformans* on *Lycopersicon esculentum* (tomato) agar after 72 hrs. of incubation.

Conc.	Cfu (Tomato)	Cfu (Sugarbeet)	Cfu (Apple)	Cfu (Carrot)
1gm	671	613	570	26
2gm	454	471	419	26
5gm	443	488	455	41
SDA	67	67	67	65
PDA	62	62	62	60

The colony forming units developed on Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA) have also been determined for comparative analysis.

In the present study, we tested four plant based media for *Cryptococcus neoformans*. Decoctions of each plant material were made by boiling the powder in distilled water and three concentrations i.e. 1%, 2% and 5% (w/v) were used to prepare agar plates. Each plant based media was inoculated with *Cryptococcus neoformans* and observed after 72 hrs for the growth and colony characteristics.

Lycopersicon esculentum (tomato) based medium was light pale in color, showed heavy growth and Brown color effect (BCE) at all the three concentrations used after 72 hrs of incubation. At lower concentration, colonies were smaller in size, numerous in numbers, and with more brown color effect (BCE) but this effect were reversed with increase in concentration. Therefore, this media could be considered as efficient selective medium for *Cryptococcus neoformans* as it exhibits brown color effect (BCE) as differential character.

Spondias dulcis (apple) based medium was transparent, and showed good and countable growth. It also exhibited brown color effect (BCE) after 72 hrs at all the three concentrations. Brown color effect and colony size both increase with increase in concentration but number of colonies decreases. This media could also be considered as selective for *Cryptococcus neoformans* as other plant based media.

Beta vulgaris (sugar beet) based medium was reddish brown in color and showed heavy growth and Brown color effect (BCE) at all the three concentrations after 72 hrs of incubation. Brown color effect increases with increase in concentration. Colony size remains constant and number of colonies decreases with increasing concentration. Therefore, this media could be considered as selective for *Cryptococcus neoformans* as it exhibits brown color effect (BCE) which is a differential characteristic of *Cryptococcus neoformans*.

Daucus corota (carrot) based medium was translucent and showed large colony size than all the plant based media studied and standard media used. It exhibits little range of Brown color effect. In light of this fact, this medium could be considered as the best medium in terms of colony size.

HPLC

The Chromatograms are presented in the Figure 3(a-d).

Using Lycopene standard, the peaks corresponding to lycopene were obtained in the samples of *Lycopersicon esculentum* (tomato), *Spondias dulcis* (apple), and *Daucus corota* (carrot).

CONCLUSION

It can be concluded from the present study that all plant based media are selective, economical and simple to prepare and offer a novel alternative for currently available synthetic media as they are supporting confluent growth of *Cryptococcus neoformans*.

Acknowledgements: Authors are thankful to the Vice Chancellor, Dr. P.K. Khosla, Shoolini University of Biotechnology and Management Sciences for providing necessary facilities and support to carry out this work. Also the help rendered by Dr. Abhishek Mathur of Sheetal Life Sciences, Dehradun regarding HPLC work is thankfully acknowledged.

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Source of Support: Nil, Conflict of Interest: None.