

A COMPARISON OF THE RADIAL GROWTH OF *ASPERGILLUS NIGER* ON VARIOUS CULTURE MEDIA PREPARED BY THE PLANT BASED EXTRACTS AND POTATO DEXTROSE AGAR

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ABSTRACT

The present study is aimed at growing *Aspergillus niger* on a variety of plant extract based culture media prepared by using various components of different plants. The extracts were used in combination with or without carbon and nitrogen sources along with the mineral salt solution (MSS) to prepare the culture media for the cultivation of *Aspergillus niger*. The growth of the fungus on these media was measured in terms of radial extension of the mycelia and the spore count, which was then compared with the growth on a conventional medium, potato dextrose agar (PDA). A maximum fungal mycelial extension, 85 mm was achieved on PDA and the extract media without supplementation and 90 mm on the media with supplementation in ten days. The spore count on PDA and extract media with and without supplementation was found to be 5.08×10^6 , 4×10^4 – 8.76×10^6 and 10^5 – 8.6×10^5 / 5mm disc/mL, respectively. The spore count of *Aspergillus niger* in supplemented media is comparable (and in some cases even better) as that of PDA.

Key words: *Aspergillus niger*, culture media, plant extracts, potato dextrose agar, radial growth, spore count

INTRODUCTION

In order to cultivate the microorganisms, suitable culture media are required which fulfill their nutritional requirements (Pelczar *et al.*, 1993) and for the growth and study of morphological and metabolic characteristics of fungi, a variety of dehydrated culture media are commercially available (Sharma and Panday, 2010). These media are routinely used for teaching, research and diagnostic purposes, by the pathological laboratories. More over some of the selective and specialized media are used for water and food analyses, antibiotic sensitivity testing, microbiological quality control in industries and other activities (Zakia *et al.*, 2012). Therefore the exact composition of a medium varies according to the purpose for which it is designed (Willey *et al.*, 2008).

With the passage of time, high costs of commodity and diagnostic items across the globe have become a distressing issue. Materials used in the microbiological laboratory, particularly the culture media are also becoming very expensive (Sidana and Farooq, 2014).

Keeping in view the importance of microbiology, the development of cost effective microbiological media is not only economically feasible for countries like Pakistan but may possibly be an alternative for the conventional media. One of the important advantages in the preparation of these media is the utilization of simple and round the year available components, (like bark, leaves or other left over of plants) hence rendering them cost effective too.

The present study evidently suggests the use of plant based components in the preparation of natural extracts. Since wood is the primary component of the plants especially trees, therefore these extracts in turn can be used as a main constituent in the preparation of liquid and solid culture media, for the cultivation of fungi. Wood is basically composed of complex carbonaceous compounds like cellulose, hemicelluloses and lignin. These compounds contribute 40-45, 15-28 and 22-32 percent respectively in wood species (Sjostrom, 1993).

Fungi are capable of producing a variety of extracellular enzymes that hydrolyze complex carbonaceous compounds. These include amylase, pectinase, chitinase, dextranase, xylanase etc. Many pathogenic and saprophytic fungi also produce a series of enzymes namely cellulases, which facilitate the degradation of cellulose to glucose units. Cellulose is the most abundantly occurring polysaccharide found in the plant tissues; serve as a potential source of carbon for fungi. In addition to cellulose, lignin is the second most abundant polymer found on the earth, which also serves as a potential source of carbon for fungi (Garraway and Evans, 1984).

Nevertheless, the complex carbonaceous compounds cannot be extracted during the conventional extraction procedures. The phytochemical analyses of various plants used in this study indicate that glycosidic compounds and/or carbohydrates in the form of reducing sugars are released when aqueous extraction of various plant materials is carried out.

For example the extracts of *Bougainvillea* sp revealed the presence of glycosides (Rashid *et al.*, 2013).

Similarly the water based date bark extracts were found to have carbohydrates and other components, when evaluated for their phytochemical properties (Al-daihan and Bhat, 2012). The phytochemical assays performed to analyze the aqueous coconut bark extracts are indicative of the presence of carbohydrates, and glycosides (Sivakumar *et al.*, 2011).

Further studies on water based neem bark and leaf extracts state that these plant components also contain glycosides (Prashant and Krishnaiah, 2014). The other study on neem leaf extracts show that the reducing sugars are also present in these extracts. (Biu *et al.*, 2009).

Glycosides are one of the groups of organic molecules in which a sugar group is bound to another functional group through a glycosidic bond. (Obla, 2013) According to the IUPAC definition, all disaccharides and polysaccharides are the glycosides (<http://www.iupac.org/>).

It means that when the glycosides are hydrolyzed the sugars are released.

Apart from sugar containing molecules, these natural extracts share some common phytochemical constituents such as saponins, steroids, flavonoids, alkaloids and tannins. Depending upon the solvents (organic or natural such as water) used for extraction; these phytochemicals may be recovered from all parts of the plants at a time or may not be extractable by these solvents from some parts. The studies also indicate that these plant extracts have very low nitrogen content which may or may not be detected by the screening methods. It is noteworthy that the plant based extracts have frequently been analyzed for their anti-microbial potential. Even some of the researchers have tried to isolate the bioactive components against bacterial or fungal pathogens.

However, in this study the potential of the plant extracts has been evaluated for their growth supporting ability for fungi. Therefore, different plant components obtained mainly from woody trees were used as the main ingredients in the preparation of extracts used for the formulation of culture media.

It has already been mentioned that the plant extracts are low in some nutrients therefore, these media were further supplemented with additional sources of carbon (extract of sugar cane peels), nitrogen (laboratory produced yeast extract) and mineral salt solution (MSS).

MATERIALS AND METHODS

Selection of plants and their components: The selection of the plants was based on its round the year availability. Accordingly, their components were selected like *Bougainvillea* branches, *Cocos nucifera* L. (coconut), *Phoenix dactylifera* L. (date palm), *Azadirachta indica* A. Juss. (neem) bark and leaves.

Preparation of extracts: Non-specified (generalized) plant extracts were prepared by using all the above listed plants and their components and individual plant or its components were used to prepare the specified plant extracts.

Plant extract 20% (w/v): The plant components which were left after cutting of trees, and pruning of hedges were collected from the University of Karachi. Small segments (approximately 0.5 cm) were cut, then rinsed with tap water, dried and weighed (20 gram/100 ml) to achieve the required concentration. Steaming was done for 15- 30 minutes at 100° C followed by filtration (Félix-Silva *et al.*, 2014). The extracts were then dispensed into rubber capped glass bottles, autoclaved and stored in the refrigerator. The working solution (2.5 percent v/v) was used for the preparation of culture media.

The other specified plant extracts were prepared in the similar manner; however sugar cane peel extract (SPEx) was prepared in a concentration of 25% (w/v) while the working solution was adjusted to 10 % (v/v) concentration.

Preparation of yeast extract (YEx): A crude preparation of yeast extract was carried out, by growing *Saccharomyces cerevisiae* in 5% (w/v) sugarcane peel extract for 24 hours. It was then centrifuged at 5000 rpm for 15 minutes and the supernatant was discarded. The cells were re-suspended in normal saline, centrifuged again at 5000 rpm for 15 minutes and the supernatant was discarded. The procedure was repeated twice and the cells were re-suspended in sterilized distilled water to achieve autolysis (www.neogen.com/Acumedica/Product). The yeast extract was then filtered and stored in screw capped bottles in the refrigerator. The protein content was found to be 1.8-2.0 g/dL as determined by Biuret's method. In the present study, yeast extract was used in 0.05 % (v/v) concentration.

Preparation of culture media without supplementation: The non-specified (generalized) and specified extract media including bougainvillea branch extract (BVEEx), coconut bark extract (CBEx), date bark extract (DBEx), neem bark extract (NBEx) and neem leaf extract (NELEEx) were prepared as mentioned below:

Generalized plant extract (GPEx) agar: Plant extract [125 mL of 20% (w/v)] was transferred to the Erlenmeyer flask and distilled water was added to make the volume up to 1000 mL. Agar-agar (15 g) was added in medium and the contents were dissolved by heating. The medium was sterilized by autoclaving (at 121°C and 15 lbs /inch²). The petri dishes were poured with the medium and after solidification retained at ambient temperature (28-30°C) for 48 hours for sterility check. Finally the plates were stored in the refrigerator till further use.

Preparation of culture media with supplementation: The generalized and specified extract media including BVEx, CBEx, DBEx, NBEx and NELEx with different combinations were prepared in the similar manner as mentioned below:

I- Supplementation of carbon source: Plant extract [125 mL of 20% (w/v)] and 400 mL of 25% (w/v) sugar cane peel extract (SPEx) was transferred to an Erlenmeyer flask. Distilled water was added to make the volume up to 1000 mL. Agar-agar (15 g) was added to prepare solid medium. The contents were dissolved by heating and finally the medium was sterilized by autoclaving.

II- Supplementation of nitrogen source: Plant extract [125 mL of 20% (w/v)] and 27 mL of 0.05 % (v/v) YEx were transferred to an Erlenmeyer flask. The rest of the procedure was followed as mentioned in section I.

III- Supplementation of carbon and nitrogen sources: Plant extract [125 mL of 20% (w/v)], 400 mL of 25% (w/v) SPEx and 27 mL of 0.05 % (v/v) of YEx were transferred to an Erlenmeyer flask. The rest of the procedure was followed as mentioned in section I.

IV- Supplementation of mineral salt solution (MSS): Plant extract, [125 mL of 20% (w/v)] and 875 mL of MSS was transferred to an Erlenmeyer flask. The rest of the procedure was followed as mentioned in section I.

V- Supplementation of carbon and MSS: Plant extract [125 mL of 20% (w/v)], 400 ml of 25% (w/v) SPEx and 475 mL of MSS was transferred to an Erlenmeyer flask. The rest of the procedure was followed as mentioned in section I.

VI- Supplementation of nitrogen and MSS: Plant extract [125 mL of 20% (w/v)], 27 mL of 0.05 % (v/v) YEx and 848 mL of MSS was transferred to an Erlenmeyer flask. The rest of the procedure was followed as mentioned in section I.

VII - Supplementation of carbon, nitrogen and MSS: Plant extract [125 mL of 20% (w/v)], 400 mL of 25% (w/v) SPEx, 27 mL of 0.05 % (v/v) YEx and 448 ml of MSS was transferred to an Erlenmeyer flask. The rest of the procedure was followed as mentioned in section I.

Preparation of potato dextrose agar: Peeled potatoes (300 g) were sliced into thin chips and boiled in 500 mL of distilled water for 30 min. The extract was then cooled and filtered. Glucose (20 g) was added in the potato extract and mixed until dissolved. In a separate flask agar-agar (15 g) was dissolved in 500 mL distilled water by heating. The contents of both flasks were mixed and the volume was made up to 1000 mL (Atlas, 2010). The rest of the procedure was followed as mentioned in section I.

The pH of all the prepared media was found to be between 5.5 - 6.9.

Isolation, identification and maintenance of fungal culture: The *Aspergillus niger* isolated from the environmental sample was identified by the standard identification methods. (Thom and Raper ,1945 ; Barnett, 1960; Barnett and Hunter, 1998). The culture was then maintained on PDA slants and stored in the refrigerator for further use.

Inoculation of fungal culture and growth measurement: *Aspergillus niger* was grown on PDA plate. Mycelial plugs (5 mm) were cut from the edge of the actively growing 3-4 day old colony (Islam *et al.*, 2003), which were then placed in the centre of all media plates along with the reference medium (PDA).

The plates were incubated at ambient temperature (16 - 45° C) and two perpendicular diameters of the growing colonies were measured in millimeter on daily basis, until the colony reached the edge of the plate (Palacios *et al.*, 2014) or for a maximum incubation period of 10 days.

Sporulation test: After the completion of incubation period, 5 mm mycelial plugs were cut randomly at 3 different places from the growth on the petri dish. The plugs were then transferred to a tube containing distilled water. The preparation was vortexed until the dislodgment of the spores. The spores were then counted in a hemocytometer and average count was reported as, spores /5mm disc / mL.

Determination of reducing sugar: The reducing sugar concentration was determined by the Dinitrosalicylic acid (DNS) method (Noelting and Bernfeld, 1948).

All the tests were conducted in triplicates.

RESULTS AND DISCUSSION

Mycelial extension of *Aspergillus niger* on different culture media: In the present study two broad categories of extract media were used for radial growth assays. Non-specified (generalized) media and the specified media.

The term generalized media, GPEx refers to the plant extracts prepared by using plants and their components as mentioned earlier. Initially the GPEx and its various combinations (both liquid and solid) were used as the screening media to evaluate their growth supporting ability for different fungi. Later the same media were used for the quantification of fungal growth. The quantitative growth analysis on solid GPEx medium with its various combinations was carried out by radial growth assay for the filamentous fungi. The individual plant extracts were used in the preparation of specified culture media after observing the growth on the GPEx media and its combinations. These media along with their combinations were evaluated for the mycelial extension of *Aspergillus niger*, isolated from the environmental sample.

A maximum mycelial extension, 85 mm was observed in case of PDA, GPEx and the specified extract media without supplementation (Fig. 1, 2, 3, 4, 5 and 6). On the other hand, mycelial extension (90 mm) was observed on the GPEx and the specified supplemented media in 10 days. The non-supplemented GPEx media were found to contain sugars which fulfilled carbon requirement of *Aspergillus niger* (Fig. 1 and 13.h). The growth in terms of mycelial extension was improved when these media were supplemented with additional carbon, nitrogen and mineral sources and reached to maximum of 90 mm in 7 days (Fig. 1 and 13.b).

NBEx, when used in combination with SPEX, maximum mycelial extension of 90 mm was attained in 6 days (Fig. 5 and 13.f). The similar results were obtained when NELEx was used in combination with SPEX, YEx and MSS (Fig. 3 and 13.e).

It is interesting and worth noting that neem and its components have already been proved to activity against a variety of fungal species (including pathogens and non-pathogens), as evident by different studies (Singh *et al.*, 1980; Biswas *et al.*, 2002; Natarajan *et al.*, 2003; Verma and Khan, 2006; Grish and Shinkara, 2008; Mondali *et al.*, 2009; Suleiman, 2010). However, the present study revealed the growth supporting activity of neem bark and leaves even when used without supplementation as shown in Fig. 3, 5, 13.i and 13. j. The mycelial extension was found to be 87 and 80 mm in NBEx and NELEx respectively.

It shows that apart from having bioactive compounds, these extracts also contain certain components which have growth supporting ability.

In a study conducted by Mahmoud *et al.* (2011), it has been reported that when aqueous extracts of neem were tested against *Aspergillus niger* at a concentration of 5% (v/v), it resulted in the reduced percent growth inhibition (35.22%). These results are in agreement with our findings, that the aqueous extracts of neem (leaf or bark) when used in a lower concentration such as 2.5 % (v/v) resulted in supporting the growth of fungi. Moreover, the methods used for the extraction (steam or chemical) and the type of specie against which it is being used may also have influence on the growth supporting potential of the extracts.

Apart from neem and its components, other plant extracts like bougainvillea branch, coconut and date bark extracts have also shown ability to support the growth of *Aspergillus niger*. The mycelial extension reached to its maximum, 84 mm in 10 days when bougainvillea and coconut bark extracts were used without supplementation (Fig. 2, 4, 13.k and 13.l). The maximum mycelial extension, 90 mm was observed in 7 days when the same extracts were supplemented with SPEX, YEx and MSS as additional nutrients (Fig. 2,4, 13.d and 13.g).

The phytochemical studies on different components of coconut and date plants clearly reveal their medicinal and nutritional importance. Nevertheless the potential of these plants have always been evaluated for their antimicrobial activity. Studies on different components of date plant by Al-daihan and Bhat, (2012) demonstrate that 10% (w/v) aqueous date bark extracts have antimicrobial activity against some bacterial pathogens. However our extracts were found to show growth promoting ability at [2.5% (v/v)] concentration (Fig. 6 and 13.m). The additional supplementation of carbon, nitrogen and mineral sources not only enhanced the mycelial extension but also produced a high spore count (Fig. 6 and 13.c). The sugar content of the date bark extract is low as depicted in Table 1.

The results on MSS supplemented media are also suggestive of the fact that these plant extracts may have low mineral content thus addition of minerals resulted in the rapid mycelial extension.

Spore production:

The spore count on PDA was found to be 5.08×10^6 spores/ 5 mm disc / mL (Fig. 7, 8, 9, 10, 11 and 12). The spore count on GPEx media was ranged between 1.52×10^5 - 8.76×10^6 . The lowest spore count was observed in

GPEX in combination with YEx and MSS, while the highest spore count in GPEX in combination with SPEX, YEx and MSS (Fig. 7). It is worth mentioning here that this is even more than the spore count on PDA. The spore count in the media with supplementation ranged between 4×10^4 – 8.28×10^6 spores /5mm disc/ mL as shown in Fig 8,9, 10, 11, and 12. Spore production can also be visualized by the dense growth on different media with various combinations as depicted in Fig. 13.b.c.d.e.f and g. The spore count in the specified media without supplementation was found to be in the range of 10^5 – 8.6×10^5 /5mm disc/mL. The highest spore count 8.28×10^6 spores /5mm disc/ mL was observed in DBEx in combination with SPEX, YEx and MSS. The same trend was observed in BVEx, which was when supplemented with SPEX and MSS resulted in second highest spore count, 7.74×10^6 spores / 5mm disc/mL (Fig. 8). The spore count in these extract media is even higher than the spore count on PDA. On the other hand the spore count on NBEx in combination with SPEX (5.08×10^6 spores/ 5mm disc/ mL) was equally comparable with the spore count on PDA.

It is clearly depicted from the results that the media without supplementation showed delayed and less spore production, however the rate of mycelial extension and spore production was enhanced in the media supplemented with MSS in addition to carbon and nitrogen sources.

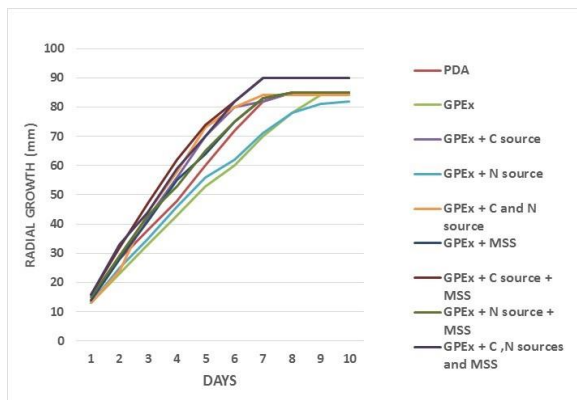


Fig. 1. Radial growth assay of *Aspergillus niger* on various combinations of generalized plant extract (GPEX) media in comparison with potato dextrose agar (PDA). The GPEX was supplemented with carbon (C) source, nitrogen (N) source, and mineral salt solution (MSS) to observe the effect of each amendment on the radial growth of *A. niger*.

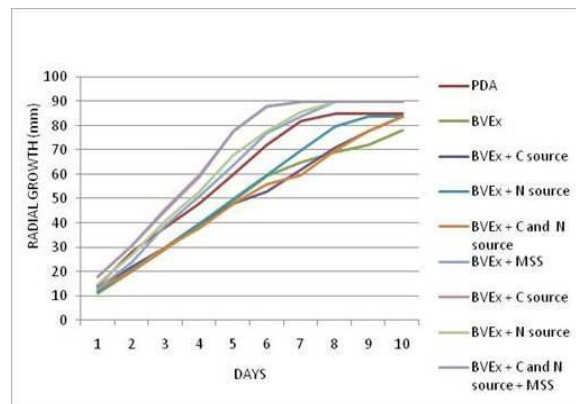


Fig. 2. Radial growth assay of *Aspergillus niger* on various combinations of bougainvillea branch extract (BVEx) media in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 1.

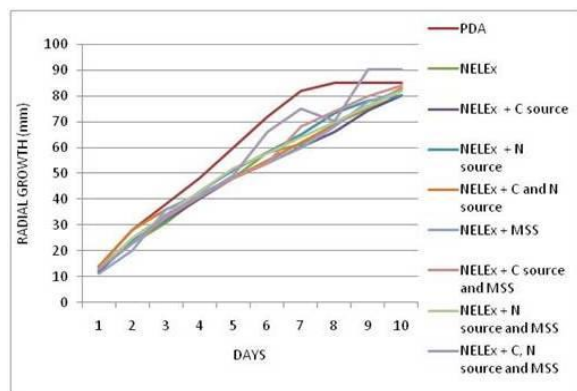


Fig. 3. Radial growth assay of *Aspergillus niger* on various combinations of neem leaf extract (NELEx) in comparison with potato dextrose (PDA). Further details as mentioned in Fig. 1.

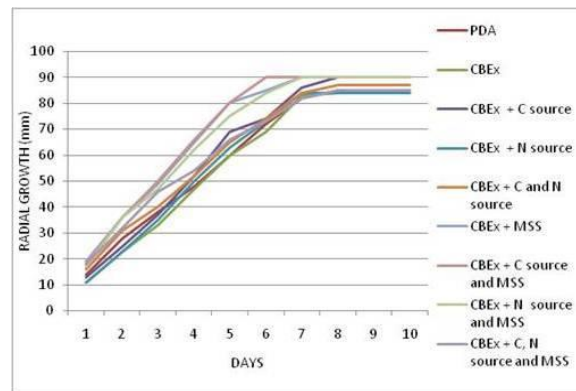


Fig. 4. Radial growth assay of *Aspergillus niger* on various combinations of coconut bark extract (CBEx) in comparison with potato dextrose (PDA). Further details as mentioned in Fig. 1.

(The numerical values depicted here represent means of triplicate and the standard deviation never exceeded 10 %)

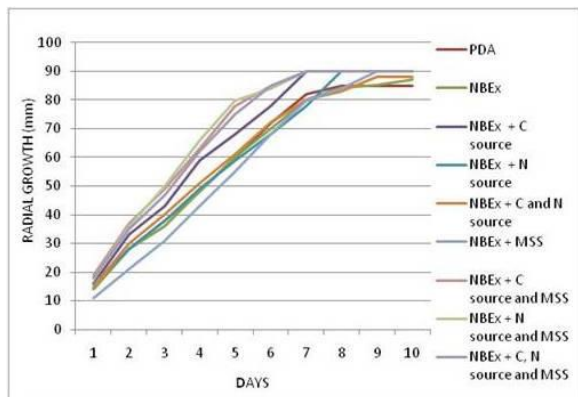


Fig.5. Radial growth assay of *Aspergillus niger* on various combinations of neem bark extract (NBEx) media in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 1.

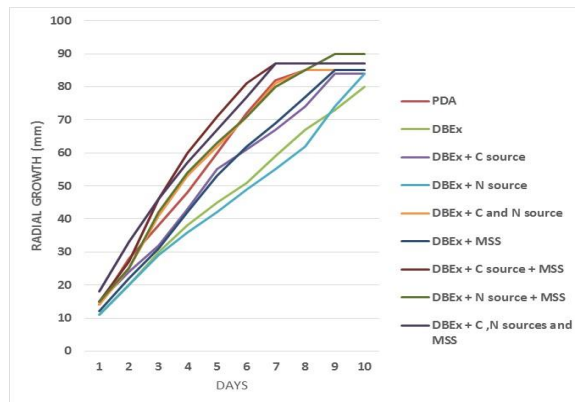


Fig.6. Radial growth assay of *Aspergillus niger* on various combinations of date bark extract (DBEx) media in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig.1

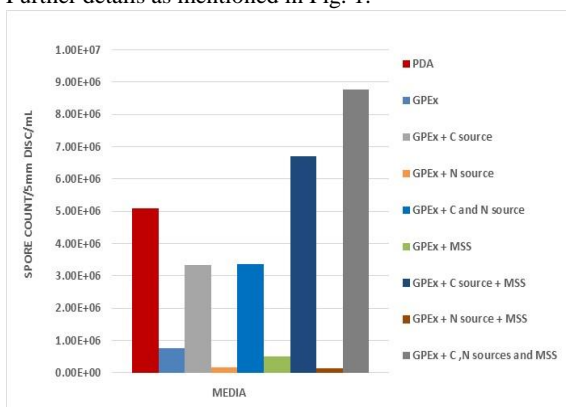


Fig.7. Spore count of *Aspergillus niger* on various combinations of generalized plant extract (GPEx) media in comparison with potato dextrose agar (PDA). The GPEx was supplemented with carbon (C) source, nitrogen (N) source and mineral salt solution (MSS) to observe the effect of each amendment on the spore count of *A. niger*.

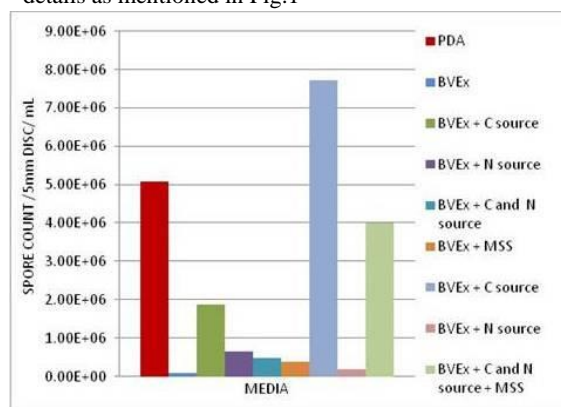


Fig.8. Spore count of *Aspergillus niger* on various combinations of bougainvillea branch extract (BVEx) media in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 7

(The numerical values depicted here represent means of triplicate and the standard deviation never exceeded 10 %)

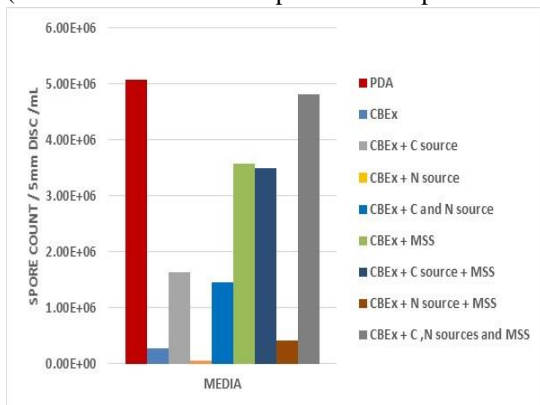


Fig.9. Spore count of *Aspergillus niger* on various combinations of coconut bark extract (CBEx) in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 7

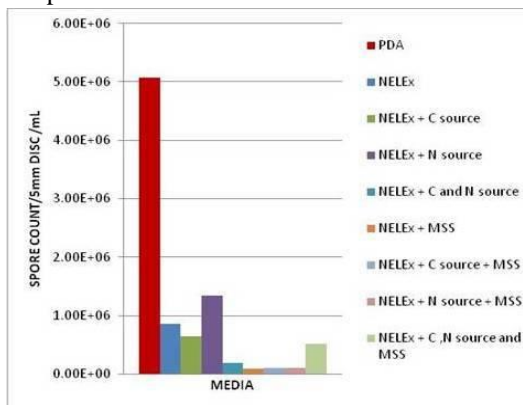


Fig.10. Spore count of *Aspergillus niger* on various combinations of neem leaf extract (NELEx) agar in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 7

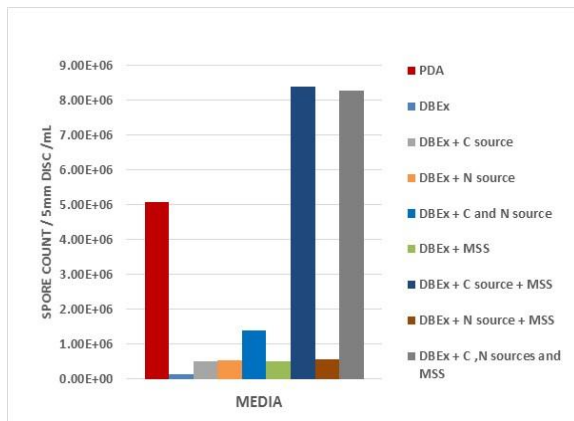


Fig.11. Spore count of *Aspergillus niger* on various combinations of date bark extract (DBEx) agar in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 7

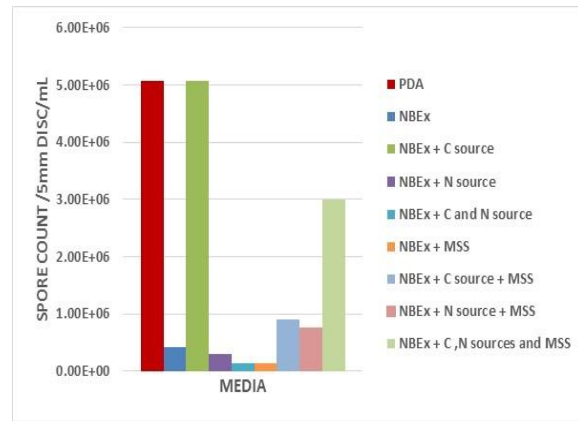


Fig.12. Spore count of *Aspergillus niger* on various combinations of neem bark extract (NBEx) agar in comparison with potato dextrose agar (PDA). Further details as mentioned in Fig. 7

(The numerical values depicted here represent means of triplicate and the standard deviation never exceeded 10 %)

Table 1. The pH and sugar content estimation in various extracts.

| S.No | Name of the extract | pH | Sugar concentration(mg/ml) |
|------|---------------------|-------------|----------------------------|
| 1. | GPEX | 5.11 ± 0.01 | 1.525 |
| 2. | BVEx | 6.42 ± 0.02 | 0.257 |
| 3. | CBEx | 5.70 ± 0.02 | 1.048 |
| 4. | DBEx | 6.03 ± 0.03 | 0.18 |
| 5. | NBEx | 6.47 ± 0.02 | 1.342 |
| 6. | NELEx | 5.71 ± 0.02 | 1.633 |
| 7. | SPEX | 5.65 ± 0.03 | 6.235 |
| 8. | YEx | 6.23 ± 0.03 | ND* |

Key: GPEX: Generalized plant extract; BVEx: Bougainvillea branch extract; CBEx: Coconut bark extract; DBEx: Date bark extract; NBEx: Neem bark extract; NELEx: Neem leaf extract; SPEX: Sugarcane peel extract; YEx: Yeast extract; *ND- Not detected.

Effect of physical factors on the mycelial extension:

The growth and sporulation of most of the fungi is favoured at pH range from 5.5 - 6.5. *Aspergillus niger* can grow at pH as low as 4.0. The temperature range for most of the fungi is 24 – 28 ° C while the optimum temperature for the growth of *Aspergillus niger* is 35 ° C (Madan and Thind, 1998).

The extract media (either generalized or specified, with or without combinations) have acidic pH. Hence the pH promoted the mycelial extension and sporulation of *Aspergillus niger*.

The incubation temperature (16- 45° C) reflects the capability of *Aspergillus niger* to grow in a wide range of temperature. The incubation period was prolonged during winter since the ambient temperature was fairly low. However the temperature variation had no effect on the mycelial extension or spore production.

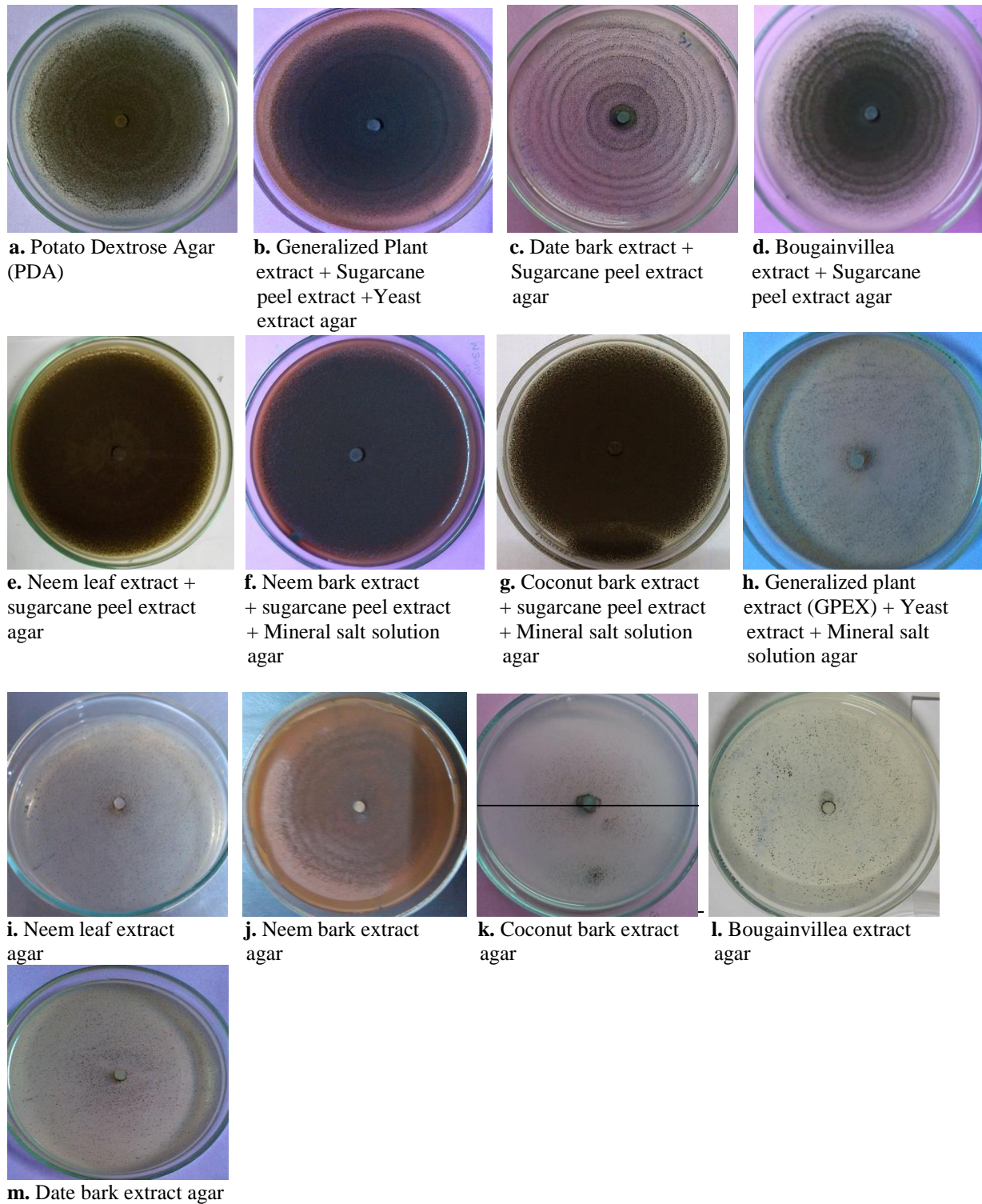


Fig. 13. Radial growth of *Aspergillus niger* on PDA and various extract media combinations.

Conclusion

In the light of the above results, it may be concluded that the plant extract based culture media used in this study are not only comparable with PDA, in terms of radial growth measurement and spore production, but in some cases,

specified media (DBEx in combination with SPEx, YEx and MSS, BVEx in combination with SPEx and MSS) have proved to be even better as compared to conventional medium (PDA).

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