



REPORT OF MYCOTOXINS PRODUCING ENDOPHYTES IN *PHYLLANTHUS EMBLICA* L. FRUITS

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Abstract

Phyllanthus emblica fruit is an important herb with the richest natural source of Vitamin C and antioxidant properties. This is a rasayan used as therapeutic agents for prevention of diseases and ailments as mentioned in Charak samhita. A variety of relationship exists between the endophytic fungi and the host plants, ranging from mutuality or symbiotic to antagonistic or slightly pathogenic because of what appears to be their contribution to the host. *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus nomius* were confirmed the production of alphatoxin B₁ whereas *Fusarium verticilloides* confirmed the production of Fumonisin B₂ according to results obtained from HPTLC method in all 19 different fungi of two divisions Ascomycota and Zygomycota followed by four classes' viz. Dothideomycetes, Eurotiomycetes, Sordariomycetes, Mucoromycetes and six families i.e. Pleosporaceae, Davidiellaceae, Trichocomaceae, Hypocreaceae, Nectriaceae, Mucoraceae identified in amla fruits sampled from Bitthal sabji market, Govindpura sabji market, Vindhya Harbal Garden, Mangalwara Market Mandideep, Piplani Hatt, Sunday Market TT Nagar, Bangrasia Sunday Market and Sehore Bajar during fruit harvesting season.

Keywords: - *Phyllanthus emblica*, endophytes, alphatoxin, Fumonisin, ailments, immunity.

Emblia officinalis (*Phyllanthus emblica* L.) is also known as Amla/aola/ Indian gooseberry is an important herb with richest natural source of high content of vitamin C, constitute of phyllembin, gallic acid, ascorbic acid, tannins etc (Ghoshal *et al.*, 1996). A variety of relationship exists between the endophytic fungi and the host plants, ranging from mutuality or symbiotic to antagonistic or slightly pathogenic because of what appears to be their contribution

to the host (Arnold, 2007). Medicinal plants are reported to harbour of endophytes (Strobel, 2002). Endophytic fungi produce a number of substances such as antioxidants, novel antibiotics, antimycotics, immunosuppressant and anticancer compounds, and thus rich source of biologically active metabolites that find wide-ranging exploitation in medicine, agriculture, and industry (Strobel *et al.*, 2003; Bhagobaty *et al.*, 2011). Mycotoxin is secondary metabolites occurs naturally in food and produced from filamentous fungi. They represent a very large group of different substances produced by different mycotoxigenic species that are capable of causing disease and death in both humans and animals (Fernández-Cruz *et al.*, 2010). Aflatoxins, ochratoxin A, patulin and *Alternaria* toxins are the most commonly found mycotoxins in fruits and their processed products (Drusch and Ragab, 2003). Aflatoxins belong to family of polyketide mycotoxins produced in agricultural products mainly by fungus *A. flavus* and *A. parasiticus*. Even though many different kinds of aflatoxins have been isolated from nature, the major aflatoxins found in agricultural commodities include aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Morton *et al.*, 1979). *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium sacchari*, *Fusarium subglutinans*, *Fusarium fujikuroi* and some other species produces Fumonisin which are derived from polyketides (Proctor *et al.*, 2003; Proctor *et al.*, 2006; Proctor *et al.*, 2008; Stepie *et al.*, 2011). Taking into consideration the economic and medicinal value of Amla, the economic loss due to fungal and mycotoxin contamination is difficult to estimate but it can only say that that loss must be large to farmers, food and tannin industries. With due thought of economic and medicinal value of amla, isolation and identification of fungi of amla as well as characterization of mycotoxin produces by isolated fungi is needed. Therefore this study had been set forth.

Materials and Methods:

Dull greenish yellow Amla / Aola (*Phyllanthus emblica* L.) fruits were collected from Bitthal sabji market, Badkheda sabji market and Piplani sabji market Bhopal (India) during their fruit harvesting season. Collected fruits were washed with tap water and leave for air dried in laboratory at room temperature. Dried samples were stored in a clean zipper poly bag for further studies. Collected fruits samples were randomly pick and surface sterilized with 2% aqueous solution of sodium hypochlorite for two minutes followed by rinsing with sterile distilled water thrice. Samples were ready to use for further studies after air dry. Dried fruits were sliced with the help of a sterile scalpel and plated on PDA (Potato Dextrose Agar) media with an antibacterial agent Chloramphenicol (50ppm). Fruits slices were placed by their inner surfaces turned up and turned down randomly. Inoculated plates were incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 7 days

and produces colonies were noted. Noted colonies were enumerated and subcultured. Repeated subcultures were performed for purification and further identification (Akhund *et al.*, 2010). Isolated fungi were identified in order to morphological characteristics viz. colony growth, aerial mycelium, colony colour, presence of wrinkles and furrows, pigment production etc. followed by staining with Lactophenol cotton blue and the reference methods of Gilman (1957), Domsch *et al.*, (1980), Barnett, (1992). Molecular characterization was performed followed by Sequencing of ITS gene, Sequence confirmation and Sequence submission.

Extraction of mycotoxin from isolated fungi:

Potato dextrose broth without Tween 80 was used for production of mycotoxin in isolated fungi. 25 ml of culture broth was dispensed into a sterile conical flask and inoculated separately with 5 ml of each isolated fungi. Inoculated flasks were incubated on a shaker at a room temperature for 8 days. 10ml of each culture was transferred in a vial. 3.5ml methanol was poured in such vial and incubated for 1hr at room temperature. After incubation mixture was centrifuged thrice at 4000rpm for 5minutes. Final supernatant was filtered with 0.22µm filter membrane and stored for further use (Bragulat *et al.*, 2002).

Extraction of mycotoxin from dried fruits:

50grams of dried aola fruit was taken in a mortar and pestle grounded till fine form powder. Chloroform extraction was performed and filtered through 0.22 filter membrane, air dried, mixed 10ml of methanol and stored for further use.

Standard was prepared as per method of Houssou *et al.*, 2009.

Detection and identification of mycotoxins:

Already developed method for detection of mycotoxins in stored food products of Sudharsan *et al.*, 2017 was followed with some modifications.

Results and Discussion:

Total 19 different fungi i.e. *Alternaria alternata*, *Cladosporium oxysporum*, *Cladosporium cladosporioides*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigates*, *Aspergillus parasiticus*, *Aspergillus Versicolor*, *Aspergillus nomius*, *Penicillium rubrum*, *Penicillium citrinum*, *Penicillium chrysogenum*, *Penicillium funiculosum*, *Acremonium implicatum*, *Aspergillus terreus*, *Fusarium solani*, *Fusarium verticilloides*, *Mucor microspores*, *Rhizopus*

stolonifer were identified from sampled amla fruits. All those were reported in two divisions Ascomycota and Zygomycota followed by four classes' viz. Dothideomycetes, Eurotiomycetes, Sordariomycetes, Mucoromycetes and six families i.e. Pleosporaceae, Davidiellaceae, Trichocomaceae, Hypocreaceae, Nectriaceae, Mucoraceae. Out of 19 identified fungal species only 4 species i.e. *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, *Fusarium verticilloides* reported as mycotoxin producers. All fungal species identified in this study belongs to two different divisions i.e. Ascomycota and Zygomycota but the species reported for mycotoxins production belongs to Ascomycota division only whereas those are distributed in four different class i.e Dothideomycetes, Eurotiomycetes, Sordariomycetes, Mucoromycetes but mycotoxin producing species limit to two classes i.e. Eurotiomycetes, Sordariomycetes. Extensively reported fungal species distributed in six different families i.e. Pleosporaceae, Davidiellaceae, Trichocomaceae, Hypocreaceae, Nectriaceae, Mucoraceae; and mycotoxin producing fungi limit to two families i.e. Trichocomaceae, Nectriaceae. Maximum numbers of contaminated samples and as well mycotoxins were found species of *Aspergillus* in Emblica powder samples (Gautam and Bhaduria, 2009). This study was also supported earlier reports of Hitokoto *et al.*, 1978, Aziz *et al.*, 1998, Bugno *et al.*, 2006. A study of Sharma and Sharma (2018) reported the presence of 25 fungal species representing 12 different genera with the 09 total number of samples colonised, 16 grand total colonies recovered, 45.00 colony forming unit (CF) %, 2.86 abundance and 0.03 A/F ratio in *Fusarium verticilloides*; 03, 06, 04 total number of samples colonised, 05, 11, 13 grand total colonies recovered, 15.00, 30.00, 20.00 colony forming unit (CF) %, 1.67, 1.86, 3.25 abundance and 0.11, 0.06, 0.16 A/F ratio in *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* respectively. Results reported in this study revealed the above study of Sharma and Sharma (2018). 692 total number of samples colonised, 2310 grand total colonies recovered, 75.88 colony forming unit (CF) %, 2.86 abundance and 0.04 A/F ratio in total isolated fungi whereas 149 total number of samples colonised, 574 grand total colonies recovered, 77.60 colony forming unit (CF) %, 3.24 abundance and 0.04 A/F ratio in all identified from different sampling stations. Division wise study reported in all species for division. *Aspergillus flavus* *Aspergillus parasiticus* *Aspergillus nomius* were confirmed the production of alphatoxin B₁ whereas *Fusarium verticilloides* confirmed the production of Fumonisin B₂ according to results obtained from HPTLC method. Alphatoxin B₁ loaded with 10µl standard giving start R_f 0.10, max R_f 0.16, max height 155.2, End R_f 0.20 and area 3197.4. VAM1, VAM2 and VAM3 sample code shows similar peak values which confirms the presence of Alphatoxin B₁ and shown start R_f 0.1, 0.1 and 0.11; max R_f 0.16, 0.16 and 0.17; max height 186.2, 211.5 and 239.5; end R_f 0.21, 0.22 and 0.21; area 3952.5, 4417.0 and 5231.1 respectively; whereas Fumonisin B₂ loaded with 20µl standard giving start R_f 0.19, max R_f

0.24, max height 156.1, End Rf 0.27 and area 4045.5. VAM14 shows nearly similar peak values with start Rf 0.2, max Rf 0.24, max height 135.9, end Rf 0.26 and area 3046.3. Studies of Sharma and his colleague (2016) support this study followed by presence of alphatoxins in dried fruits of amla. Rajeshwari and Raveesha (2015) also found alfatoxins in their study on alfatoxin contamination in raw herbal materials. Sharma and Sharma (2018) earlier reported the presence of Fumonisin B₂ producer *Fusarium verticilloides* in aola fruits as this study also reported same species. Camardo *et al.*, 2019 reported presence of mycotoxins Alphatoxin B₁ and Fumonisin B₂ in *Aspergillus flavus* and *Fusarium verticillioides* respectively.

Fig. No. 01: Image visualisation of HPTLC plate for Alphatoxin B₁

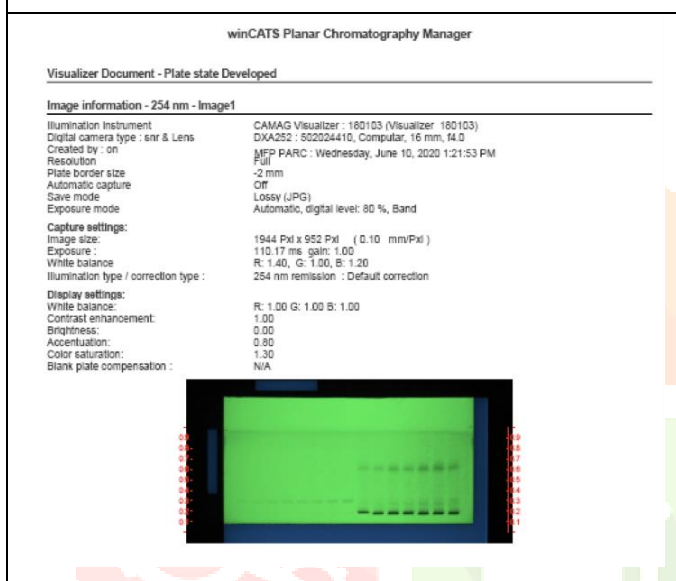


Fig. No. 03: Image visualisation of HPTLC plate for Fumonisin B₂

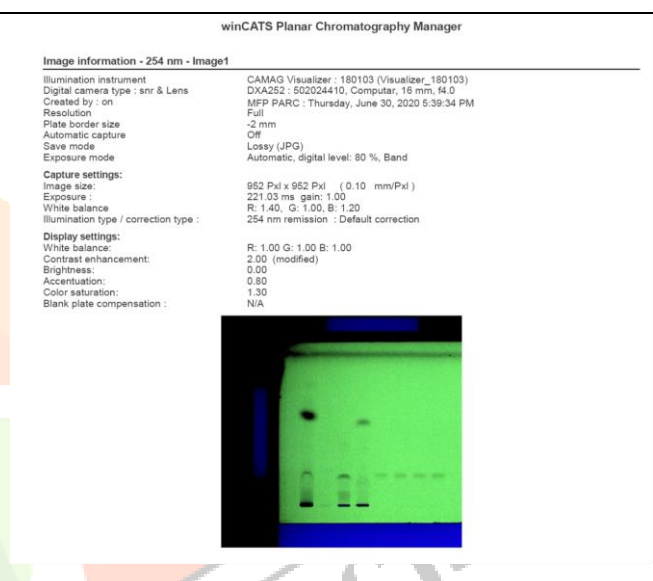


Fig. No. 02: Graph plot of detection for Alphatoxin B₁

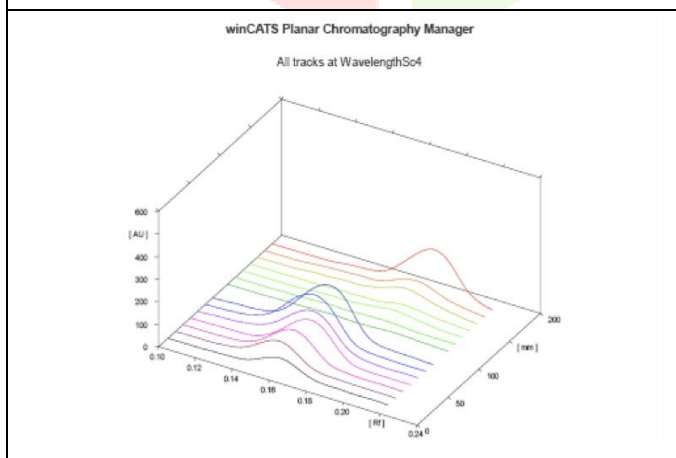
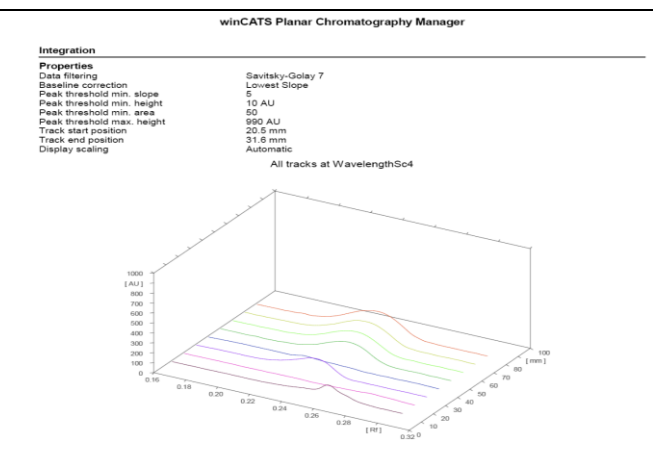


Fig. No 04 Graph plot of detection for Fumonisin B₂



Conclusions:

This study stated that fruit surface is most valuable habitat that influences the microorganism to grow and evolve. Some species are grown well whereas some are killed due to antagonistic

properties of fruits. 19 different species of fungus are detected from samples of amla (*Phyllanthus emblica*) fruits collected from different location of Bhopal and adjoin areas. There was three *Aspergillus* and one *Fussarium* species are identified as micotoxins alfatoxins B₁ and Fumosinin B₂ respectively. This study suggests that there are urgent need to make awareness for proper collection and storage of amla fruit. Due to lack of proper storage and transportation, mycotoxin contamination occur in fruits leads diseases to consumer and also loss the economy followed by rot.

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