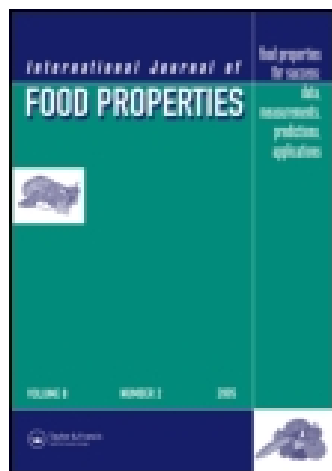


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### Evaluation of *Phyllanthus polyphyllus* L. Extract and its Active Constituent as a Source of Antifungal, Anti-Aflatoxigenic, and Antioxidant Activities

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## Evaluation of *Phyllanthus polyphyllus* L. Extract and its Active Constituent as a Source of Antifungal, Anti-Aflatoxigenic, and Antioxidant Activities

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The present investigation was carried out to evaluate the antifungal activities of *Phyllanthus polyphyllus* L. leaf extract, to isolate its active constituent 4-*o*-methyl gallic acid (4-*o*-MGA), and to determine the antioxidant and antiaflatoxigenic properties. The bioassay-guided fractionation of methanol extract led to the isolation of active compound 4-*o*-methylgallic acid. The methanol extract showed the highest amount of phenolic content (290 mg GAE/g dry extract), which indicated the involvement of phenolic compounds in the radical scavenging activity observed by the methanol extract. The antioxidant capacity of 4-*o*-MGA was greater than the standard butylated hydroxytoluene, followed by methanol extract. The concentration-dependent growth inhibitory activity was observed against the tested fungi, in which the field fungi were susceptible, while the storage fungi were found to be more resistant including aflatoxigenic *A. flavus*. A correlation was observed between fungal biomass and aflatoxin production in control and treatment, there was a decrease in biomass of *A. flavus* and aflatoxin production with increasing concentration. The aflatoxin production was completely inhibited *in vitro* by methanol extract at 1 mg/mL and 4-*o*-MGA at 2 mg/mL, but the mycelial growth was not inhibited completely. The inhibition of aflatoxin production was relatively higher than the mycelial growth inhibition of *A. flavus*, such behaviors might have been determined by the presence of hydrolysable tannin 4-*o*-MGA in the extract, which is known to inhibit the aflatoxin biosynthesis. The significant antioxidant and aflatoxin inhibitory activities of *P. polyphyllus* could be exploited for its application in preventing oxidative deterioration and fungal spoilage of food products.

*Keywords:* *Phyllanthus polyphyllus*, Antifungal, Antiaflatoxigenic, Antioxidant, 4-*o*-Methylgallic acid.

### INTRODUCTION

Moulds are responsible for off-flavor formation, production of allergenic compounds, and mycotoxin contamination, which are a risk for human and animal health. Mycotoxins are produced in grains in the field, during transport, and storage where conditions are suitable for their

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production.<sup>[11]</sup> Many fungi produce mycotoxins such as aflatoxins, ochratoxins, and fumonisins, which are mutagenic, teratogenic, and hepatotoxic secondary metabolites.<sup>[21]</sup> The fungi cause food spoilage, which causes reduction in quality and quantity. Food quality has been guaranteed by controlling fungi that produce mycotoxins.<sup>[3]</sup> Fungi have also unfavorable effects on quality, safety, and preservation of food. Synthetic chemicals are widely used for control, however, these chemicals may cause toxic residues in treated products.<sup>[4]</sup> Several strategies are used at controlling fungal growth and the mycotoxin synthesis in stored grains by chemical treatments. According to Wu and Khlanguwiset,<sup>[5]</sup> interventions to reduce mycotoxin-induced illness can be grouped into three categories; agricultural, dietary, and clinical. Agricultural interventions are methods that can be applied either in the field (pre-harvest) or in drying, storage, and transportation (post-harvest) to reduce mycotoxin levels in food. The dietary and clinical interventions are considered as secondary interventions by which the aflatoxin related illness can be reduced.

*Aspergillus flavus* is an important mycotoxigenic mould that colonizes food grains and their by-products, it is an important aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) producer and can infect food grains during pre- and post-harvest; aflatoxin contaminations can occur mainly due to the poor management of drying and storage phases. It is estimated that around a 25% of world's crops are annually affected by mycotoxins, mainly those produced by the genera *Aspergillus*, *Fusarium*, and *Penicillium*.<sup>[1,6]</sup> Furthermore, the reactive oxygen species (ROS) molecules cause oxidative stress by damaging the proteins, lipids, nucleic acids and also stimulate aflatoxin biosynthesis. Lipid peroxidation is one of the major causes of deterioration of food products during processing and storage, which alter taste and aroma, and cause undesirable effects on the human health.<sup>[7]</sup> Use of synthetic chemicals to control biodeterioration is concerned to the risk of toxicity and negative impact on human health. Furthermore, synthetic preservatives can induce generation of ROS, leading to oxidative damage of metabolites and enhancing aflatoxin biosynthesis.<sup>[8]</sup> Synthetic antioxidants are mainly used to prolong the storage stability of foods, however, some synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate have been revealed to be toxic, carcinogenic, and other side effects.<sup>[7,9–11]</sup> Hence, interest has been increased for finding antioxidants and antifungal compounds of natural source, which are suitable for use in food and/or medicine.

A large number of synthetic chemicals are used to control of fungal contamination, however, these synthetic chemicals are restricted, due to their possible carcinogenicity, teratogenicity, high and acute toxicity, long degradation periods, environmental pollution, and their effects on human beings.<sup>[4,7,12]</sup> Therefore, there is an increased interest in finding natural antifungals of plant origin alternative to synthetic chemicals. Phytocompounds are expected to be safer than synthetic pesticides, as they are easily decomposable, not environmental pollutants, and possess no residual or phytotoxic properties.<sup>[13]</sup>

Plant extracts are potentially useful additives for food preservation as they are likely to prolong shelf life and improve the quality of stored food products.<sup>[14]</sup> Plant extracts contain various antioxidant compounds such as polyphenols, phenols, flavonoids, etc. which could be the bioactive basis of their antimicrobial properties. Phenolic compounds have been shown to have an inhibitory effect on growth and toxin production in *Aspergillus* spp. by their antioxidant potencies.<sup>[15–17]</sup> Plant extracts with antifungal properties could potentially be used to control mycotoxigenic fungi in foods and feeds. Due to the environmental effects of synthetic pesticides and their side-effects associated with health risks, natural pesticides of plant origin are being evaluated in order to control fungal spoilage and mycotoxins production in different storage grains.<sup>[6]</sup> In this way, researchers have focused on the potentiality of plants and their metabolites to inhibit toxigenic fungus growth and/or toxin production as a useful tool for controlling mycotoxin contamination of crops and agricultural commodities.

*Phyllanthus polyphyllus* L. (Euphorbiaceae) is a deciduous shrub widely distributed in tropical and subtropical regions in India and Srilanka.<sup>[18]</sup> In India, the raw drugs of *Phyllanthus* species

have been extensively used for treating liver disorders, intestinal infections, jaundice, and diabetes in traditional medicine.<sup>[19]</sup> The genus *Phyllanthus* contains highly valued medicinal plants which produce a variety of secondary metabolites including flavonoids, phenolic acids, alkaloids, lignans, and tannins, have long been extensively used in folk medicine all over the world.<sup>[20]</sup> *P. polyphyllus* has been investigated for their various bioactive properties such as anti-inflammatory,<sup>[18]</sup> anti-tumour,<sup>[21]</sup> cytotoxic,<sup>[21,22]</sup> and hepatoprotective activities.<sup>[23,24]</sup> Species of these plant genera are used in folk medicine and consumed as healthy ingredients, they could also be useful in preventing mould growth and mycotoxin contamination due to their antifungal properties. To the best of the authors knowledge, the antifungal and antiaflatoxic activities of *P. polyphyllus* have not been evaluated so far. Therefore, the present study was aimed at the investigation of antifungal and antiaflatoxic, antioxidant activities of *P. polyphyllus*, and isolation of the active constituents responsible for such activities.

## MATERIALS AND METHODS

### Chemicals and Reagents

All the culture media were purchased from Hi-Media, Mumbai (India). All solvents, reagents, BHT and iodo-nitro-tetrazolium (INT) were procured from SRL (Mumbai, India). All chemicals and solvents used were of analytical grade. Microtiter-plates (96-well) were purchased from Axiva, New Delhi (India). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (Steinheim, Germany) and thin layer chromatography (TLC, Silica gel 60) plates from Merck (Darmstadt, Germany).

### Collection of Plant Materials

Fresh disease free leaves of *P. polyphyllus* were collected from the Jnanabharathi Campus, Bangalore University, Bangalore (India) in the month of July, 2011. The collected plant material was washed with distilled water and shade-dried at room temperature. The dried leaves were ground well and stored in air-tight containers. An authenticated voucher specimen (Voch.No.MB&BT.DCM.59) of the plant was deposited in the Herbarium of Department of Microbiology and Biotechnology, Bangalore University, Bangalore, India.

### Preparation of Plant Extracts

Fifty grams of the powdered plant sample was successively extracted with 200 mL of petroleum ether (60°C), toluene (95°C), chloroform (60°C), methanol (70°C), and ethanol (70°C) using a Soxhlet apparatus. The solvents were used sequentially with their increasing polarity. All the solvent extracts were concentrated separately under reduced pressure using a rotary flash evaporator and stored in air tight glass tubes.<sup>[25]</sup>

### Estimation of Total Phenolic Contents

The amount of phenolic content present in the solvent extracts was estimated by following Folin-Ciocalteu method with minor modifications.<sup>[26]</sup> Briefly, 1 mL of each extract (1 mg/mL) was added to 0.1 mL of Folin-Ciocalteu reagent and kept for 5 min at room temperature in dark conditions. After incubation, 2 mL of 15% sodium carbonate was added and diluted to 10 mL by adding distilled water, then incubated at room temperature for 90 min in dark conditions. Absorbance was measured at 720 nm using double beam UV-VIS spectrophotometer (Elico, India). Total phenolic content was expressed as gallic acid equivalent (mg GAE/g dry extract) based on the calibration curve.

## Isolation and Identification of the Bioactive Compound

The methanol extract (ME) which showed highest antimicrobial activity was fractionated into four different fractions viz., acidic, phenolic, basic, and neutral.<sup>[27]</sup> The acidic fraction which showed highest antifungal activity was subjected to further purification by column chromatography (CC). The column of silica mesh 60-120 (SRL, Mumbai, India) was eluted with solvents of increasing polarity using a gradient of  $\text{CHCl}_3$  and MeOH (10:0, 9:1  $\rightarrow$  0:10, v/v). The eluates were collected in different fractions and concentrated. Based on antifungal activity and TLC analysis, the CC fractions of similar profile were pooled together. The active compound was obtained as crystals, then analyzed for purity of the active compound following TLC analysis and tested with ferric chloride ( $\text{FeCl}_3$ ). The compound was identified based on the comparison of infrared (IR), electrospray ionization-mass spectrometry (ESI-MS),  $^1\text{H}$  &  $^{13}\text{C}$  NMR data with the published values.<sup>[18,28]</sup>

## Antifungal Activity

### *Test microorganisms*

A total of 15 seed-borne pathogenic fungi viz., *Alternaria brassicola*, *A. geophila*, *Aspergillus flavus*, *A. tamari*, *A. terreus*, *A. fumigatus*, *Curvularia tetramera*, *Fusarium equiseti*, *F. lateratium*, *F. udum*, *Penicillium chrysogenum*, and *P. citrinum*, which were isolated from food grains in previous studies. *Fusarium moniliforme* and *F. oxysporum* were procured from National Collection of Industrial Microorganisms (NCIM, Pune, India). Sabouraud dextrose agar (SDA) was used as the media for fungal cultures.

### *Poisoned food technique*

Antifungal activity of the solvent extracts and 4-*o*-MGA was determined by the poison food technique as described by Mohana et al.<sup>[25]</sup> All the test samples were dissolved in methanol and incorporated into SDA to achieve the media of requisite concentrations. The control media without test sample was added by the methanol, the solvent which was used for dissolving the samples. The prepared media were autoclaved, poured into Petri dishes (20 mL), and allowed to cool. Five millimeter discs of 7-day-old culture of test fungi were placed at the center of the Petri dishes. The inoculated plates were incubated at  $28 \pm 1^\circ\text{C}$  for 7 days. Triplicates were maintained for each concentration and control. The fungitoxicity of test samples in terms of percentage of mycelia inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{dC - dT}{dC} \times 100$$

where, dC = average increase in mycelia growth in control and dT = average increase in mycelial growth in treated.

### *Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC)*

The microbroth dilution method was employed to determine the MIC and MFC of ME and 4-*o*-MGA following standard procedures.<sup>[9,29]</sup> Two-fold serial dilutions of test samples with different concentrations (0.062 to 8.0 mg/mL) were prepared in 96-well microtitre plate with sauboraud dextrose broth (SDB). The overnight incubated test fungi in broth culture were adjusted to concentrations of  $10^4$  cfu  $\text{mL}^{-1}$ . Each well of 96-well microtitre plate was containing 200  $\mu\text{L}$  of two-fold diluted broth of different concentrations and inoculated with 15  $\mu\text{L}$  of test organisms. The inoculated microtitre plate was sealed with parafilm, then agitated with a microtitre plate shaker and incubated at  $30^\circ\text{C}$  for 72 h. The inoculated plates were observed for the presence or absence of

fungal growth. After macroscopic observation, a 10  $\mu\text{L}$  of treated broths were streaked radially onto the SDS plates and incubated at 30°C for 72 h. After the incubation period, the lowest concentration at which the growth was inhibited that value was recorded as MIC. The complete absence of growth on the agar surface in the lowest concentration of the sample was defined as MFC.<sup>[9]</sup> After streaking on to agar plates, 50  $\mu\text{L}$  of INT (0.2 mg/mL) was added to each well and incubated at 37°C for 30 min. The pale yellow-colored INT was reduced to a pink color which indicated the presence of viable microbial cells, while the yellow color remained the same where the fungal growth was inhibited.<sup>[30]</sup>

### *Comparison with some synthetic fungicides*

The fungitoxic activities of ME and 4-*o*-MGA were compared with of some synthetic fungicides viz., copper oxychloride 50% WP (Fungicop-50, Karnatak Agrochemicals Pvt. Ltd., Bangalore, India) and zinc ethylene bithiocarbamate 75% WP (Indifil Z-75, Indofil Chemicals Company, Mumbai, India) by recording the MIC and MFC following the micro-broth dilution method as described above.

### Anti-Aflatoxigenic Activity

#### *In vitro assay*

Sucrose-magnesium sulphate-potassium sulphate-yeast extract (SMKY) liquid medium was used to determine the efficacy of ME and 4-*o*-MGA against AFB<sub>1</sub> production.<sup>[14]</sup> SMKY medium (25 mL) was taken in 100 mL flasks, to which requisite amount of test samples were added to get 0.125, 0.25, 0.50, 1.0, and 2.0 mg/mL concentrations. The flasks were aseptically inoculated with suspension of toxigenic isolates of *A. flavus* (10<sup>6</sup> spores/mL, 100  $\mu\text{L}$ /flask) and incubated at 28°C for 10 days. The flask containing 0.5 mL of dimethyl sulphoxide (DMSO) was maintained as control. The broth cultures were filtered through Whatman No. 1 filter paper, mycelia dried at 100°C for 12 h and mycelial dry weight (MDW) was recorded. The MDW of ME and 4-*o*-MGA treated samples and control were compared for their MDW losses. The filtrate was used for the isolation of AFB<sub>1</sub> by adding equal volume of CHCl<sub>3</sub> (20 mL) and shaken well in a separating funnel. The CHCl<sub>3</sub> layer was passed through anhydrous sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) and evaporated in dark conditions at room temperature. The residue was re-dissolved 1 mL of CHCl<sub>3</sub> and 10  $\mu\text{L}$  of sample was spotted onto the TLC plate adjacent to AFB<sub>1</sub> standard. The plate was developed in CHCl<sub>3</sub>-acetone (96:4, v/v) solvent system, air-dried, and visualized under ultra-violet (365 nm) light (UV-cabinet, Labline, India). Qualitative identification of AFB<sub>1</sub> content was done by visual comparison of intensity of fluorescence of the samples with standard spots. For quantitative estimation, the fluorescent spots were scraped off the plates, dissolved in 5 mL cold CH<sub>3</sub>OH, and centrifuged at 3000 rpm for 5 min. The absorbance of supernatant was measured at 265 nm using a spectrophotometer (Systronics, India) and the amount of AFB<sub>1</sub> content was calculated following formula:

$$\text{Aflatoxin content } (\mu\text{g/kg}) = [D \times M/E \times L] 1000$$

where  $D$  is absorbance;  $M$  is molecular weight of AFB<sub>1</sub> (312);  $E$  is molar extinction coefficient of AFB<sub>1</sub> (21,800); and  $L$  is path length (1 cm).

#### *In vivo assay*

The efficacy of ME and 4-*o*-MGA on aflatoxin production in viable (*in vivo*) maize was determined by following the procedure of Garcia et al.<sup>[1,31]</sup> and Probst and Cotty<sup>[32]</sup> with slight modifications. The maize seeds were treated with different concentrations of ME and 4-*o*-MGA



ranging from 0.125 to 4.0 mg per gram of maize seeds. The maize seeds without sample treatment were maintained as control. The sample-treated and untreated maizes were inoculated with 100  $\mu$ L spore suspension of *A. flavus* toxigenic strain containing  $10^4$  spores/mL. The water activity ( $a_w$  0.95) of maize was adjusted by aseptically adding sterile distilled water to maize kernels in sterile container as described by Garcia et al.<sup>[11]</sup> The inoculated maizes were kept for incubation at room temperature for 15 days. After incubation, the maize seeds (5 g) were milled and extracted with 15 mL of acetonitrile + water (60 + 40, v/v) and shaken for 10 min. The extract was filtered through Whatman No. 1 filter paper and the filtrate was extracted with equal volume of  $\text{CHCl}_3$ . Further, the extracted AFB<sub>1</sub> was estimated qualitatively and quantitatively as described above.

### DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of ME and 4-*o*-MGA was determined by using the method described by Ebrahimabadi.<sup>[26]</sup> Briefly, plant extract of different concentrations ranging from 156 to 2000  $\mu$ g/mL were prepared by using two-fold dilution method. A solution of 0.1 mM 2,2-DPPH was prepared in methanol. The reaction was containing 1 mL of sample and 3 mL of 0.1 mM 2,2-DPPH, allowed for incubation in dark conditions for 30 min (28°C). Methanol was used as negative control and BHT as standard. The absorbance of the reaction mixture was measured at 517 nm by using double beam UV-VIS spectrophotometer (Elico, India). The ability of the plant sample to scavenge 2,2-DPPH was calculated as:

$$\text{Radical scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where,  $A_{\text{control}}$  is the absorbance of the control (DPPH+methanol) and  $A_{\text{sample}}$  is the absorbance of the sample (DPPH + methanol + sample). The sample concentrations providing 50% inhibition ( $\text{IC}_{50}$ ) were calculated by plotting the inhibition percentages against concentrations of the samples.

### Statistical Analysis

Experiments were performed in triplicate and data given were analysed by SPSS 20 (SPSS Inc., IBM, USA). Differences between means were determined using Tukey's univariate comparisons ( $p \leq 0.05$ ).

## RESULTS

The plant materials were sequentially extracted using a Soxhlet apparatus, all the solvent extracts were concentrated *in vacuo* and tested for their antifungal activities. The ME was obtained as brown-gummy residue (yield: 0.48%/100 g of dry plant material, w/w), which showed the highest antifungal activity. The phenolic compounds were found to be present in the ME and the total phenolic content was estimated as 290 mg GAE/g. The ME of *P. polyphyllus* was subjected to fractionation to give four different fractions viz., acidic, phenolic, basic, and neutral. The active acidic fraction showed antifungal activity, which was further purified by CC with a gradient elution of  $\text{CHCl}_3$  and MeOH (10:0, 9:1  $\rightarrow$  0:10, v/v) to afford 8 fractions. The third and fourth fractions of CC showed similar chromatographic profile, which were pooled together, and the  $\text{FeCl}_3$  test confirmed that the compound belongs to phenolic group. In the negative ion mode  $[\text{M} + \text{H}]^-$  of ESI – MS, the isolated active compound showed a molecular ion (m/z) peak at 184.3 corresponding to the molecular formula  $\text{C}_8\text{H}_8\text{O}_5$ . The <sup>1</sup>H-NMR [ $\delta$  7.03 (2H, s, H-2, H-6), 4.86 (br s, OH), 3.80 (3H, s, OCH<sub>3</sub>)], <sup>13</sup>C-NMR [ $\delta$  121.47 (C-1), 110.05 (C-2), 146.49 (C-3), 139.74 (C-4), 146.49 (C-5), 110.05



(C-6), 52.24 (OCH<sub>3</sub>), 169.02 (C = O)], and IR spectral analysis revealed that the compound is 4-*o*-methylgallic acid (IUPAC name: 4-methoxy-3, 5-dihydroxybenzoic acid) based on the comparison of its spectral data with the reported values in literature.

The growth inhibitory effects of ME and 4-*o*-MGA were screened against a panel of 15 fungal pathogens containing storage and field fungi are given in Table 1. The percentage of growth inhibition by the ME and 4-*o*-MGA was estimated by measuring the growth diameter of the colony grown in the medium with the treatment and control. Most of the treated field fungi were susceptible to 2 mg/mL concentration of ME and 4-*o*-MGA with inhibition of spore germination. Among the fungi tested, *A. tamari*, *A. flavus*, and *P. citrinum* were found to be most resistant, with the mycelial growth inhibition 18, 12, and 0%, respectively. Moderate inhibitory activity was observed against the field fungi such as *F. moniliforme* (51.72%), *C. tetramera* (39.32%), and *F. equiseti* (36.68%), with inhibition of spore germination at a concentration (2 mg/mL). In micro-broth dilution assay, the ME showed significant inhibitory activity against most of the pathogenic fungi tested with lower MIC and MFC than that of 4-*o*-MGA and synthetic copper oxychloride. Zinc ethylene bithiocarbamate showed highest inhibitory activity against wide range of fungi. On comparative evaluation with synthetic fungicides, ME and 4-*o*-MGA showed varying degrees of MIC and MFC values against different fungi tested. The results demonstrated that the ME has remarkable antifungal activities than its active constituent 4-*o*-MGA.

Antiaflatoxic activities of ME and 4-*o*-MGA were tested *in vitro* and *in vivo* against an aflatoxic strain of *A. flavus*. The concentration-dependent antiaflatoxic activities of ME and MGA are presented in Table 2. The ME completely inhibited the AFB<sub>1</sub> production at 1 mg/mL *in vitro*, while the 4-*o*-MGA showed moderate inhibition at 2 mg/mL *in vitro*, but failed to inhibit completely. Fungal biomass and AFB<sub>1</sub> production in treatment sets were found to be decreased with increasing concentrations of ME and 4-*o*-MGA, whereas, the control set showed the highest AFB<sub>1</sub> (1500 µg/L) content with highest biomass (MDW). The ME treated set showed moderate inhibition at 4 mg/mL with 1000 µg/Kg of AFB<sub>1</sub> content *in vivo*, while the 4-*o*-MGA treated set showed 1500 µg/Kg of AFB<sub>1</sub> content at 4 mg/mL. *In vivo* assay demonstrated that the ME is having moderate AFB<sub>1</sub> inhibitory activity than that of 4-*o*-MGA, and highest AFB<sub>1</sub> (2500 µg/Kg) content was detected in control set.

In the DPPH radical scavenging assay, the IC<sub>50</sub> values of ME and 4-*o*-MGA were found to be 15.6 and 7.8 µg/mL, respectively (Fig. 1). The antioxidant activity of the synthetic antioxidant BHT (10.0 µg/mL) was also determined and compared with the IC<sub>50</sub> values of ME and 4-*o*-MGA. A lower IC<sub>50</sub> value indicates the greater antioxidant activity. The 4-*o*-MGA exhibited higher antioxidant activity with lower IC<sub>50</sub> value than the BHT, and ME showed moderate activity with higher IC<sub>50</sub> value. The order of antioxidant activity was MGA > BHT > ME.

## DISCUSSION

Recently, several phytochemicals have been reported for their antifungal and aflatoxin inhibitory properties, especially the aflatoxin inhibitory activities are associated with the antioxidant potential of phytochemicals.<sup>[15,17,33]</sup> Hence, the antioxidants have become an essential part of contemporary food preservation technology. In order to evaluate the various bioactive properties of natural products, it is necessary to investigate the major phytoconstituents, which are involved in such activities. In this context, the *P. polyphyllus* extract and its active constituent 4-*o*-MGA were investigated for their antifungal, antiaflatoxic and antioxidant activities.

The interesting biological activities and the no reports on antifungal activities of *P. polyphyllus* encouraged the authors to pursue an investigation to evaluate its antifungal properties, to isolate the bioactive antifungal component(s) and to determine the antiaflatoxic properties. In this

TABLE 1  
Antifungal activities of methanol extract of *P. polyphyllus*, 4-*o*-MGA, and synthetic fungicides against seed-borne pathogenic fungi

Organisms	Methanol extract			4- <i>O</i> -MGA			<i>CO</i> <sup>b</sup>			<i>ZEB</i> <sup>c</sup>		
	% Mycelial inhibition <sup>a</sup>	MIC (mg/mL)	MFC (mg/mL)	% Mycelial inhibition <sup>a</sup>	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)
<i>A. brassicicola</i>	29.4 ± 0.32	0.12	0.25	23.8 ± 0.42	2.0	4.0	0.03	0.50	0.03	0.03	0.06	
<i>A. geophila</i>	26.4 ± 0.26	0.12	0.25	15.4 ± 0.14	2.0	4.0	0.12	2.00	0.03	0.03	0.50	
<i>A. flavus</i>	12.7 ± 0.14	0.25	2.0	1.1 ± 0.33	4.0	>4.0	0.25	2.00	0.12	0.12	1.00	
<i>A. fumigatus</i>	33.2 ± 0.76	0.25	2.0	6.4 ± 0.06	2.0	>4.0	0.25	2.00	0.25	0.25	1.00	
<i>A. ochraceus</i>	29.5 ± 0.08	0.12	1.0	20.6 ± 0.23	2.0	>4.0	0.25	0.50	0.06	0.06	0.50	
<i>A. tamari</i>	18.0 ± 0.12	0.06	0.25	0.0 ± 0.00	2.0	>4.0	0.50	2.00	0.12	0.12	0.25	
<i>A. terreus</i>	22.1 ± 0.45	0.06	0.25	24.0 ± 0.13	2.0	>4.0	0.25	0.50	0.12	0.12	0.25	
<i>C. tetramera</i>	39.3 ± 0.76	0.03	0.12	39.9 ± 0.55	2.0	2.0	0.25	2.00	0.01	0.01	0.50	
<i>F. equiseti</i>	36.6 ± 0.33	0.06	0.12	0.0 ± 0.00	2.0	2.0	0.25	2.00	0.01	0.01	0.12	
<i>F. lateritium</i>	28.5 ± 0.14	0.12	0.25	29.8 ± 0.32	2.0	2.0	0.50	2.00	0.03	0.03	0.12	
<i>F. moniliforme</i>	21.1 ± 0.27	0.12	0.25	0.0 ± 0.00	1.0	2.0	1.00	2.00	0.06	0.06	0.25	
<i>F. oxysporum</i>	28.7 ± 0.43	0.12	0.25	57.2 ± 0.24	2.0	2.0	0.50	2.00	0.03	0.03	0.12	
<i>F. udum</i>	35.0 ± 0.22	0.06	0.125	43.4 ± 0.73	1.0	2.0	0.25	2.00	0.01	0.01	0.12	
<i>P. chrysogenum</i>	29.9 ± 0.11	2.00	>4.0	9.3 ± 0.23	2.0	>8.0	0.50	2.00	0.50	0.50	2.00	
<i>P. citrinum</i>	0.0 ± 0.00	2.00	>4.0	2.5 ± 0.14	4.0	>8.0	1.00	2.00	0.50	0.50	2.00	

<sup>a</sup>Percent (%) mycelial inhibition at 2 mg/mL.

<sup>b</sup>Copper oxychloride 50%.

<sup>c</sup>Zinc ethylene bisthiocarbamate 75%.

Data given are the mean of three replicates ± standard error ( $P \leq 0.05$ ).

TABLE 2  
*In vitro* and *in vivo* aflatoxin B<sub>1</sub> inhibitory activity of methanol extract of *P. polyphyllus* and 4-*o*-MGA

Sample treatments at different concentrations	Methanol extract			4- <i>o</i> -MGA		
	MDW (µg/20 mL)	AFB <sub>1</sub> (µg/L) <i>in vitro</i> *	AFB <sub>1</sub> (µg/Kg) <i>in vivo</i> **	MDW (µg/20 mL)	AFB <sub>1</sub> (µg/L) <i>in vitro</i> *	AFB <sub>1</sub> (µg/Kg) <i>in vivo</i> **
Control	1047 ± 0.32	1500 ± 20	2500 ± 32	1047 ± 0.32	1500 ± 24	2500 ± 36
0.125	1007 ± 0.39	180 ± 12	2000 ± 28	1173 ± 0.41	1000 ± 20	2000 ± 28
0.250	973 ± 0.24	60 ± 8	2000 ± 24	978 ± 0.22	800 ± 14	2000 ± 30
0.500	948 ± 0.29	50 ± 8	1500 ± 18	943 ± 0.32	400 ± 16	2000 ± 28
1.000	963 ± 0.19	—	1500 ± 16	843 ± 0.15	100 ± 12	1500 ± 16
2.000	878 ± 0.14	—	1500 ± 18	818 ± 0.18	—	1500 ± 18
4.000	ND	ND	1000 ± 12	ND	ND	1500 ± 22

\**In vitro* treatment with samples at different concentrations (mg/mL).

\*\**In vivo* treatment with samples at different concentrations (mg/g).

ND: not determined; —: absent.

Data given are the mean of three replicates ± standard error ( $P \leq 0.05$ ).

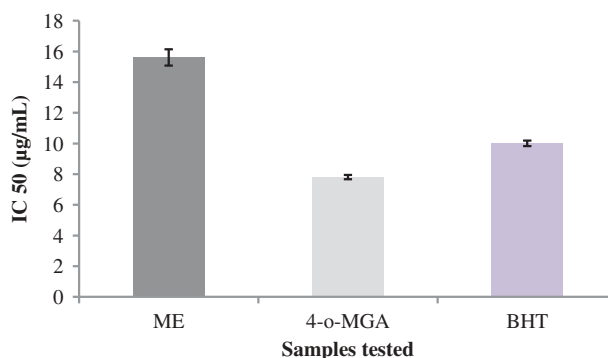


FIGURE 1 DPPH radical scavenging activity of methanol extract of *P. polyphyllus*, 4-*o*-MGA, and BHT; data given are the mean of three replicates ± standard error ( $P \leq 0.05$ ).

article, the potential antifungal activity of *P. polyphyllus* and inhibitory activity towards aflatoxin biosynthesis is discussed, with special reference to its bioactive component. Several prior studies have highlighted the *P. polyphyllus* for its various bioactivities such as anti-inflammatory,<sup>[18]</sup> antitumour,<sup>[21]</sup> cytotoxic,<sup>[21,22]</sup> and hepatoprotective activities.<sup>[23,24]</sup> The 4-*o*-MGA has been well reported as active constituent for such bioactivities. Oliveira et al.<sup>[34]</sup> reported the potential anti-atherogenic effect of standard 4-*o*-MGA on foam cell formation and showed that it was most effective among the standard gallic acid derivatives used. The 4-*o*-MGA has been reported in the plant *Rhus glabra* (Anacardiaceae) as antimicrobial constituent against bacteria by Saxena et al. (1994).<sup>[28]</sup> To the best of the authors knowledge, antifungal and antimycotoxigenic activities of *P. polyphyllus* and its active constituent 4-*o*-MGA against *A. flavus* growth and aflatoxin production are not evaluated so far. In this investigation, the bioassay-guided fractionation of ME ion led to the isolation of active compound from acidic fraction. The active crystalline compound was identified as benzenoid compound 4-*o*-methylgallic acid (IUPAC name: 4-methoxy-3,5-dihydroxybenzoic acid) belongs to the phenolic group.<sup>[18,28]</sup>

The concentration-dependent growth inhibitory activity was observed against the tested fungi, in which the field fungi were susceptible, while the storage fungi were found to be more resistant. The MIC of ME was comparatively lower than that of the synthetic fungicides tested, thereby, its potency as antifungal agent of plant origin would be economical. In AFB<sub>1</sub> inhibitory assay, a correlation was found between fungal biomass and AFB<sub>1</sub> production in control and treatment sets comprising different concentrations of ME and 4-*o*-MGA. At higher concentrations, there was a decrease in biomass of the toxigenic *A. flavus* with decrease in AFB<sub>1</sub> production. However, the AFB<sub>1</sub> production was completely inhibited *in vitro* by ME at 1 mg/mL, while it was moderately inhibited by 4-*o*-MGA, but not completely. Hence, the ME was found to be more efficacious in inhibition of AFB<sub>1</sub> production than its constituent 4-*o*-MGA. Even though, the ME showed moderate inhibitory activity than that of 4-*o*-MGA *in vivo* assay, both ME and 4-*o*-MGA failed to inhibit toxin production completely, this may be due to diffusion of the samples treated. The ME showed remarkable activity in suppression of AFB<sub>1</sub> than the 4-*o*-MGA, it is evident from the results that this may be due to synergetic effect of other phytochemicals present in the extract.

On comparative evaluation, both ME and 4-*o*-MGA inhibited AFB<sub>1</sub> production *in vitro*, but they did not show higher mycelial growth inhibitory activity. The maximum mycelial growth inhibition observed was only 57.24% in poisoned food technique, with moderate inhibition against field fungi and lower activity against storage fungi. The ME and 4-*o*-MGA did not show any significant activity against *A. flavus* aflatoxigenic strain (12%) and the colony diameters did not present a significant difference relative to controls. It is interesting to note that the inhibition of AFB<sub>1</sub> production was relatively higher than the mycelial growth inhibition of *A. flavus*. Such behaviors might have been determined by the presence of hydrolysable tannin 4-*o*-MGA in the extract, which is known to inhibit the aflatoxin biosynthesis in the growth medium.<sup>[35]</sup>

Mahoney et al.<sup>[17]</sup> demonstrated the involvement of oxidative stress in stimulating aflatoxin production by *Aspergillus* sp. and overcome of such stress by natural antioxidants suppresses or eliminates aflatoxin formation. He reported that the effect of tannins in reducing oxidative-induced aflatoxin formation was due to their antioxidant properties, thereby over-riding the stimulative effect on aflatoxigenes. It is reported that the ochratoxin A production by *Aspergillus* spp. was inhibited by gallic acid derived phenolic compounds having antioxidant potencies, with no inhibition of fungal growth.<sup>[33]</sup> Recent research suggests potential use of polyphenolics in food processing for improving quality, safety, and stability of food products, with human health benefits.<sup>[36]</sup> Several prior reports stated that the antioxidant phenolic and polyphenolic compounds are responsible for the inhibition of aflatoxin biosynthesis in *A. flavus*. Such considerations have guided us to hypothesize that the inhibitory activity of *P. polyphyllus* extract against AFB<sub>1</sub> production by *A. flavus* is due to presence of polyphenolic 4-*o*-MGA, which is able to relieve oxidative stress thereby suppress or eliminate aflatoxin biosynthesis.

The ME showed the highest amount of phenolic content (290 mg GAE/g), which indicate the involvement of phenolic compounds in the radical scavenging activity observed by the ME. The antioxidant capacity of 4-*o*-MGA was greater than the standard BHT, however, the activity of ME was close to the BHT with regardless of the concentration conditions. Raj Kapoor et al.<sup>[21]</sup> reported the hepatoprotective and antioxidant activity of *P. polyphyllus* in acetaminophen-induced hepatotoxicity in rat model. Srirama et al.<sup>[22]</sup> demonstrated the antioxidant activity of crude extract of *P. polyphyllus*, but the active constituent responsible for such activities are not reported. *P. polyphyllus* has earlier been reported for its antioxidant properties based on preliminary investigations of crude extract. However, this is the first report on *P. polyphyllus* showing inhibitory effect on AFB<sub>1</sub> synthesis, in checking fungal deterioration of viable maize (*in vivo*) and its constituent 4-*o*-MGA as an antioxidant.

## CONCLUSION

The results of this study indicate the interesting bioactive properties of polyphenolic compounds of *P. polyphyllus*. The high contents of polyphenolic composition made *P. polyphyllus* as a promising and inexpensive source of biologically active polyphenolics. The high polyphenolic potential, and significant antioxidant and aflatoxin inhibitory activities of *P. polyphyllus* leaf, offer possibilities for its application in preventing oxidative deterioration and fungal spoilage of food products. Further investigations are necessary to resolve the question of whether the atoxigenicity of aflatoxigenic fungus when exposed to hydrolyzable tannins occurs permanently or not.

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