Antifungal Potential of Propolis Against Carcinogenic 
Citrinin Produced by Aspergillus terreus Thom

Hashem Abeer¹ and Abd_Allah EF²*

¹Department of Botany and Microbiology, Faculty of Science, 
King Saud University, Riyadh 11451, Saudi Arabia.
²Department of Plant Production, Faculty of Food and Agricultural Sciences, 
King Saud University, Riyadh - 11451, Saudi Arabia.

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A laboratory experiment was conducted to examine the propolis-induced changes 
on Aspergillus terreus as common producer of carcinogenic citrinin in seeds, feed, food 
and foodstuff. Keeping in view the attributes of growth criteria and citrinin production 
of A. terreus, propolis was very effective significantly in decreasing mold growth and 
mycotoxin production. The alterations in elements accumulation were negatively due to 
impact of propolis. The amino acids metabolism as key physio-biochemical attributes of 
fungal metabolism directly influenced by propolis negatively. The accumulation of both 
glutamic acid and proline due to propolis was observed, signalize to the activation of 
glutamate kinase followed by glutamine synthetase (for glutamic acid) and ³- 
semialdehyde dehydrogenase (for proline), respectively. The alteration in amino acids 
metabolism indicated clearly too significant modifications in mold metabolic process 
towards catabolism.

Key words: Propolis; Aspergillus terreus; Citrinin; amino acids; elements accumulation.

Aspergillus terreus is the major producer 
of carcinogenic citrinin in crops worldwide in 
addition to its importance as opportunistic human 
pathogen in aspergillosis (Pasqualotto, 2009; Horn 
and Moore, 2009; Hashem et al., 2013). The 
common clinical syndromes caused by A. terreus 
include chronic granulomatous sinusitis, keratitis, 
cutaneous aspergillosis, wound infections and 
osteomyelitis following trauma and inoculation of 
the fungus (Hedayati et al., 2007). However, 
balanoposthitis caused by A. terreus has rarely 
been reported. In this report, we describe a case of 
asevere penile A. terreus infection, as well as the 
treatment administered to this patient (Li et al., 
2005). Antifungal resistance is a broad concept 
describing failure of a fungal infection to respond 
to antifungal therapy (Kontoyiannis and Lewis, 
2002). Consequently, there is urgent need to 
develop nonchemical alternative strategies to 
dissolve this clinical problem via alternative means 
with biotic origin.

Propolis [PR] (Bee glue) is a mixture of 
beewax and resins collected by the honeybee (Apis 
mellifera) from parts of plant, buds, flower and 
exudates (Ghisalberti, 1979). Propolis has been 
used to seal holes, exclude draught, and protect 
the beehive against external invaders. The main 
function of propolis is to prevent the 
decomposition of organic matter within beehive 
by inhibiting microbial growth and activity 
(Quiroga et al., 2006). Many biological activities
of propolis have been reported such as medicinal (Orsolic et al., 2003) antibacterial (Uzel et al., 2005), antifungal (Buchta et al., 2011; Hashem et al., 2013) and antitumor (Frozza et al., 2013). Generally, many biological active compounds have been identified in propolis such as polyphenols, phenolic aldehyde, sequiterpene quinines, coumarins, amino acids, steroids, and inorganic compounds (Katircioglu and Mercan, 2006). The biological activities of propolis vary depend up on geographical origin and bearing plants (Kujumgiev et al., 1999).

The objective of this study was to observe the antifungal impact of effect of propolis on growth and some metabolic process of \textit{A. terreus} under laboratory conditions.

**MATERIALS AND METHODS**

The experimental microorganisms

\textit{Aspergillus terreus} Thom was isolated from rice grain sample collected from Zagazig city, Egypt. The identification of experimental mold was carried out according to Domsch et al. (1993). Citrinin-sensitive strain of \textit{Bacillus brevis} was kindly provided by Dr. Gamal El-Didamony (Botany Department, Faculty of Science, Zagazig University, Egypt) was used as biological indicator of citrinin.

**Preparation of PR extract**

Propolis was collected from colonies at Abha city, Saudi Arabia and it was scrappedoff the top of frames and inner wall boxes of bee colonies. PR was extracted with aqueous:ethanol (10:90, v/v) as described by Hashem et al., (2012). Based on preliminary experiment, three concentrations (0.0, 0.3 and 0.5; g/100ml) of PR were selected.

**Culture growth media and general growth conditions of \textit{A. terreus}**

Czapek-Dox agar medium (Raper and Fennel, 1965) was used for the growth of experimental mold (\textit{A. terreus}). Broth cultures (100 ml medium in 250 ml capacity Erlenmeyer flasks) were inoculated with 0.5 mm agar disc of \textit{A. terreus} as described by Abd_Allah and Ezzat (2005). Mycelial dry weight was estimated by filtration the broth mold culture after ten days of incubation at 28\(^+\) \degree C in dark at static state. Mycelium growth was washed carefully with distilled water, dried at 105\(^o\)C up to two successive constant weights, then the mycelia dry weight was recorded. The test fungus was also grown in agar medium in 9.0 cm diameter Petri-dishes for seven days incubated at 28\(^+\) \degree C in dark and the radial growth was measured. Conidial production and their germination were studied according to Roberts and Selitrenikoff (1988).

**Estimation of mycotoxin citrinin**

The extraction and clean-up of citrinin were carried out according to Jackson and Ciegler (1978). Fifty ml of culture filtrate was shaken with an equal volume of chloroform for 30 min. The chloroform layer was separated over a bed of anhydrous sodium sulfate. Clean-up was carried out using concentrated HCl and 0.1 M NaHCO\(_3\). The quantitative determination of citrinin was carded out fluorometrically according to Trantham and Wilson24 using spectrofluorometer Varian model 330 with detector (Varian Associates,ouston, TX, USA). The excitation wavelength of the fluorometer was 330 nm and the emission wavelength was 500 nm. The chemical and biological confirmations were carries out according to Hald and Krogh (1973) and Abd_Allah and Ezzat (2005), respectively.

**Estimation of ion accumulation**

The oven dry mycelial samples of \textit{A. terreus} were digested and the ions were estimated according to the method of Wolf (1982) using a flame photometer Jenway Flame Photometer, Bibby Scientific Ltd-Stone-Staffs-St15 OSA – UK. Standard curve of each mineral (10- 100 µg/ml) used as reference.

**Amino acids analysis**

Free amino acids were extracted from dry mycelial samples of \textit{A. terreus} with absolute ethanol. The qualitative as well as quantitative determination of amino acids was carried out using LKB 415 alpha plus Amino Acid Analyzer (AAA) according to Christias et al., (1975). Standard amino acids (BHD Chemicals, Poole, UK) were used as reference.

**Statistical analysis**

All data were subjected to statistical analysis. Treatments were compared using Fisher’s Least Significant Difference (LSD) analysis. Cluster analysis was performed using SPSS 20 software package.
RESULTS

In the current study, a preliminary test was carried out to elucidate the antifungal potential of PR on local (Egyptian) isolate of *A. terreus* isolated from rice grain sample. Such isolate was able to produce citrinin *in vitro* and *in vivo* (in rice grains). It is necessary to mention that such rice grain sample was contaminated with mycotoxin citrinin.

Data recorded for different criteria related to growth of *A. terreus* showed that application of varying levels of PR (0.0, 0.3, and 0.5 g/100ml) caused significant decrease in growth criteria was directly proportional with the concentration of PR (Table1). The concentration of 0.3 (g/100 ml) of PR caused significant decrease in conidial production; conidial germination; radial growth and mycelial growth of *A. terreus* by percent of 22.25; 39.29; 17.44 and 37.74, respectively (Table 1). The application of further concentration of PR (0.5, g/100 ml) was accompanied with significant inhibition in all growth criteria compared with both the previous concentration (0.3, g/100 ml) and control (Non) as shown in Table 1.

PR stress (0.0, 0.3, and 0.5 g/100ml) significantly reduced the production of mycotoxin citrinin in solid media (Petri dishes) and liquid media (conical flasks) (Figure 1). Citrinin production by *A. terreus* in both Petri dishes and conical flasks was inhibited significantly under concentration of (0.3, g/100 ml) of PR. At the further concentration of PR (0.5, g/100 ml), significant inhibition in production of citrinin in conical flasks was observed, whilst citrinin production in Petri dishes completely inhibited (Fig. 1).

The results for element contents in the mycelial growth *A. terreus* under the biotic stress of PR were shown in Table 2. Mycelial Na⁺ contents increased considerably (p<0.01) in *A. terreus* under all PR levels and a maximum increase in this ion was observed at 0.5 g/100ml (Table 2). A considerable reduction in K⁺ concentrations was observed under PR stress, a maximum reduction in K⁺ was observed at 0.5 g/100ml. Mycelial Ca²⁺ contents were also affected due to imposition of varying levels PR stress to the growth medium. In the same context, the Mg²⁺ contents were significantly decreased in directly proportional with PR concentration (Table 2).

The chromatographic analysis revealed the presence of 20 free amino acids in mycelial control of *A. terreus*. The amino acids were alanine, arginine, asparagine, aspartic acid, cysteine,

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### Table 1. Effect of different concentrations of propolis (w/v) on number of germinated conidia (conidia in number); conidial production (conidia mm²X10⁴); radial growth (cm/plate) and mycelial growth (g/100 ml culture medium) of *A. terreus*

<table>
<thead>
<tr>
<th>Concentration of propolis (g/100 ml)</th>
<th>Number of germinated conidia (out 100 conidia)</th>
<th>Conidial production (conidia mm²X10⁴)</th>
<th>Radial growth (cm/plate)</th>
<th>Mycelial growth (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Non)</td>
<td>95.34</td>
<td>9.34</td>
<td>9.00</td>
<td>2.04</td>
</tr>
<tr>
<td>0.3</td>
<td>74.12</td>
<td>5.67</td>
<td>7.43</td>
<td>1.27</td>
</tr>
<tr>
<td>0.5</td>
<td>35.87</td>
<td>1.25</td>
<td>3.72</td>
<td>0.64</td>
</tr>
<tr>
<td>LSD at: 0.05</td>
<td>10.14</td>
<td>0.86</td>
<td>1.23</td>
<td>0.38</td>
</tr>
</tbody>
</table>

### Table 2. Effect of different concentrations of propolis (w/v) on elements accumulation (mg/g dry wt) of *A. terreus.*

<table>
<thead>
<tr>
<th>Propolis (g/100 ml)</th>
<th>Sodium</th>
<th>Potassium</th>
<th>Calcium</th>
<th>Magnesium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Non)</td>
<td>0.182</td>
<td>2.573</td>
<td>0.132</td>
<td>0.307</td>
</tr>
<tr>
<td>0.3</td>
<td>0.237</td>
<td>1.782</td>
<td>0.077</td>
<td>0.183</td>
</tr>
<tr>
<td>0.5</td>
<td>0.416</td>
<td>1.043</td>
<td>0.034</td>
<td>0.104</td>
</tr>
<tr>
<td>LSD at: 0.05</td>
<td>0.07</td>
<td>0.23</td>
<td>0.04</td>
<td>0.08</td>
</tr>
</tbody>
</table>

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glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, Tyrosine and valine in concentration of 0.41, 0.58, 1.02, 0.16, 0.08, 0.15, 0.82, 0.12, 0.11, 0.18, 0.34, 0.64, 0.07, 0.43, 0.24, 0.48, 0.13, 0.17, 0.63 and 0.21, respectively with total free amino acids equal 18.46 mg/g mycelial dry weight (Table 3). As shown in Table 1, the application of PR at concentration of 0.3 g/100ml caused the absence of methionine, and significant decrease in alanine, cysteine and lysine by percent of 48.7, 50.0 and 34.3 respectively as compared with control A. terreus. On the other hand, the same concentration of PR (0.3 g/100ml) caused significant increase in total free amino acids of A. terreus was accompanied with significant increase in the rest amino acids. The higher concentration of PR (0.5 g/100ml) caused significant increase in total free amino acids by percent of 108.0, as compared with control A. terreus. Such increase in total free amino acids caused by PR at concentration of (0.5 g/100ml) was accompanied with significant increment in all free amino acids except cysteine as well as methionine which were disappeared (not detected under the experimental conditions) and lysine which decreased by percent of 62.5 as compared with control A. terreus.

**DISCUSSION**

PR (or bee glue) is a natural resinous substance produced by Apis mellifera bees made up from parts of the plants, buds and exudates. In the beehive, PR is thought to be used to seal holes,
exclude draught and protect against external invaders. Its main function, however, is to prevent the decomposition of organic matter within the hive by inhibiting microbial growth (Quiroga et al., 2006). Long ago, it was reported that PR was very well known to the priests who had monopolized medicine, chemistry and art of mummmifying corpses (Lotfy, 2006). In the present study, the criteria related attributes growth of A. terreus inhabited significantly due to application of PR. Similar antifungal activities of PR on growth of different fungi such as Trichophyton mentagrophytes (Buclha et al., 2011); Candida albicans (Afrouzan et al., 2012); Aspergillus parasiticus (Hashem et al., 2012) and Penicillium viridicatum (Hashem et al., 2013) have been reported. The phenols (Ghaly et al., 1988) and flavonoids (Cushnie and Lamb, 2005) has considered the main carriers of antifungal properties of PR. Also, it has been reported that PR might inhibit DNA replication hence caused an inhibition in cell division Takaisi-Kikuni and Schilcher (1994). As another suggested mechanism, clear catabolic repression of lipids metabolism (total lipids, neutral lipids, phospholipids) of A. parasiticus has been reported by PR in our previous study (Hashem et al., 2012). Moreover, Hashem et al., (2013) reported that PR caused significant alteration in amino acids metabolism of Penicillium viridicatum towards clear catabolic repression. A marked reduction in production of citrinin in both solid media and liquid media by A. terreus was observed due to different PR concentrations (0.0, 0.3 and 0.5 g/100ml). Although mycotoxins reduction production by most mycotoxigenic fungi is a common effect of PR stress (Khezri et al., 2006; Li et al., 2007; Hashem et al., 2012; Hashem et al., 2013), however, the actual physiological and biochemical mechanisms involved in mycotoxins reduction are still not well especially according to molecular approach. The accumulation of Na+ contents in mycelia of A. terreus increased considerably under PR stress, however K+, Ca2+ and Mg2+ contents were significantly decreased in directly proportional with PR concentrations. The accumulation excessive accumulation of Na+ ions in fungal mycelia under biotic and abiotic stress is defense mechanism (Monns and Tester, 2008; Alqarawi et al., 2012; Souza et al., 2013) which further leads to alteration of mycelia osmotic potential thereby reducing cell turgidity in fungal cells due to water loss caused by PR (Sripriya et al., 2009). Study done by Mello et al., (2006) suggested that the antifungal activity of propolis is due to changes in the cell wall permeability leading to disturbance of volume and hence membrane rupture. In the same context, it was reported that Release of cytochrome c from mitochondria is therefore considered a key initial step in the apoptotic process (Ott et al., 2002). Furthermore, de Castro et al., (2011) reported that propolis was able to induce an apoptosis cell death response of Saccharomyces cerevisiae via alteration of cytochrome c. The potential of PR to increase the direct potential-dependent permeabilization of biological membranes suggest it to use as anticancer polyarginine-KLA peptides (Lemeshko, 2013). The data indicated the presence of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine amino acids in mycelial control of A. terreus. This result confirmed those of Vyas et al., (2005); Alqarawi et al., (2012); Hashem et al., (2012) who reported parallel results. PR stress remarkably enhanced the absence of methionine, and significant decrease in alanine, cysteine and lysine, however glutamate amino acids (glutamic acid and proline) and total free amino acids of A. terreus significantly increased. The consumption of such amino acids decreased due to PR to support more energy (Adenosine Tri-phosphate, ATP) required for mold resistance against antifungal potential as described by Yamaguchi and Fujimura (2005); Chen et al., (2008) and Alqarawi et al., (2012). The accumulation of glutamic acid and proline due to PR means activation of their biosynthesis from glutamate via both glutamate kinase followed by α-semialdehyde dehydrogenase (for proline) and glutamine synthetase (for glutamic acid), respectively as reported by Albert et al., (2002). Such alteration in amino acids metabolism is parallel to Perea and Patterson (2002); El-Melahawy et al., (2008); AbdEl-Ghany et al., (2009) and Hashem et al., (2012) who reported that such alteration in amino acids metabolism as sensitive monitor for antifungal resistance against abiotic stress of fungicides. In another study on Aspergillus flavus, Alqarawi et al., (2012) found that the antifungal from biological
origin caused significant increase in glutamic acid, proline, serine, leucine, phenyl alanine and total free amino acids. Hashem et al., (2013) reported similar evidences regard the effect of PR on the amino acids composition of Penicillium viridicatum.

In conclusion, PR caused a marked suppression in growth criteria, production of mycotoxin citrinin and induced significant alterations in minerals accumulation and amino acids metabolism of A. terreus towards catabolism. It is now evident that PR can be used as alternative biotic antifungal with good and acceptable results will be suitable for future prospects.

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