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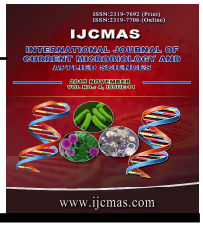

Original Research Article

Antifungal Evaluation of Iraqi Propolis against *Penicillium expansum* and Mycotoxin Production in Apple

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A B S T R A C T

This study aimed to evaluate Iraqi propolis to control Penicillium apple decay and toxin production. Three concentration of PEE used in storage experiment for three week, EPP treatment suppress disease expending and maintain apples fruit that treated with 1, 2 and 3% of PEE compared with pathogen treatment, it was 3.64, 3.42, 3.08 , 4.35 cm and 6.03, 5.72, 5.70, 8.53 cm respectively for the 3rd week. Also the depth of molded area was decreased for the treatment 2 and 3% EPP comparison with pathogen treatment by 3.23, 3.00 and 4.07 cm respectively. Results showed that patulin reduced significantly in the 2 and 3% of PEE compared with pathogen treatment only it was 0.50, 0.50 and 1.30 µg/kg respectively, Also reated the apple with EPP showed decrease in citrinin production and accumulation in fruit tissues by 5, 5.83, 4.17 and 9.63 µg/kg for the 1, 2, 3% PEE and pathogen treatment respectively.

Keywords

Patulin, Citrinin, Friut Decay, ELISA

Introduction

Penicillium expansum is phytopathogenic fungus that causes fruit losses ranging from 5% to 20% in world (Cappellini and Ceponis, 1984) and up to 50% in developing countries (El-Ghaouth, 1997). *Penicillium* spp mycotoxin is a common secondary metabolites contaminated fruits and vegetables, both patulin and citrinin can be founded in infected fresh fruit (Van, 1994). *P.expansum* is the major causal agent of apple soft rot and blue mold rot (Sommer., et al 1974). The pathogen can infected the fruit during agricultural and marketing operations dames like: Trauma, skin breaks and other physical damage during harvesting of apples fruit provide entry for *P.expansum* and other fungal fruit decay (Draughon and Ayres, 1980).

Patulin may occur in fruits and fruit juices such as apple juice and other juices, *P.expansum* is known for its production patulin and citrinin in moldy fruits (Vinas et al., 1993). Chemically Patulin (4-hydroxy-4H-furo [3,2c] pyran-2[6H]-one), is a water-soluble lactone, first time discovered was

used as an antibiotic during the 1940s (Stott and Bullerman, 1975). After several studies results showed that patulin is toxic to fungi, bacteria, animals and higher plants (Berestets'kyi and Synyts'kyi, 1973). The chronic health risks of patulin exposure and consumption can caused neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic, and carcinogenic effects (Wichmann, et al 2002). Patulin has been classified as a carcinogen group 3 (IARC, 1987). Citrinin can caused a chronic disease to animals and humans, It is known as a nephrotoxin and several studies reported the potential immunotoxicity (Sharma, 1993). Phytotoxic effects of citrinin have also been reported (Betina, 1989).

Many studies report that propolis used to extending fruits shelf life and preventing fungal decay during storage (Çandir., et al 2009; Özdemir., et al 2010). The propolis contain about 50% resin, 30% wax, 10% essential oils, 5% pollen and 5% other organic compounds (Falcão et al., 2010). Curifuta., et al (2012) found tested four concentrations (0.5, 1.0, 2.5 and 5.0 %) of propolis ethanoic extract (PEE) that showed significant antifungal inhibition effects to *P.expansum* on agar medium. Matny., et al (2014) reported that snap bean treated with 5% of EEP prevent white mold *Sclerotinia sclerotiorum* in storage conditions for more than two weeks.

Contamination with mycotoxin produce by *P.expansum* specially patulin and citrinon is big problem in apple industry. Many studies found fruit and juice contamination with mycotoxin. In Korea Paik et al (2000) showed Patulin was detected in apple storage conditions at 5.68-47.8 µg/g for in his study.

One hundred samples of apples were collected from households in Croatia 78 % of the samples were infected by *P. expansum*, citrinin was detected in 19 % of apple samples, in concentrations ranging from 50 to 240 µg/kg (Pepeljnjak et al., 2002). Katerere et al (2007) exam apple juice samples collected from south Africa cape town to patulin occurrence, eight of ten samples were contaminated with patulin at 75 ppb. Martins et al., (2002) report in his study that there are no correlation between the concentrations of patulin and citrinin and the size of fruit rotten area. The United States has been much slower to set regulation on patulin, but today the U.S. Food and Drug Administration limits patulin to 50 µg/L for apple juice (USFDA 2004).

This study aim to investigate the efficacy of Iraqi propolis against *P. expansum* that caused apple decay and prevent mycotoxin production by use natural materials that have no effected on human healt.

Materials and Methods

Fungus isolation

Apple samples were collected from three different location (local market) in Baghdad (5 sample /location), all samples were infected with *Penicillium* spp. In the laboratory, pathogen was isolated by taken spore wipe by the tip of sterilize needle and streak on PDA media on 9 cm Petri dish and incubated at 25 C for 2 days. Single spore technique were used by pick up a single colony and relocated in new PDA Petri dish and incubation at 25 C for 7 day.

Pathogenicity test

Ten isolates were tested to show the pathogenicity on apple fruits. Three apples for each isolate were performed, surface sterilize by 10% bleach were used, for 10 min dipping after that wash with sterilize water. With sterilize knife 0.5 ×0.5cm bores were performed for each apple, 100 µl of

4×10^4 spore suspension to each isolate were inoculated and place it in plastic box 15×15×10 dim and incubate in 25 C for 7 days. The aggressive isolate were used in the storage experiment.

Propolis preparation

Iraqi bee propels was collected from the bee hives in Baghdad (2014-2015), 100 g of propolis were kept in the freezer for 1h and grinded by the coffee grinder. Propolis ethanolic extract (PEE) were prepared by add 250 ml of 96% ethanol in a conical flask with shaking 150 rpm for overnight. The solution was filtrated by using centrifugation at 5000 rpm for 5 min and the supernatant were collect. In room temperature, the filtrated solutions were dried.

Storage experiment

Nine apples for each treatment (three apple/ replication) were performed, surfaces sterilized by using 10% bleach for 5 min followed by water washing. All apples were dry and with sterilize knife 0.5×0.5 cm dim each fruit were pure from the side. Three concentration of PEE were prepared 1, 2 and 3% by weight 1, 2 and 3 g respectively of a dry resin of PEE in water. Each treatment was immersion in the prepared concentration of PEE and left to dry. One hundred micro liter of spore suspension were placed in each apple pore. Both the control treatment (without pathogen) and pathogen control (pathogen only) were immersion in water only, and the control treatment inoculated with sterilize water, and the pathogen control inoculated with spore suspension. All treatments were place in plastic box 15×15×10 dim and kept in 25 C for 3 weeks.

Molded area and depth of mold were calculated by using a ruler and the secondary metabolite patulin were measured by using ELISA kite from Glory Science Co. Ltd (USA). All the data were statistically analysis by using GenStat software.

Patulin and citrinin detection

Patulin and Citrinin were measured by using ELISA test kit provides by Glory Science Co. Ltd (USA) kit for each toxin. Each apple fruit was blending with 100 mL distilled water. Ten milliliters of apple juice were transfer to a separate funnel, 20 mL of acetonitrile with slow shaking for 1 min. Carefully take 100 µL of the supernatant and mix with 300 µL of 1 X patulin dilution buffer, 100 µL from each sample were used to measured patulin. All samples were measured by following company protocol and readed by Bio-Tek ELx800 Microplate Reader. The patulin and Citrinin concentrations were calculated by draw standard carve (company kit protocol).

Results and Discussion

Results showed a significant disease reduce on apple fruit in storage condition, in the first week molded area for 2 and 3% EPP was significantly reduced compared with the pathogen treatment; it was 2.14, 1.54 and 2.67 cm respectively. Both the 2^{th} and 3^{rd} week the EPP treatment suppress disease expending and maintain apples fruit that treated with 1, 2 and 3% of PEE compared with pathogen treatment, it was 3.64, 3.42, 3.08 , 4.35 cm and 6.03, 5.72, 5.70, 8.53 cm respectively table 1, figure 1.

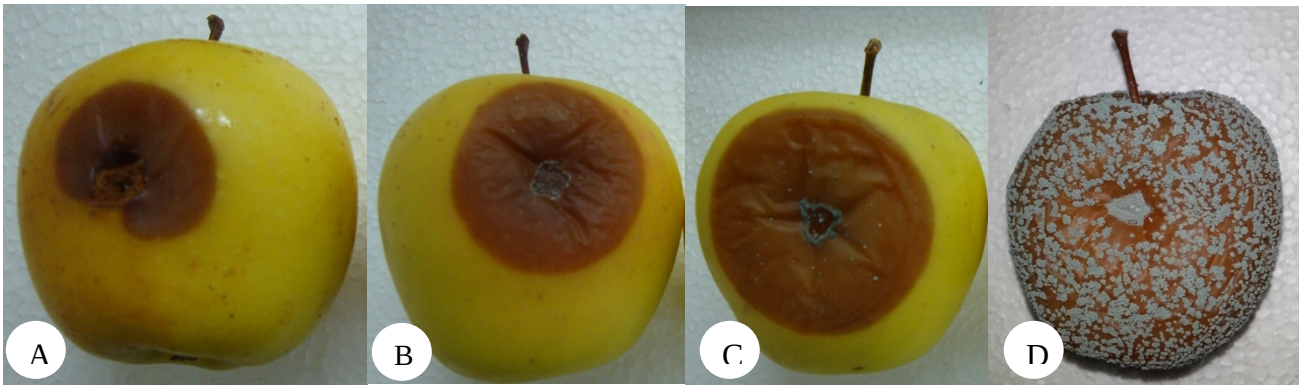
Depth of the molded area were measured in the end of experiment to estimate the ability of EPP to prevent decay inside the fruits. Result indicated that 2 and 3% EPP decrease the depth of molded area in comparison with pathogen treatment at 3.23, 3.00 and 4.07 cm respectively.

Mycotoxin test for detection patulin and citrinin perform. Results showed that patulin reduced significantly in the 2 and 3% of PEE compared with pathogene treatment only it was 0.50, 0.50 and 1.30 µg/kg respectively, while there are no significant different between 1% PEE and pathogen treatment it was 1.40 and 1.30 µg/kg respectively. Also citrinin toxin showed higher level compared with patulin. Treated the apple with EPP showed decrease in citrinin production and accumulation in fruit tissues, it was 5, 5.83, 4.17 and 9.63 µg/kg for the 1, 2, 3% PEE and pathogen treatment respectively.

Tabl.1 Effect of different concentrations of PEE on Penicillium apple decay, mycotoxin production in storage conditions

Treatments	Mold area dim / cm			Depth of mold area /cm	Patulin µg/kg	Citrinin µg/kg
	1 week	2 week	3 week			
Control	0.00	0.00	0.00	0.00	0.00	0.00
Pathogen	2.67	4.35	8.53	4.07	1.30	9.63
Propolis 1%	2.17	3.46	6.03	3.63	1.40	5.00
Propolis 2%	2.14	3.42	5.72	3.23	0.50	5.83
Propolis 3%	1.54	3.08	5.70	3.00	0.50	4.17
LSD 0.05	0.53	0.43	0.73	0.47	0.21	4.24

Figure.1 Effect of EPP on apple fruit decay. A- 1% EPP. B- 2% EPP. C 3%EPP and D- Pathogen only



Fruit decay disease caused by fungus is a large problem in marketing and storage stage in the world wide, and use of chemical product to control fruit decay losses is serious effect that caused health problem for the consumers. Founded new natural products and new methods to control diseases, on the others side its safe on consumers health. This study showed the efficacy of Iraqi propolis to control and reduce apple decay caused by *P.expansum*, results founded that the 3% EPP reduce molded area on apple fruit after 3 week to 66.8% compared with pathogen treatment, that indicate the Iraqi propolis may be contain many and high concentration of

active ingredients that control the pathogen, according on the types of trees that grow in the region of propolis collected. Many studies mention about the chemicals compounds in propolis and biological activities depend on different factors such as the geographical regions, collection time and plant source (Bankova, 2005; Hegazi., et al 2014). Antimicrobial activity of bee propolis was be mention by many studies (Matny et al, 2014; Matny, 2015)

In this study mycotoxin production by *P.expansum* were evaluated. Patulin and citrinin concentration in apple juice of treated fruit with PEE showed reduction in toxin production. Reduction in mycotoxin production it may be consequent on the antifungul activity of PEE effected directly on the fungal grow that leaded to inhibition toxin production or the PEE interaction with the phase of the biosyntheses of toxin production.

Several study mention about the mycotoxin inhibition by PEE. Peng et al (2012) found that pinocembrin one of propolis ingredients play role in the mode of action of propolis antimicrobial activity against fungal through inhibition the respiration of hyphal cells, which led to energy deficit and cell membranes damage that accelerated cell death. Silici and Kevser (2014) found that treated apple juice 2 mg/ml PEE of Turkish propolis after inoculation with *P. expansum* for 84h incubation, Patulin was reduce to 27.63ppb compared to 56.40 ppb in control treatment. Also another study found that treated the liquid medium with PEE 4 g/L reduce spore germination and dry mass of *Aspergillus flavus* 80% and aflatoxin production to 100% (Ghaly et al, 1998). Ezzat (1992) previous study showed citrinin production by *A. terreus* was inhibited by 29-100 % by using PEE 3-48 mg /100 mL in culture medium and by 12-93 % when aqueous propolis extract 50-350 mg/100 mL.

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