

**Research Article**

## TESTING THE TOXICITY OF THE EXTRACTS OF APPLE FRUIT INFECTED BY *VENTURIA INAEQUALIS* AGAINST BRINE SHRIMP (*ARTEMIA SALINA*) AND TERMITES (*COPTOTERMES FORMOSANUS*)

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### ABSTRACT

*Venturia inaequalis* overwinters in the fruit Apple. In the present study crude and partially purified extracts of the infected fruit were tested against the larvae of *Artemia salina* (Brine shrimp) and the adults of *Coptotermes formosanus* (Termites) to determine the toxicity of these extracts. The extract in water showed 5% mortality of Brine shrimp after 12 hours of exposure and 10% after 24 hours while there was no effect on termites after 12 hours but the mortality was 5% after 24 hours. In partially purified extracts Brine shrimp showed a mortality percentage of 5%, 10% and 10% after exposure for 12 hours to 6000ppm, 8000ppm and 1000ppm respectively which did not cross 10% after 24 hours, while in termites the mortality remained at 5% after 24 hours. The study indicates that there is no serious mycotoxins activity seen in the infected apple fruits.

**Keywords:** *Venturia inaequalis*, mycotoxins, *Artemia salina*, extracts, *Coptotermes formosanus*

### INTRODUCTION

Apple scab is one of the most common orchard diseases in temperate regions, it is caused by the fungus *Venturia inaequalis*, and is the most important disease of cultivated apple (*Malus x domestica*) worldwide in relation to economic cost of control (Carisse and Bernier, 2002). *V. inaequalis* has a wide geographical range and is found in almost all areas in which apples are grown commercially. However, the disease is more severe in temperate countries with cool, moist climates during early spring (MacHardy, 1996).

Most major commercial apple cultivars are highly susceptible to this disease (Gessler *et al.*, 2012). The fungal infection causes damages to leaves and fruits, which results in severe reductions in fruit quality and yield. In case of insufficient control of apple scab, the economic losses can increase up to 70 % of the production value (Gupta, 1992). The life cycle of this fungus is divided into a primary and a secondary stage. In the secondary stage infections continue throughout the summer, until the leaves and fruit fall from the tree at the onset of winter. *V. inaequalis* overwinters mostly as immature Perithecia, where sexual reproduction takes place, producing a new generation of ascospores that are released the following spring. Scab lesions located on the woody tissues may also overwinter in place, but will not undergo a sexual reproduction cycle; these lesions can still produce infective conidial spores in the spring.

The fruit may look perfectly healthy externally but the fungal mycelium and spores can be present in the fruit due to the over wintering phase of the life cycle of this pathogen. Apple juice is a very common beverage which is mass produced in most countries, the chances of any mycotoxin produced by the fungus or its spores entering such a food product are therefore very high. The present study undertakes to test the toxicity of fruits infected by *Venturia inaequalis* on the larvae of *Artemia salina* (Brine shrimp) and the adult of the insect *Coptotermes formosanus* (Termites). This will help us identify any harmful mycotoxin secreted by the fungi during overwintering in the fruit.

### Review of Literature

The ascomycete *Venturia inaequalis* Cooke (Wint.) (anamorph: *Fusicladium pomi* (Fr.) Lind or *Spilocaea pomi* (Fr.)) infects members of the subfamily Maloideae, and causes the disease apple scab, the most important disease of apple worldwide. *V. inaequalis* is a hemibiotrophic fungus, which means that it does not only grow on/in living leaves, but also has a necrotrophic phase. The life cycle of *V. inaequalis* can be subdivided into two phases: a sexual or primary phase and an asexual or secondary (MacHardy *et al.*,

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2001; Verma and Sharma, 1999). Although our understanding of the life cycle and epidemiology of apple scab is complete, most of the research has been on the management of the disease in which we still rely heavily upon chemical control (Bowen *et al.*, 2011). The increased use of fungicides has led to the development of new, durable scab-resistant apple cultivars will most likely gain importance in future apple scab management strategies. One of the latest area of research is the isolation and introduction of scab resistance genes and the most famous is the well-known *Rvi6/Vf* 'gene' Joshi *et al.*, (2011).

In the area of protein analysis also the major work is being carried out in the area of special proteins called pathogenesis-related (pr) proteins (Gessler and Pertot, 2012). The work on mycotoxins has been restricted to their detection from the fruit post harvest especially in the juice which is a popular drink, many studies have been done on this aspect (IARC, 1986; Egmond, 1989). Not much work has been done on the toxicology of *Venturia inaequalis* therefore this study was undertaken.

## MATERIAL AND METHODS

Apple fruits (*Malus x domestica*) var. Red Delicious infected with the pathogen and showing complete symptoms were collected from the orchards in the apple belt of Kullu and Manali region of the state of Himachal Pradesh, India. This formed the basic raw material for preparation of extracts for toxicity testing.

### Preparation of Crude Extracts

Four different solvents Water, Methanol, Chloroform and Hexane were used to prepare extracts from the diseased portions of the fruit infected with *Venturia inaequalis*. The diseased portion of the collected Apples was excised out and a weighed quantity (50gms) cut into small pieces was placed in a separating funnel and the solvents poured into the funnel. This mixture was allowed to stand for 24 hours. The extract was then evaporated in vacuo and the residue left behind was used as the crude extract. Different concentrations of these extracts were prepared and used for ascertaining the toxicity of these crude extracts.

### Test of the Effect of Extract on Artemia Salina Shrimp Larvae

50 mg of shrimp eggs was sprinkled into container containing 250ml of artificial sea water. The container was placed beside a light ray precisely the window blind for rays of light and proper ventilation, After 48 hours, brine shrimp larvae were collected by dropping pipette. About 4.5ml of brine solution (sea water) into each test tube and the 0.5ml diluted test solution of the extracts was added to the test tubes. The corresponding concentrations of the extracts were 5000ppm, 1000ppm, 100ppm and 10ppm respectively. Fifty (50) active brine shrimp (nauplii) were transferred into each of these vials using Pasteur pipette. Replicates of each of the dose levels were prepared, using seawater and the solvent as control the number of survivors, and dead, nauplii were recorded after 12 and 24 hours (Ogugua, *et al.*, 2012).

### Filter Paper Test for Effect of Extract on Termites Coptotermes Formosanus

Whatman filter paper No. 42, 9 cm in diameter was soaked in 5000ppm, 1000 ppm, 100ppm and 10ppm concentrations of the crude extracts air dried and placed in Petri dishes (95 × 15 mm). 20 adult termites (*Coptotermes formosanus*) collected from the field were released in the Petri dishes having treated filter paper. A similar petri dish with untreated filter paper was used as control. The Petri dishes having filter paper were placed in growth chamber under controlled conditions of 28±2°C and 80%±5 humidity. Data for mortality were recorded after 12 hours and 24 hours of exposure (Ahmed *et al.*, 2011.).

### Purification of Crude Extract

The crude aqueous extract which showed positive result against the insects was concentrated to 0.5 ml and then loaded onto silica gel plates for TLC. The TLC plates were developed using different solvent systems according to the methods of Hargwig & Scott 1971; Carpentier *et al.*, 2008b. The separated fractions were tried out against the insects and the active fraction was used to further study the effect on the target insects. In this study the target insects were exposed to 4000ppm, 6000ppm, 8000ppm and 10000ppm solutions of the active fraction for 12 and 24 hours.

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### RESULTS AND DISCUSSION

The two insects were exposed to four different concentrations of crude extracts prepared from diseased Apple fruits. The crude extracts of water, Methanol, Hexane and Chloroform in concentrations of 10ppm, 100ppm, 1000ppm and 5000ppm were administered to the two target insects and results noted after 12 hours and 24 hours. The experiments showed that there was no mortality in any concentration of all the four extracts except in the 5000ppm concentration of the aqueous extract (Table1&2). The extract in water showed 5% mortality of Brine shrimp after 12 hours of exposure while there was no effect on termites after 12hours. This mortality increased to 10% after 24 hours in Brine shrimps, similarly in the case of Termites the aqueous extract gave a mortality percentage of 5% after 24 hours of exposure.

The partially purified extract obtained after TLC contained a protein fraction which showed activity against the target insects, different concentrations of the fraction were prepared and the target insects were exposed to them. The concentrations were 4000ppm, 6000ppm, 8000ppm and 10000ppm. The Brine shrimp showed a mortality percentage of 5%, 10% and 10% after exposure for 12 hours to 6000ppm, 8000ppm and 1000ppm respectively (Table 3) where as a mortality percentage of 5% was seen in termites exposed to 8000ppm and 1000ppm for the same time period. After 24 hours all the mortality percentage for brine shrimp was 10% in 6000ppm, 8000ppm and 10000ppm. Termites showed a mortality percentage of 5% at 6000ppm and 10% at 8000ppm and 10000ppm after 24 hours.

**Table1: Effect of different concentrations of crude extracts on the mortality % of the target insects after 12 hours of exposure**

Type of Extract	Mortality% of Brine Shrimp				Mortality% of Termites			
	10ppm	100ppm	1000ppm	5000ppm	10ppm	100ppm	1000ppm	5000ppm
AQUEOUS	NIL	NIL	NIL	5%	NIL	NIL	NIL	NIL
METHANOL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
CHOLOROFORM	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
HEXANE	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL

**Table2: Effect of different concentrations of crude extracts on the mortality % of the target insects after 24 hours of exposure**

TYPE OF EXTRACT	MORTALITY% OF BRINE SHRIMP				MORTALITY% OF TERMITES			
	10ppm	100ppm	1000ppm	5000ppm	10ppm	100ppm	1000ppm	5000ppm
AQUEOUS	NIL	NIL	NIL	10%	NIL	NIL	NIL	5%
METHANOL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
CHOLOROFORM	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
HEXANE	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL

**Table3: Effect of different concentrations of purified extract on the mortality % of the target insects after 12 & 24 hours of exposure**

Concentration of Purified Extract	Mortality% of Brine shrimp		24	Mortality% of Termites	
	AFTER 12 HOURS	AFTER 24 HOURS		AFTER 12 HOURS	AFTER 24 HOURS
4000 PPM	NIL	NIL		NIL	NIL
6000PPM	5	10		NIL	5
8000 PPM	10	10		5	10
10000 PPM	10	10		5	10

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The study was conducted to ascertain if the apple fruit infected by the fungi *Venturia inaequalis* contains toxins which can cause mortality in the two test insects Brine shrimp and Termites. Mycotoxins have been isolated from many fungi and they are known to cause serious problems for humans and animals in addition to plants in which they are produced. Mycotoxins are toxic secondary metabolites produced by molds and can contaminate a wide variety of foods and feeds. Human cases of ergotism or St. Anthony's Fire have been described in Europe since the Middle Ages and are now known to be caused by alkaloids produced in rye by the old *Claviceps purpurea*. In 1960, an outbreak of Turkey X disease in England and the subsequent discovery of the aflatoxins stimulated great interest in the field of mycotoxin research (Bullerman, 1979). Since then many more mycotoxins, such as thricothecenes, zearelenone, ochratoxins and fumonisins have been discovered. Apples are one of the most important temperate fruits which are put to many different uses. Post harvest fungi and mycotoxins have been isolated from the fruit and from the juice. Patulin is a mycotoxin is produced by *Penicillium expansum* which has been isolated from apples and apple juice (Brause *et al.*, 1997). In addition to this toxin producing fungi *Aspergillus flavus*, *A. niger*, and *Rhizopus stolonifer* were isolated from harvested apple fruit (Hasan, 2013). The disease apple scab is of common occurrence in all apple growing regions of the world including India, and the disease cycle is such that the mycelium and spores remain in/on the fruit, therefore there are chances of toxins if any remaining with the fruit. In this study it was found that the Methanolic, Hexane, and chloroform extracts did not show any mortality of any of the two target insects. It was aqueous protein fraction extracted from the infected fruit which produced 10% mortality at a concentration of 6000ppm in the larvae of Brine shrimp, which is a very sensitive to any toxic compound present in its environment. The other insect termite against which this extract was tested showed a mortality percentage of 10% at a concentration of 8000ppm when exposed for 24 hours. In both the insects the mortality percentage remained stable even when the concentration was increased to 10000ppm this means that the levels of toxicity of the extracts from this fungus are not very to the test insect even at this extremely high concentration. There is a need for further investigation to check if the protein extracted was from the fungus or it was secreted as a response to the attack by the plant.

## REFERENCES

- Ahmed S, Zafar MI, Hussain A, Riaz MA and Shahid M (2011). Evaluation of Plant Extracts on Mortality and Tunneling Activities of Subterranean Termites in Pakistan. *Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment*. Edited by Dr. Margarita Stoytcheva (InTech) 457- 63.
- Bowen JK, Mesarich CH, Bus VG, Beresford RM, Plummer KM and Templeton MD (2011). *Venturia inaequalis*: the causal agent of apple scab. *Molecular Plant Pathology* 12(2) 105-22.
- Brause R, Trucksess MW, Thomas FS and Page SW (1997). Determination of patulin in apple juice by liquid chromatography: collaborative study. *Journal of AOAC International* 79 451-455.
- Bullerman LB (1979). Significance of mycotoxins to food safety and human health. *Journal of Food Protection* 42 65-86.
- Carisse O and Bernier J (2002). Effect of environmental factors on growth, pycnidial production and spore germination of *Microsphaeropsis* isolates with biocontrol potential against apple scab. *Mycological Research* 106 1455-1462.
- Carpentier S, Panis B, Vertommen A, Swennen R, Seargeant K, Renaut J, Laukens K, Witters E, Samyn B and Devreese B (2008b). Proteome analysis of non-model plants: a challenging but powerful approach. *Mass Spectrometry Reviews* 27 354-377.
- Gessler C and Pertot I (2012). Vf scab resistance of *Malus*. *Trees* 26 95-108.
- Gupta GK (1992). Apple scab (*Venturia inaequalis* (Che.) Wint.). In: *Plant Diseases of International Importance. Diseases of Fruit Crops*. Edited by Kumar J, Chaube HS, Singh US and Mukhopadhyay AN (Prentice Hall, Englewood Cliffs, New Jersey) 3 1-31.
- Hargwig J and Scott PM (1971). Brine Shrimp (*Artemia salina* L.) Larvae as a Screening System for Fungal Toxins. *Applied Microbiology* 21(6) 1011-1016.

### Research Article

**Hasan HAH (2013).** Patulin and aflatoxin in brown rot lesion of apple fruits and their regulation. *World Journal of Microbiology and Biotechnology* **26**(7) 607-612.

**IARC (1986).** Some naturally occurring and synthetic food components, furocoumarins and ultraviolet radiation. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (IARC: Lyon, France) **40** 83-98.

**Joshi SG, Schaart JG, Groenwold R, Jacobsen E, Schouten HJ and Krens FA (2011).** Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. *Plant Molecular Biology* **75** 579–591.

**MacHardy WE (1996).** *Apple Scab: Biology, Epidemiology and Management* (The American Phytopathological Society Press, St. Paul) 546.

**MacHardy WE, Gadoury DM and Gessler C (2001).** Parasitic and biological fitness of *Venturia inaequalis*: relationship to disease management strategies. *Plant Diseases* **85** 1036-1051.

**Ogugua SE, Kehindeb AJ, James BI and Pauld DK (2012).** Assessment of cytotoxic effects of methanol extract of *Calliandra portoricensis* using brine shrimp (*artemia salina*) lethality bioassay. *Global Journal of Biosciences and Bio-Technology* **1**(2) 257-260.

**Van Egmond HP (1989).** Current situation on regulations for mycotoxins. Overview of tolerances and status of standard methods of sampling and analysis. *Food Additives & Contaminants* **6** 139-188.

**Verma LR and Sharma RC (1999).** *Diseases of Horticultural Crops: Fruits* (Indus Publishing Company, New Delhi) 718.