THE EFFECT OF SOME FUNGICIDES AND TOTAL SAPONIN ON SELECTED CHEMICAL COMPONENTS OF CERTAIN FUNGI

WAHEID, M. A.,[1] S. SH. RAMSES [2] AND RANIA A. ABDOU[2]

1. *Biochemistry Dept., Fac. of Agric., Ain Shams University*
2. *Central Agricultural Pesticide Laboratory, ARC, Dokki, Giza*

(Manuscript received 31 May 2009)

Abstract

The present study aimed to evaluate the effect of total saponin (TS) extracted from mulberry root bark and some fungicides using EC_{50} for each on the chemical components (total soluble sugars, free amino acids or total protein) and some enzymes (amylase, peroxidase, catalase, polyphenol oxidase (PPO), protease, polygalacturonase (PG), polymethyl esterase (PME) and cellulase of certain fungi i.e. *Al. alternata, B. cinerea, F. oxysporum, R. solani* and *S. rolfsii.* Electrophoresis studies were carried out on selected fungi grown in liquid media treated with total saponin at EC_{50} comparing with untreated media. Results concluded that, TS and carboxin fungicide decreased the biochemical components *F. oxysporum, R. solani* and *S. rolfsii.* Thiophanate-methyl and TS decreased total soluble sugars, free amino acids and total protein. The inhibition percentage of free amino acids and total protein in *B. cinerea* treated with thiophanate-methyl were 37.0 and 43.1, respectively, compared with the untreated controls. Amylase activity produced by *B. cinerea* treated with TS or thiophanate-methyl was decreased comparing with the untreated control. PPO activity was completely inhibited in *B. cinerea* when liquid media was treated with thiophanate-methyl. The reduction of catalase and protease activities was recorded in *B. cinerea* treated with TS or thiophanate-methyl and compared with the untreated control. Thiophanate-methyl induced a significant ($p<0.01$) decrease in peroxidase activity produced by *B. cinerea* comparing with the untreatment, while the treatment with TS induced non significant decrease in peroxidase activity compared with the untreated control. Adding TS to liquid medium was more effective than thiophanate-methyl in reducing cellulase and PG activities produced by *B. cinerea.* Also, the treatment of liquid media with TS or thiophanate-methyl caused highly inhibition in PME activity, which reached 62.3 and 65.4%, respectively. The reduction of free amino acids and total protein were observed in *Al. alternata* treated with TS or copper sulfate, which recorded 24.3, 32.0% and 29.9, 46.2%, respectively, of that produced in untreated media. Amylase activity produced by *Al. alternata* was completely inhibited other treatment with TS, while copper sulfate caused significant ($p<0.001$) decrease in this enzyme compared to the untreated control. In contract, TS expressed non significant decrease in PPO activity, but, copper sulfate expressed a significant ($p<0.01$) decrease in PPO activity produced by *Al. alternata.* TS and copper sulfate caused greatly decrease and significant ($p<0.01$) protease activity comparing with the untreated control. The inhibition of protease activity for treated *Al. alternata* recorded 48.8 and 53.5%, respectively. TS induced non significant decrease in preoxidase

activity, while, copper sulfate caused a low significant ($p<0.05$) decrease in the enzyme activity produced by *Al. alternata* compared to the untreatment. In contract, TS was more effective than copper sulfate on the reduction of cellulase and PG activities. The electrophoresis studies showed that *S. rolfsii* was more sensitive to TS comparing with the other selected fungi.

INTRODUCTION

Saponins are widely distributed in nature, being present in more than 1730 plant species belonging to 104 families. Of these species 627 were found to contain triterpenoid saponins and 127 to contain steroidal saponins i.e. triterpenoidal saponins are most abundant in plant kingdom. The pentacyclic triterpenoid saponins are of rare occurrence in monocot-yledons. They are more frequent in dicotyledons, being abundant in Caryophyllaceae, Sapindaceae, Polygolaceae, Sapotaceae and of common occurrence in Phytolaccaceae, Zygophyllaceae, Oleaceae, Papaveraceae, Araliaceae, Linaceae, Rutaceae (Fenwick *et al.*, 1991).

Until few years it was customary to classify the pentacyclic triterpenoids, according to Waffo-Teguo *et al.*,(2004), into three groups:-

1- Oleanane, β-amyrin or oleanolic acid group.

2- Ursane, α-amyrin or ursolic acid group.

3- Lupane or lupeol-betulic acid group.

Several biological effects have been ascribed to saponins. Extensive research has been carried out into the membrane-permeability, immunostimulant, hypocholesterolaemic, antimicrobial, hepatoprotective, antiviral and anticarcenogenic properties of saponins and they have also been found to significantly affect growth, feed intake and reproduction in animals. These components can thus affect animals in a host of different ways both positive and negative.

MATERIALS AND METHODS

Total Saponin

Total saponin was prepared according to Ukpabi and Ukpabi, (2003). Root bark of mulberry was air-dried and ground to fine powder. The powder was soaked in petroleum ether (40-60°C) for 24 hours to remove fats. The defatted powder was extracted with 50% aqueous methanol till exhaustion. The methanolic extract was re-extracted whit *n*-butanol several times. The butanolic extract was evaporated till dryness. The residue was dissolved in small amount of alcohol. Then, the total saponin precipitated by addition of large amount of acetone (1:5 v/v).

Fungal strains

Antifungal activity of total saponin and fungicides (shown in Table 1) was investigated against *Alternaria alternata, Botrytis cinerea, Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii.* All the tested fungal strains were maintained on Potato Dextrose Agar (PDA) medium (Oxiod CM 139) and were subcultured every two weeks.

Table 1. Trade, common and chemical names and active ingredient of tested fungicides.

Traditional name	Common name & Active ingredient	Chemical name	Formulation
Del cup 6%	Copper sulfate (23.5%)	Copper sulfate pentahydrate	Liquid
Topsin-M 70%	Thiophanate-methyl (70%)	Dimethyle(1,2phenylene) bis (iminocarbono-thioye) bis cabonate	Waterable powder
Vitavax-T	Carboxin (75%)	5,6-dihydro-2-methyl-N-phenyl-1,4-oxathiin-3-carboxyamide-bis (dimethyl thio-carbamayl) disulfide	Waterable powder

Methods:

Laboratory experiments

Effect of the tested fungicides and total saponin (TS) at EC_{50} on chemical components and enzymes produced by pathogenic fungi was studied.

In this experiment 2ml of total saponin or tested fungicides at EC_{50} Table (2) were added to 100ml of sterilized PD medium inoculated with 4 discs (5mm) of any tested fungi and incubated at (25±2°C). Five flasks were used for each particular treatment as replicates. When the mycelial growth covered the surface media in untreated flask (control), the mycelial matrix was excluded by filtration and dried at the room temperature over night. The dry mycelial mates were homogenated and chemical determinations were carried out.

***Table 2. Effective concentration inhibited 50% (EC_{50}) of linear growth of each fungus**

Fungi	EC_{50} in mm	
	Carbixn	Total saponin
R. solani	5.02	1056.9
S. rolfsii	1.30	118.9
F. oxysporum	10.01	2160.26
	Thiophenate-methyl	Total saponin
B. cinerea	0.89	938.1
	Copper-sulfate	Total saponin
Al. alternata	182.81	2000.0

*previous study by Sami and Rania

Chemical determinations of fungi

Determination of total soluble sugars.

Total soluble sugars of fungi were determined according to Shaffer-Somogi micro method described in **A.O.A.C. (1995).**

Quantitative assay of free amino acids as lysine

Samples were prepared by extraction 0.5g of each fungus by 25ml ethanol 80% **(Jayaraman, 1985)**. A standard solution of lysine was prepared by dissolving 0.02g lysine in 100ml of 80% ethanol. The color developed was measured using a spectrophotometer at wave length 570nm. The concentrations of free amino acids were calculated as lysine from the standard curve Fig.(1).

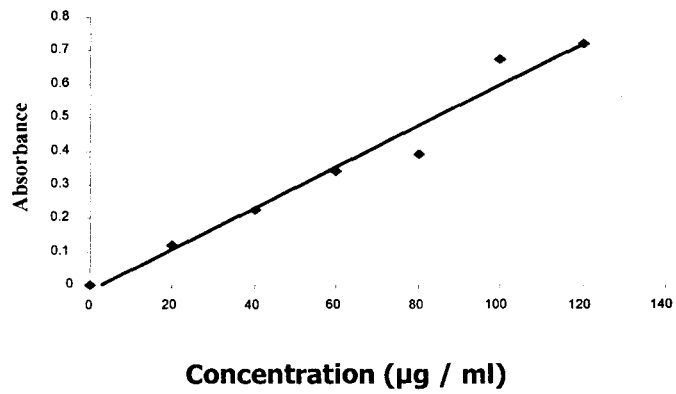

Fig. 1. Standard curve of free amino acids determined colorimetrically as lysine at 570nm.

Determination of total protein.

A ratio of 1:2.5 (w/v) of each fungus to extraction buffer (0.125M Tris-borate, pH 8.9) was used. The soluble protein concentration was spectroscopically determined by referring to a calibration curve relating the concentration of authentically albumin bovine at 546nm (Fig.2) according to Lowery *et al.,* (1951).

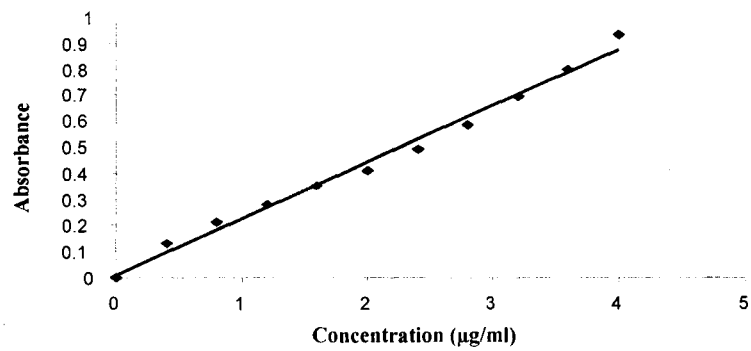

Fig. 2.) Standard curve of albumin bovine determined colorimetrically at 546 nm.

Determination of total amylase activity.

One gram of each fungus was homogenized in mortar with 4ml 0.01M Tris-HCl buffer pH 8.0 containing 0.02M NaCl and $CaCl_2$. The supernatant was used for total amylase activity according to the method described by Dewez *et al.,* (2005). The total amylase activity is expressed as mg starch consumed/ 15min /1g fungus (Fig.3).

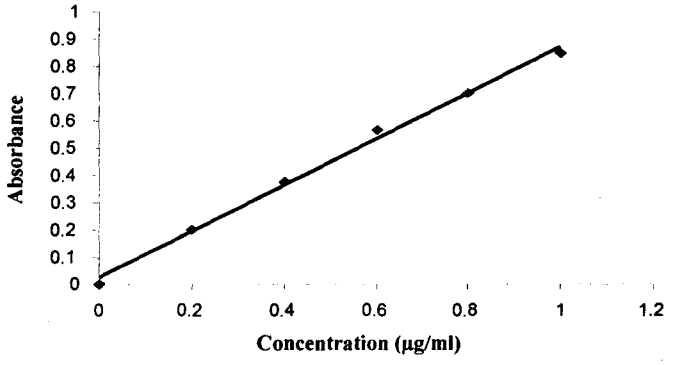

Fig. 3. Standard curve of starch determined colorimetrically at 660 nm .

Determination of peroxidase activity.

One gram of each fungus was extracted two times with 0.1M potassium phosphate buffer pH 4.7 containing 0.25M sucrose. The colorimetric assay of the total peroxidase in extract was conducted as described by Sreenivasulu *et al.,* (1999). The peroxidase activity was expressed in a unit of the increase of absorption at 470nm for 5min. per g fungus, designated as Δ O.D. 470nm/5min/g fungus.

Determination of catalase activity.

One gram of each fungus was homogenized two times with 0.01M phosphate buffer (pH 7.0). Catalase activity was determined according to the method described by Dewez *et al.,* (2005). The enzyme activity was determined as the change in absorbance at 240nm per one gram fungus / 1min.

Determination of polyphenol oxidase (PPO) activity.

One gram of each fungus was extracted with citrate-phosphate buffer (pH 6.2) in ratio 1:2 (w/v). Enzyme assay was accomplished according to method described by Dewez *et al.,* (2005). Results were expressed in units of PPO. One unit of PPO activity is defined as an increase of 0.1 unit absorbance per min at 420nm.

Determination of protease activity.

Protease activity was determined by method described by Dewez *et al.,* (2005). The blue color developed was determined after 5min. at 625nm.

Determination of polygalacturonase (PG) activity.

PG activity was assayed by estimating the loss in viscosity of 1.2% aqueous high methoxy pectin at 37°C by the method described by Echandi *et al.,* (1957).

Determination of poly methyl esterase (PME) activity.

PME activity was determined by the titration method described by Matta and Dimond, (1963).

Determination of cellulase activity.

Cellulase activity was determined by measuring the loss in viscosity of carboxymethyl cellulase (CMC) solution at 37°C. The method described by Matta and Dimond, (1963).

Electrophoretic studies.

Electrophoresis of fungal filtrates (untreated and treated with total saponin) of soluble proteins was done. After incubation period of all pervious fungi which was grown on PD medium, the mycelial mats were separated using cheesecloth and ground in liquid nitrogen to a fine powder. The fungal powder (0.5g) was mixed with 5ml of extraction buffer (0.05M Tris-HCl pH 7.5, 5% glycerol, 0.1% β-mercaptoethanol). The samples were centrifuged at 10000rpm for 45min., at 4°C and the supernatant was collected. Fifty microlitter of protein fraction was added to the same volume of SDS sample buffer pH 6.8 (Laemmli, 1970) in eppendorf tube and boiled in water bath for 2min.

Electrophoresis was performed with a SDS-PAGE system (Laemmli, 1970) using 15.0% resolving gel pH 8.8, 3.9% stacking gel pH 6.8 and carried out at 150V at 10°C. After Electrophoresis, the gel was stained overnight in a general protein stain (Coomassie Brilliant Blue). It was distained in a solution of 50% methanol and 7% acetic acid in water and photographed.

Approximate molecular weights of protein bands (kDa), were calculated according to standard proteins with known molecular weights and Rm values of the main protein bands were calculated according to the following formula:-

$$Rm = \frac{\text{Distance of band from the origin}}{\text{Distance of bromophenol blue from the origin}}$$

RESULTS AND DISCUSSION

Effect of total saponin (TS) and carboxin on the biochemical components produced by *F. oxysporum, R. solani* and *S. rolfsii*.

Tables (3), (4) and (5) showed the effect of the treatment liquid PD media with TS or carboxin fungicide at EC_{50} of each on total soluble sugars, free amino acids, total protein and enzyme activities produced by *F. oxysporum, R. solani* and *S. rolfsii*.

Results in Tables (3), (4) and (5) proved that, the PD liquid media treated with TS or carboxin induced significant decrease in total soluble sugars for *F. oxysporum* and *R. solani* comparing with the untreated media. Such effect was highly pronounced in case of treated *R. solani* with TS or carboxin, which reached 50 and 25%, respectively of that recoded in the untreatment. No significant decreases in total soluble sugars for *S. rolfsii* were observed when the liquid media was treated with TS or carboxin at EC_{50}, respectively.

Data also showed that, the reduction of free amino acids for the three fungi was observed due to the treatment of PD medium with TS or carboxin. Such effect was significant ($p>0.001$) and pronounced in case of *S. rolfsii* was treated with carboxin comparing with untreated media reached 9.4±1.3 and 19.4±1.6mg/1g fungus, respectively. The reduction percentages of free amino acids for *F. oxysporum, R. solani* and *S. rolfsii* were (34.7, 32.1), (31.7, 22.0) and (51.5, 33.5) when liquid media was treated with carboxin or TS, respectively.

The same results trend was found with the inhibition of total protein for the three fungi. The inhibition of total protein was significant ($p<0.001$) and pronounced when *S. rolfsii* was treated with TS or carboxin, reaching 41.1 and 62.5%, respectively, compared to the untreated control. These results were similar to that reported by Lalitha and Venkataraman, (1991). They suggested that, the antifungal activity of saponins isolated from *Madhuca butyracea* seeds on *Pythium sp., R. solani, S. rolfsii* and *F. oxysporum,* may be due to reduction of biochemical components such as free amino acids and total protein which play role in growth and pathogenicity of their fungi.

Amylase activity in *S. rolfsii* and *F. oxysporum* was sharply reduced due to the treatment with TS (0.8±0.11 and 0.28±0.03) and carboxin (0.8±0.01 and 0.56±0.2) comparing with untreatment (2.6±0.31 and 2.04±0.1mg starch/1g fungus), respectively. Such effect was more noticeable in *F. oxyspourum* treated with TS and carboxin which equal 86.2 and 72.5% of that observed in untreated. The lowest effect was observed in case of *R. solani* treated with TS or carboxin which recorded 20.7 and 35.9%, respectively, of that obtained in untreated.

On the other hand, the reduction of polyphenol oxidase (PPO) activity was non significant in *S. rolfsii* and *R. solani* when the liquid media was treated with TS. PPO activity decreased significant in *S. rolfsii* and *R. solani* when it was treated with carboxin reaching 38.9 and 36.6% comparing with untreated control, while, PPO activity was not detected in *F. oxysporum.*

Data in Tables (3), (4) and (5) indicated that, the treatment with TS or carboxin exerted non significant decrease in catalase activity for *F. oxysporum.* While, it caused significant decrease in the enzyme activity for *S. rolfsii* and *R. solani,* respectively. The reduction of catalase activity was more pronounced in *R. solani* treated with TS or carboxin reaching 51.0 and 35.0%, respectively, of that obtained in untreated PD media.

Protease activity for *F. oxysporum* and *R. solani* was greatly inhibited result of TS or carboxin treatment at EC_{50}. Such effect was significant ($p<0.001$) carboxin treatment. The reduction of the enzyme activity in *F. oxysporum* and *R. solani* treated with carboxin was pronounced being 74.7 and 59.4%, respectively, compared with the untreated control, but it was not detected in case of *S. rolfsii.*

The treated PD liquid media with TS at EC_{50} for the three fungi induced non significant decrease in peroxidase activity compared with the untreated control. While, the treatment with carboxin expressed significant ($p<0.001$) decrease in the enzyme activity produced by these fungi. Such effect was greatly noticeable in *S. rolfsii* which recorded completely inhibition in peroxidase activity due to the treatment with carboxin. While, the reduction of the enzyme activity for *R. solani* and *F. oxysporum* treated with carboxin was 38.2 and 29.0%, respectively, compared with the untreated medium.

Contrarly, of that, the treatment with TS on the three fungi was more affective than carboxin on cellulase and polygalacturonase (PG) activities. The reduction of cellulase and PG activities were highly significant ($p<0.001$) in case of *S. rolfsii* (3.0±0.02 and 7.0±0.8) or *R. solani* (30.0±1.3 and 25.0±2.0) treated with TS comparing with the untreated control which equal (13.0±2.4 and 10.0±1.6) or (54.0±2.3 and 40.0±1.7 RA units/1ml), respectively. *F. oxysporum* treated with TS at EC_{50}, showed significant decrease ($p<0.05$) in cellulase and PG activities compared with the untreated control reaching (24.0±1.8, 29.2±2.6) and (30.0±2.1, 35.0±3.2 RA units/1ml), respectively. Cellulase activity in *S. rolfsii* treated with TS was greatly, being 76.9% of the untreated control.

On the other hand, the treatment with carboxin to *R. solani* and *F. oxysporum* caused non significant decrease in cellulase and PG activities comparing with the untreated control. *S. rolfsii* treated with carboxin showed significant reduction of

cellulase and PG activities comparing with the untreated control. The inhibition of cellulase and PG activities in *S. rolfsii* treated with carboxin were pronounced reaching 60.8 and 20.0%, respectively, of that detected in the untreated control, (Tables 3, 4 and 5).

Table 3. Effect of total saponin (A) and carboxin (B) at (EC_{50}) on total soluble sugars, free amino acids, total protein and enzymes activities produced by *R. solani*

Parameters	*Untreated*	*(A)-treated*	(B)-treated
Total soluble sugars (g/1g fungus)	0.4±0.001	0.02±0.001***	0.03±0.001*
%	0.0	50.0	25.0
Free amino acids (mg/1g fungus)	4.1±0.1	3.2±0.2**	2.8±0.1**
%	0.0	22.0	31.7
Total protein (mg/1g fungus)	11.0±1.3	8.5±1.5*	7.0±0.8**
%	0.0	22.7	63.4
Amylase activity (mg starch /1g)	14.5±1.5	11.5±1.1*	9.3±1.3*
%	0.0	20.7	35.9
PPO activity (O.D. /5min)	0.737±0.1	0.697±0.1	0.467±0.09*
%	0.0	5.4	36.6
Catalase activity (O.D. /1min)	0.617±0.08	0.401±0.01**	0.532±0.02*
%	0.0	51.0	35.0
Protease activity (O.D. /5min)	0.704±0.2	0.365±0.1**	0.286±0.01**
%	0.0	48.2	59.4
Peroxidase activity (O.D. /5min)	0.432±0.1	0.401±0.02	0.267±0.01**
%	0.0	7.2	38.2
Cellulase activity (RA units /1ml)	54.0±2.3	30.0±1.3***	40.0±2.5*
%	0.0	44.4	25.9
PG activity (RA units /1ml)	40.0±1.7	25.0±2.0***	34.0±3.5
%	0.0	37.5	15.0
PME activity (meq acid /1ml/ hr)	0.36±0.04	0.24±0.01***	0.20±0.01***
%	0.0	33.3	44.4

Results are expressed as mean ± SE for 4 replicates in each group.

%: the percentage of inhibition.

*significant $p<0.05$ **$p<0.01$ ***$p<0.001$

The treatment with TS or carboxin at EC_{50} in the liquid medium induced significant decrease in pectin methyl esterase (PME) activity in the three fungi except *F. oxysporum* treated with TS. In general, carboxin fungicide was more effective than TS in reducing the PME activity in the three fungi. Such effect was highly significant ($p<0.001$) and pronounced in *R. solani,* followed by *F. oxysporum* and *S. rolfsii,* which

the inhibition percentage equal to 44.4. 26.1 and 23.6, respectively, of that recorded in untreated

R. solani, S. rolfsii and *F. oxysporum* produces PG, cellulase and PME adaptively. A high correlation exists between the ability of *R. solani* to produce large quantities of PG *in vitro* and their pathogenicity. PG was primarily responsible for tissue maceration. Whereas, cellulase seemed to play a secondary role in the process. There was no consistent relation between PME activity and tissue maceration (Bateman, 1993).

Table 4. Effect of total saponin (A) and carboxin (B) at (EC_{50}) on total soluble sugars, free amino acids, total protein and enzymes activities produced by *S. rolfsii*

Parameters	*Untreated*	*(A)-treated*	(B)-treated
Total soluble sugars (g/1g fungus)	0.05±0.001	0.048±0.01	0.05±0.01
%	0.0	4.0	0.0
Free amino acids (mg/1g fungus)	19.4±1.6	12.9±2.0*	9.4±1.3***
%	0.0	33.5	51.5
Total protein (mg/1g fungus)	5.6±0.8	3.3±0.5***	2.1±0.1***
%	0.0	41.1	62.5
Amylase activity (mg starch /1g)	2.6±0.31	0.8±0.11**	0.8±0.1**
%	0.0	66.4	66.4
PPO activity (O.D. /5min)	0.656±0.1	0.532±0.1	0.401±0.01**
%	0.0	18.9	38.9
Catalase activity (O.D. /1min)	0.617±0.01	0.510±0.01*	0.500±0.02*
%	0.0	17.3	19.0
Protease activity (O.D. /5min)	n.d.	n.d.	n.d.
Peroxidase activity (O.D. /5min)	0.723±0.2	0.548±0.01**	n.d.
%	0.0	24.2	100
Cellulase activity (RA units /1ml)	13.0±2.4	3.0±0.02***	5.1±0.1**
%	0.0	76.9	60.8
PG activity (RA units /1ml)	10.0±1.6	7.0±0.8***	8.0±1.1*
%	0.0	30.0	20.0
PME activity (meq acid /1ml/ hr)	0.55±0.02	0.42±0.01**	0.47±0.01*
%	0.0	14.5	23.6

Results are expressed as mean ± SE for 4 replicates in each group.

%: the percentage of inhibition. n.d: not detected

*significant $p<0.05$ **$p<0.01$ ***$p<0.001$

Table 5. Effect of total saponin (A) and carboxin (B) at (EC_{50}) on total soluble sugars, free amino acids, total protein and enzymes activities produced by *F. oxysporum*

PARAMETERS	UNTREATED	(A)-TREATED	(B)-TREATED
Total soluble sugars (g/1g fungus)	0.018±0.001	0.014±0.001*	0.015±0.001*
%	0.0	22.2	16.7
Free amino acids (mg/1g fungus)	47.6±3.5	32.3±0.15**	31.1±1.3**
%	0.0	32.1	34.7
Total protein (mg/1g fungus)	4.8±1.1	3.3±0.5*	3.0±0.8**
%	0.0	33.3	37.5
Amylase activity (mg starch /1g)	2.04±0.1	0.28±0.03**	0.56±0.2*
%	0.0	86.2	72.5
PPO activity (O.D. /5min)	n.d.	n.d.	n.d.
Catalase activity (O.D. /1min)	0.732±0.01	0.701±0.01	0.691±0.02*
%	0.0	4.2	5.6
Protease activity (O.D. /5min)	0.407±0.03	0.192±0.01**	0.103±0.01**
%	0.0	52.8	74.7
Peroxidase activity (O.D. /5min)	0.334±0.002	0.313±0.001	0.237±0.01**
%	0.0	6.3	29.0
Cellulase activity (RA units /1ml)	30.0±2.1	24.0±1.8*	28.0±1.9
%	0.0	20.0	6.7
PG activity (RA units /1ml)	35.0±3.2	29.2±2.6*	33.0±2.6
%	0.0	17.1	5.7
PME activity (meq acid /1ml/ hr)	0.23±0.01	0.21±0.001	0.17±0.001**
%	0.0	8.7	26.1

Results are expressed as mean ± SE for 4 replicates in each group.
%: the percentage of inhibition. n.d: not detected
*significant $p<0.05$ **$p<0.01$

From these results we can conclude that TS or carboxin fungicide may inhibit the growth of the three fungi and pathogenicity by decreasing their biochemical components. This conclusion was in agreement with that reported by Muhsin *et al.,* (2000). They recommended using saponin extracted from garlic extract to inhibit enzyme production and activity in order to suppress fungal pathogenicity

The effect of TS and thiophanate-methyl fungicide on the biochemical components produced by *B. cinerea.*

Table (6) shows the effect of the treatment of liquid media with TS or thiophanate-methyl fungicide (EC_{50}) each on total soluble sugars, free amino acids, total protein and enzymes activities produced by *B. cinerea.*

The liquid medium PD treated with TS or thiophanate-methyl at EC_{50} caused significant decrease in total soluble sugars of *B. cinerea* comparing with untreatment. Such decrease was a significant ($p<0.01$) and pronounced in case of the treatment with TS comparing with untreatment which reached 0.04±0.001 and 0.061±0.001g/1g fungus, respectively. The inhibition of total soluble sugars in fungus treated with TS was 34.4%, of the untreated control.

Adding TS or thiophanate-methyl (EC_{50}) to liquid medium resulted a significant decrease in free amino acids and total protein for *B. cinerea.* This result was significant ($p<0.001$) and pronounced when the liquid media was treated with thiophanate-methyl comparing with untreatment. The inhibition percentage of free amino acids and total protein in *B. cinerea* treated with thiophanate-methyl were 37.0 and 43.1, respectively, comparing with untreatment, (Table 6).

Amylase activity in *B. cinerea* treated with TS or thiophanate-methyl showed significant ($p<0.05$) decrease comparing with the untreated control, reaching 0.69±0.01, 0.70±0.01 and 0.81±0.07mg starch /1g fungus, respectively. On the other hand, completely inhibition in PPO activity for *B. cinerea* occurred when liquid media was treated with thiophanate-methyl (EC_{50}), while, the treatment with TS expressed non significant decrease in PPO activity compared to the untreated control.

Results (Table 6) illustrated the reduction of catalase and protease activities in *B. cinerea* treated with TS or thiophanate-methyl comparing with the untreated control. Such effect was highly significant ($p<0.001$) and pronounced in the thiophanate-methyl treatment. The inhibition of catalase and protease activities reached 23.0 and 50.8%, of the untreated control respectively. Similarly, thiophanate-methyl (EC_{50}) induced significant ($p<0.01$) decrease in peroxidase activity produced by *B. cinerea* comparing with the untreated control which equal to 0.401±0.02 and 0.516±0.03 O.D/5min, respectively. The treatment with TS showed non significant decrease in peroxidase activity compared to untreated control.

These results were in agreement with that recorded by (Adrian *et al.,* 1997). They indicated that, Resveratrol, a stilbene produced by grapes (*Vitis spp.*), inhibited the spread of *B. cinerea* infection. Laccase produced by this fungus is assumed to detoxify resveratrol. In recent studies, it was shown that a specific laccase of *B.*

cinerea dose not detoxify resveratrol but converts it into compounds more toxic for the fungus (Schouten *et al.,* 2002).

Adding TS (EC_{50}) to liquid medium was more effective than thiophanate-methyl on the reduction of cellulase and PG activities produced by *B. cinerea.* The inhibition of cellulase and PG activities was 40.0 and 37.5%, respectively, of that obtained in the untreated control. Also, the treatment with TS or thiophanate-methyl at EC_{50} to liquid media caused highly inhibition in PME activity, reaching 62.3 and 65.4%, respectively, of that recorded in the untreated control. These results were similar to that reported by Muhsin *et al.,* (2000).

Table 6. Effect of total saponin (A) and thiophanate-methyl (B) (EC_{50}) on total soluble sugars, free amino acids, total protein and enzymes activities produced by *B. cinerea*

Parameters	Untreated	(A)-treated	(B)-treated
Total soluble sugars (g/1g fungus)	0.061±0.001	0.040±0.001*	0.55±0.001
%	0.0	34.4	9.8
Free amino acids (mg/1g fungus)	16.2±0.5	12.6±1.5**	10.2±1.0**
%	0.0	22.2	37.0
Total protein (mg/1g fungus)	10.2±1.5	8.6±0.5	5.8±0.4***
%	0.0	15.7	43.1
Amylase activity (mg starch /1g)	0.81±0.07	0.69±0.01*	0.70±0.01
%	0.0	14.8	13.6
PPO activity (O.D. /5min)	0.529±0.12	0.415±0.1	n.d.
%	0.0	21.6	100???
Catalase activity (O.D. /1min)	0.731±0.02	0.570±0.01*	0.563±0.01***
%	0.0	32.0	50.8
Protease activity (O.D. /5min)	0.309±0.2	0.207±0.1*	0.152±0.01***
%	0.0	48.2	59.4
Peroxidase activity (O.D. /5min)	0.516±0.03	0.500±0.01	0.401±0.02***
%	0.0	3.1	22.3
Cellulase activity (RA units /1ml)	40.0±4.2	24.0±3.5**	35.0±2.5
%	0.0	40.0	12.5
PG activity (RA units /1ml)	32.0±2.7	20.0±5.6**	32.0±2.6
%	0.0	37.5	0.0
PME activity (meq acid /1ml/ hr)	0.28±0.01	0.10±0.01***	0.097±0.01***
%	0.0	62.3	65.4

Results are expressed as mean ± SE for 4 replicates in each group.

%: the percentage of inhibition. n.d: not detected

*significant $p<0.05$ **$p<0.01$ ***$p<0.001$

Effect of total saponin (TS) and copper sulfate fungicide on the biochemical components produced by *Al. alternata.*

Table (7) illustrated that, the effect of the treatment on PD liquid media with TS and copper sulfate fungicide at EC_{50} of each on total soluble sugars, free amino acids, total protein and enzymes activities produced by *Al. alternata.*

TS and copper sulfate at EC_{50} induced significant decrease in total soluble sugars in *Al. alternata*. Such effect was highly significant ($p<0.001$) and noticeable in case of the treatment with TS, which reached 47.4% of the untreated control. As well as, the reduction of free amino acids and total protein were observed in *Al. alternata* treated with TS or copper sulfate, which recorded 24.3, 32.0% and 29.9, 46.2%, respectively, of that produced in untreated media.

Data in Table (7) proved that, amylase activity produced by *Al. alternata* was completely inhibited when it was treated with TS. Copper sulfate caused highly significant ($p<0.001$) decrease in this enzyme compared with the untreated control, reaching 0.36±0.01 and 0.96±0.03mg starch/ 1g fungus, respectively, (i.e. 62.5% of the untreated media). Contrarily, TS expressed non significant decrease in PPO activity, but, copper sulfate showed significant ($p<0.01$) decrease in PPO activity in *Al. alternata.*

A significant reduction of catalase activity in *Al. alternata* treated with TS or copper sulfated was found when TS was added to the media comparing with the untreated control being 0.513±0.01 and 0.801±0.11 O.D/1min., respectively. Additionally, TS and copper sulfate at EC_{50} caused significant reduction ($p<0.01$) in protease activity comparing with the untreated control. The inhibition of protease activity in *Al. alternata* treatment recorded 48.8 and 53.5%, respectively, of that detected in the untreatment, (Table 7).

TS induced non significant decrease in preoxidase activity, while, copper sulfate caused significant ($p<0.05$) decrease in this enzyme produced by *Al. alternata* compared to the untreated control, being 0.491±0.03 and 0.683±0.02 O.D/ 5min., respectively. Contrarily, the treatment of liquid media with TS at EC_{50} was more effective than copper sulfate on the reduction of cellulase and PG activities. The inhibition rates of cellulase and PG activities were 56.8 and 31.4% in *Al. alternata* treated with TS, comparing with 18.9 and 14.3%, respectively, of that recorded in untreatment, in copper sulfate treatment. PME activity was not detected in *Al. alternata.* These results were similar to that reported by Muhsin *et al.,* (2000).

Table 7. Effect of total saponin (A) and copper sulfate (B) at (EC_{50}) on total soluble sugars, free amino acids, total protein and enzymes activities produced by *Al. alternata.*

Parameters	Untreated	(A)-treated	(B)-treated
Total soluble sugars (g/1g fungus)	0.019±0.001	0.010±0.01***	0.015±0.001*
%	0.0	47.4	21.1
Free amino acids (mg/1g fungus)	21.4±1.8	16.2±2.0*	15.0±1.5**
%	0.0	24.3	29.9
Total protein (mg/1g fungus)	5.2±0.7	4.0±0.2*	2.8±0.1***
%	0.0	32.0	46.2
Amylase activity (mg starch /1g)	0.96±0.03	N.D.	0.36±0.01***
%	0.0	100???	62.5
PPO activity (O.D. /5min)	0.489±0.08	0.411±0.1	0.304±0.05**
%	0.0	16.0	38.0
Catalase activity (O.D. /1min)	0.801±0.11	0.513±0.01**	0.721±0.1
%	0.0	36.0	10.0
Protease activity (O.D. /5min)	0.619±0.17	0.317±0.08**	0.288±0.05**
%	0.0	48.8	53.5
Peroxidase activity (O.D. /5min)	0.683±0.02	0.600±0.05	0.491±0.03*
%	0.0	12.2	28.1
Cellulase activity (RA units /1ml)	37.0±4.2	16.0±5.8**	30.0±3.6
%	0.0	56.8	18.9
PG activity (RA units /1ml)	35.0±3.8	24.0±2.5**	30.0±5.3
%	0.0	31.4	14.3
PME activity (meq acid /1ml/ hr)	n.d.	n.d.	n.d.

Results are expressed as mean ± SE for 4 replicates in each group.

%: the percentage of inhibition. n.d: not detected

*significant $p<0.05$ **$p<0.01$ ***$p<0.001$

Electrophoresis study

Electrophoresis studies were carried out on selected fungi grown in liquid media treated with TS at EC_{50} comparing with the untreated media, (Table 8).

The electrophoretic protein pattern of *B. cinerea* treated with TS and untreatment was presented in lane 2 and 1, respectively. Results showed that, the intensity of the four bands with MW about 90.0, 66.0, 58.0 and 20.0kDa decreased in the treated *B. cinerea* comparing with the untreated control. The absence the band with MW 116.0kDa and the new synthesized band with MW 64.0kDa were observed

resulting from the treatment of the liquid media with TS comparing with the untreated control.

The electrophoretic protein pattern of *S. rolfsii* treated with TS and the untreatment is presented in lane 4 and 3, respectively. The results indicated that the intensity of the three bands with MW about 90.0, 66.0 and 45.0kDa decreased in the treated *S. rolfsii* comparing with untreated control. The absent bands with MW 116.0 and 97.0kDa with the new synthesized bands with MW 36.0 and 20.0kDa were detected due to the treatment of the liquid media with TS comparing with the untreated control.

In case of *R. solani,* the electrophoretic protein pattern of fungus treated with TS and the untreatment are presented in lane 6 and 5, respectively. Data in Table (8) showed that the intensity of the four bands with MW about 97.0, 36.0, 10.0 and 6.5kDa decreased in the treated *R. solani* comparing with the untreated control. While, absence bands with MW 90.0, 84.0, 66.0, 64.0, 49.0 and 14.2kDa were observed resulting from the treatment of the liquid media with TS comparing with the untreated control.

The electrophoretic protein pattern of *F. oxysporum* treated with TS and the untreated is presented in lane 8 and 7, respectively. The results demonstrated that the intensity band with MW about 116.0kDa decreased in the treatment comparing with the untreated control. The increasing in intensity of bands with MW of about 64.0 and 58.0kDa with the new synthesized band with MW 20.0kDa observed as a result of the treatment of the liquid media with TS comparing with the untreated control (Table 8).

Finally, the electrophretic protein pattern of *Al. alternata* treated with TS and untreated was presented in lane 10 and 9, respectively. These results indicated that there was no change in protein pattern of *Al. alternata* when liquid media was treated with TS at EC_{50} comparing with untreated medium.

From these results it is concluded that *S. rolfsii* was more sensitive to TS comparing with the other selected fungi.

Table 8. SDS-PAGE patterns of soluble protein fraction extracted from selected fungi treated with TS (T) and untreated media (C).

M.W. (kDa)	M	*B. cinerea*		*S. rolfsii*		*R. solani*		*F. oxysporum*		*Al. alternata*	
		C	T	C	T	C	T	C	T	C	T
205.0	---									---	---
116.0	---	---		---				+++	---	---	---
97.0	---			---		+++	---	---	---	---	---
90.0		+++	---	+++	---	---			---		
84.0	---	---	---			---		---	---		
66.0	---	+++	---	+++	---	---					
64.0			---			---		---	+++	---	---
58.0		+++	---					---	+++		
55.0	---	---	---			---	---			---	---
49.0						---		---	---		
45.0	---			+++	---						
36.0	---				---	+++	---	---	---		
30.0	---										
24.0	---										
20.0	---	+++	---		---	---	---		---	---	---
14.2	---					---					
10.0						+++	---	---	---		
6.5	---					+++	---	---	---		

+++: increase in intensity when compared C with T both of fungus.

The study showed that the treatment of the fungi with saponin extract caused decrease in the amount of the secreted enzymes (amaylase, PPO, catalase, cellulase, peroxidase and PG) of the fungi compared with the untreated control of the extract which may be related to inhibition of mycilium growth of these fungi.

REFERENCES

1. A.O.A.C. 1995. Official methods of analysis of the association of official analytical chemists.15th ED. Published by the association of official analytical chemists. INC. suite 400,200 Wilson Baulevard. Arligton Virgina 22201 USA., pp.69-90.
2. Adrian, M., P. Jeandet, J. Veneau, L. Weston and R. Bessis. 1997. Biological activity of resveratrol, a stilbenic compound from grapevines, against *Botrytis cinerea* the causal agent for gray mold. J. Chem. Ecol.23:1689-1702.

3. Bateman, D.F. 1993. The "Macerating Enzyme" of *Rhizoctonia solani.* Phytopathol.14:1179-1185.
4. Dewez, D., L. Geoffroy, G. Vernet and R. Popovic. 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga *Scenedesmus obliquus.* Aquatic Toxicol. 74:150-159.
5. Echandi, E.S., D. Van-Gundy and J.C. Walker. 1957. Pectolytic enzymes secreted by soft rot bacteria. Phytopathol. 47:549-552.
6. Fenwick, G.R., K.R. Price, C. Tsukamoto and K. Okubo. 1991. Saponins in Toxic Substances in Crop Plants, (Dmello F.J.P., Duffus C.M. and J.H. Duffus, editors). Cambridge: The Royal Society of Chemistry. PP.361,362.
7. Jayaraman, J. 1985. Laboratory Manual in Biochemistry. PP.132-135. Wiley Eastern Limited. New Delhi, N.Y.
8. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature Lond. 227,680-685.
9. Lalitha, T. and L.V. Venkataraman. 1991. Antifungal activity and mode of action of saponins from *Madhuca butyracea.* Indian J. Experimental Biolo, 29(6):558-562.
10. Lowery, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.
11. Matta, A. and A.F. Dimond. 1963. Symptoms of *Fusarium sp.* in relation to quantity of fungus and enzyme activity in tomato stems. Phytopathol. 53:574-578.
12. Muhsin, T.M., S.R. Al-Zubaidy and E.T. Ali. 2000. Effect of garlic bulb extract on the growth and enzymatic activities of rhizosphere and rhizoplane fungi. Mycopathologia 152:143-146.
13. Schouten, A., L. Wagemarers, F.T. Stefanata, R.M.V.D. Kaaij and J.A.L.V. Kan. 2002. Resveratrol acts a natural pro fungicide and induces self-intoxication by a specific laccase. Mol. Microbio. 43:883-894.
14. Sreenivasulu, N., S. Ramanjulu, K. Ramachandra-Kini, H.S. Prakash and C. Sudhakar. 1999. Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet differential salt tolerance. Plant Sci. 141:1-9.
15. Ukpabi, U.H. and U.J. Ukpabi. 2003. Potential of seeds of *Napoleona imperialis* (*P.beauv*) as a source of hemolytic saponin and feed ingredients. Lifestock Research for Rural Development 15(12):103-112.
16. Waffo-Teguo, P., L. Voutquenne, O. Thoison, V. Dumontet, V.H. Nguyen and C. Lavaud. 2004. Acetylated glucuronide triterpene bidesmosidic saponins from *Symplocos glomerata.* Phytochem. 65: 741-750.

تأثير بعض المبيدات الفطرية والصابونين الكلي علي المحتوي الكيميائي لبعض الفطريات

وحيد محمد عفيفي[1] ، سامي شفيق رمسيس[2] ، رانيا عبده عبده حسين[2]

١. *قسم الكيمياء الحيوية ، كلية الزراعة ، جامعة عين شمس*

٢. *المعمل المركزي للمبيدات ، مركز البحوث الزراعية – الدقى – الجيزة*

تهدف هذه الدراسة الي قياس تأثير استخدام المبيدات الفطرية ومركب الصابونين الكلي المستخلص من قلف جذر شجرة التوت علي المحتوي الكيميائي للعديد من الفطريات الممرضة وذلك للوصول الي طريقة ومكان فعل هذه المركبات داخل الفطر حيث تم في هذه الدراسة استخدام المبيدات الفطرية كربوكسين و كبريتات النحاس وثيوفينات الميثيل وكذلك مركب الصابونين الكلي بالتركيز المثبط لنصف النمو الميسليومي (EC_{50}) للفطريات *Al. alternata, B. cinarea, F. oxysporum, R. solani* and *S. rolfsii* وذلك باضافة هذه المركبات كل علي حده الي بيئه انماء الفطريات موضع الدراسة مع وجود بيئة غبر معاملة بأى من هذه المركبات للمقارنة. تم تقدير كل من السكريات الذائبة الكلية والأحماض الأمينيه الحرة والبروتين الكلي وكذلك انزيمات الأميليز، البيروكسيديز، الكتاليز، البولي فينول أوكسديز، البروتيز، البولي جلاكتوييورونيز، البولي ميثيل استيريز والسليلوزالتي تنتجها الفطريات موضع الدراسة وقد أوضحت النتائج أن أضافة الصابونين الكلي أو المبيدات الفطرية الي البيئة السائلة أحدثت أنخفاض في السكريات الذائبة الكلية، البروتين الكلي، الأحماض الأمينية الحرة والأنزيمات التي تنتج بواسطة هذه الفطريات والتي لها دور في نموها وتسممها هذه الفطريات وقد لوحظ أن المبيدات الفطرية المختبرة لها تأثير واضح علي أنخفاض نشاط أنزيم البيروكسيديز والبولي فينول اوكسيدز بينما كان تأثير الصابونين الكلي واضح علي أنخفاض نشاط أنزيمات تحلل السليلوز، بولي جلاكتوييورونيز، بولي ميثيل أستريز المنتجة بواسطة الفطريات النامية علي بيئة معاملة مقارنة بالغير معاملة. أثبتت دراسة الألكتروفوريسيس أن أضافة الصابونين الكلي بالتركيز المثبط لـ ٥٠٪ من نمو الفطر الي البيئة السائلة أحدثت أنخفاض في كثافة بعض الحزم البروتينة وغياب بعضها في البروتينات المستخلصة من الفطريات النامية علي بيئة معاملة مقارنة بالغير معاملة وكانت هذه النتيجة أكثر وضوحا في حالة فطري *B. cinarea* و *S. rolfsii* .