IMPACT OF TWO PHENOLIC COMPOUNDS ON THE DIGESTIVE PHYSIOLOGY OF A NOCTUID HERBIVORE, *SPODOPTERA LITTORALIS* (BOISD.)

AMIN, T. R.[1], S. A. ABO-EL-ENEIN[2], AND TAKWA H. ELLAKWA[1]

1. *Plant- Protection Research Institute, ARC, Dokki, Giza, Egypt*
2. *Chemistry Dept., Faculty of Science, Ain-Shams University*

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Abstract

The ability of two benzoic acid derivatives; tannic and salicylic acids to affect digestive enzymes and their substrates was evaluated. Phenolic acids were incorporated into artificial diet at concentrations of $20X10^{-3}$ *M* through $160X\ 10^{-3}$ *M*. *In vivo* studies demonstrated that treatment of *Spodoptera littoralis* (Boisd.) 4th larval instar for 5 days with phenolic acids; significantly reduced growth, main metabolites and digestive enzymes. *In vitro* experiments indicated that phenolic acids not only had the ability to affect protein (casein), but also affected carbohydrates (sucrose) and their specific enzymes; protease and invertase, indicating their possible ability to get food less digestible. The observed reduction in weight gain could be attributed, at least in part, to the effect on digestion, but not excluding the presence of other additional mechanisms. We suggested that the oxidative stress of phenolic acids could affect digestive enzymes and their dietary substrates, which ultimately could reduce larval growth.

INTRODUCTION

Allelochemicals or allelochemics are non-nutrient compounds produced by one organism and affect another species (Whittakar, 1970). They occur in plant tissues as phenolics, polyphenols, flavonoids and tannins and appear to be involved in insect resistance of crop plants. Phenolics are considered as important components of both constitutive and induced defenses against herbivores and pathogens, by acting as antinutritive compounds affecting the growth and development of a variety of insects (Reese and Beck, 1976; Duffey, 1986; Abdel-Baky *et al.,* 2005).

The protective effects of phenolics are thought to be due to their oxidative stress. Plant chemicals contain quinones and phenolics that can oxidize and form toxic O-quinones and other reactive oxygen species (Hodnick *et al.,* 1989). Reactive products from phenolic oxidation can reduce the quality of dietary protein for insects by alkylating nucleophilic sites, decreasing lysing content, and causing protein (including enzymes) polymerization and fragmentation (Felton *et al.,* 1992), lipid peroxidation and nucleic acids oxidation (Summers and Felton, 1994), and damage to the midgut cells of the feeding insect (Ahmad, 1992; Bi and Felton, 1995). However, it is not clear from the previous literatures that phenolics can affect other dietary components such as carbohydrates. Felton *et al.* (1992) reported that the toxicity of

quinones may not be limited to interactions with proteins. Also, to the best of our knowledge, no one has studied the physiological effect of phenolic acids on the digestive enzymes of insects.

We chose the cotton leafworm, *Spodoptera littoralis* larvae as our experimental insect. It is highly polyphagous pest, so it exposed to variety of allelochemicals. Two benzoic acids derivatives; salicylic acid and tannic acid are common phenolics and known to occur in plant tissues as barely, were incorporated into the diets of the fourth larval instar, in an attempt to evaluate their effects. Our research probes to answer the following questions: 1) Do phenolics inhibit digestive enzymes of the larvae either *in vivo* or *in vitro*? 2) If they can significantly reduce total proteins, what is the cause? Is their ability to conjugate with dietary proteins or their ability to inhibit digestive enzymes or anything else? 3) Can these allelochemicals affect other nutritional compounds, besides dietary proteins?

MATERIALS AND METHODS

Insects and preparation of diets:

Neonates of *S. littoralis* are a laboratory breeding strain; they were reared on artificial diet of Shorey and Hale (1965). Two commercially available phenolic compounds were obtained (El-Nasr Pharmaceutical Chemicals Co., Egypt). They were salicylic acid and tannic acid. They were incorporated into the diets of the newly hatched fourth larval instar for five days at concentrations of $20X10^{-3}$ *M* through $160X10^{-3}$ *M*. The tested phenolics were relatively insoluble in water, and were first dissolved in acetone (500 mg phenolic acid/1 ml acetone). The control diets were received acetone alone, then stirred mechanically with the other ingredients of the diet. Each diet was replaced every day. All bioassay experiments were replicated 5 times with 10 larvae/replicate. The fresh weight and the number of survivors were daily recorded.

Preparation of larvae and main metabolites assays:

The larvae were homogenized in distilled water (5 larvae/5 ml). Homogenates were centrifuged at 8000 r.p.m. for 15 min at 5°C in a refrigerated centrifuge. The deposits were discarded and the supernatant was kept in a deep freezer till use. Total proteins were determined by the method of Bradford (1976). Total carbohydrates were extracted as described by Crompton and Birt (1967), and were determined by the phenol sulphuric acid method (Dubios *et al.,* 1956). Homogenization was done using a chilled glass Teflon tissue grinder, while centrifugation was carried out using a refrigerated centrifuge (GS-6r, Beckman, USA). The end products of the reactions were estimated using a spectrophotometer (Spectronic 1201, Milton Roy Co., USA).

Dissection of guts and digestive enzymes assays:

The larval guts were dissected by immersing larvae in isoosmotic saline (0.15 M Kcl, pH7). With the aid of sharp razor, the dorsal side of the body was longitudinally opened, exposing the alimentary canal, cutting it first slightly anterior to the oesophagous and again at the posterior end of the rectum. Then the guts were rinsed twice with saline. The guts were homogenized (5 guts/ 2 ml distilled water) as described before and the supernatant was analyzed for tryptic (protease) and sucrose (invertase) activity. For the general protease activity assay, casein was used as substrate as described by Birk *et al.* (1962), while invertase activity was determined using sucrose as the enzyme substrate (Ishaaya and Swiriski, 1976), and glucose resultant from digestion of sucrose was determined by the method of Barham and Trinder (1972).

Enzymes and substrates *in vitro* inhibition by phenolics:

Phenolic acids were first dissolved in acetone (0.5 gm/ml) and diluted by distilled water, preparing salicylic and tannic acid solutions (5 and 0.05%, respectively). The acetone concentration in the assays did not affect the enzyme activity, since enzyme activity determined in specimens containing the corresponding amount of acetone, were not differ from that containing ΔH_2O instead of the solvent. Levels of 10 and 100 µl of the phenolics were added to the gut enzyme-buffer solution and incubated at 37°C for 10 min prior to the initiation of the reaction.

Controls of the reaction received the same amount of phenolics, but without incubation, since it was observed that, especially in the protease reaction (read at an absorbancy of 280°), phenolics affect optical density of the produced color. Also, we chose 0.2 M glycine-NaOH buffer (pH 8) for protease activity. Pierpoint (1983) mentioned that the hydrogen bonding between phenolics and proteins (probably enzymes) do not form at pH greater than 8. The ability of phenolic acids to affect proteins (casein) or carbohydrates (sucrose), as substrates, *in vitro* was also evaluated following the same formentioned procedures.

All experiments were in 3-5 replicates. The values were shown as means ± standard deviations. Data were subjected to analysis of variance (ANOVA), and Duncan's multiple range test to differentiate between the means at $P< 0.05$.

RESULTS AND DISCUSSION

Although acute exposure to dietary phenolic acids does not constitute a serious challenge to the growth rates of *Helicoverpa zea,* a chronic exposure does result in significantly reduced growth (Summers and Felton, 1994). It is not known exactly concentrations of phenolic compounds in common host plants of *S. littoralis* larvae. However, phenolic acids may incorporated into diet at concentration of 37.5 mM as done by Reese and Beck (1976) on a lepidopteran species; *Agrotis ipsilon.* They

treated this insect chronically for 28 days. During the present work phenolics concentrations were raised up to 160 mM, to allow phenolics to exert their effect in a relatively short period (5 days).

Weight gain of the cotton leafworm was significantly reduced by the tested phenolics (Fig. 1). There were high significant correlation between weight gain, and both tannic and salicylic acid (r = -0.93 and -0.883, respectively). Salicylic acid reduced weight gain more than tannic acid. Another biological activity of these compounds is the effect on survival of feeding insects.

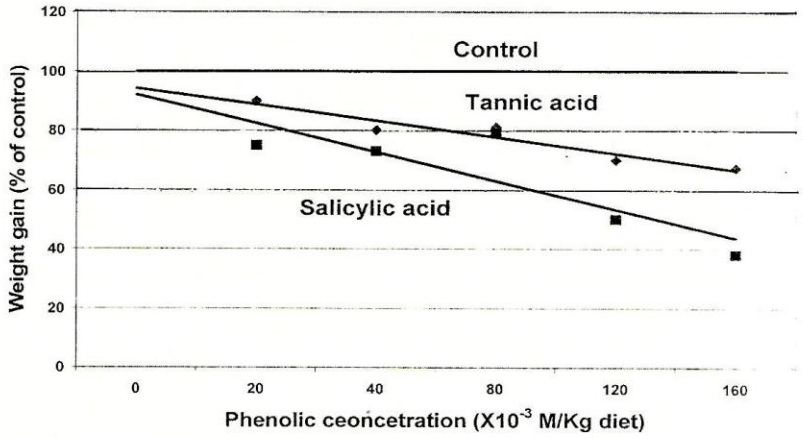

Fig. 1. Effect of phenolic acids on weight gain at 5 days of *S. littoralis* larvae.

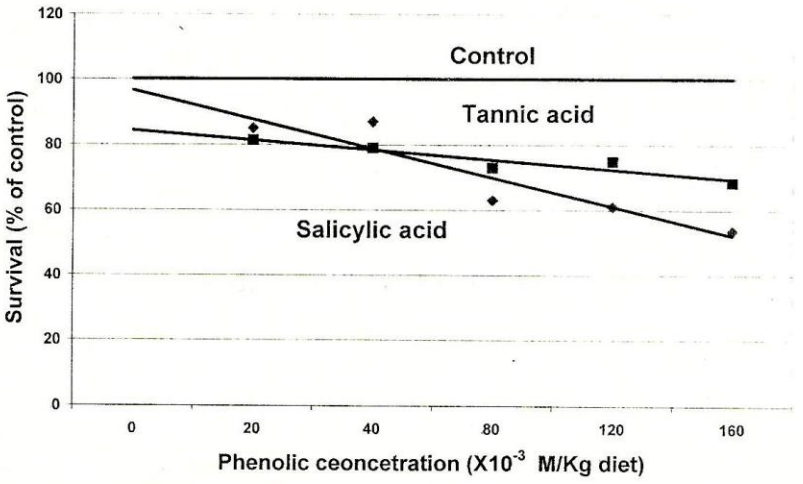

Fig. 2. Effect of phenolic acids on survival at 5 days of *S. littoralis* larvae.

Fig. 2 demonstrates that phenolics significantly suppress insect survival. Tannic acid increased larval mortality more than salicylic acid, but the mortality was not increased more than 46.15% as compared to control. The effect of phenols on survival and growth was observed in many insect species and *S. littoralis*. Total phenols in tomato leaflets were positively correlated with mortality of cotton leafworm (Antonious *et al.,* 1999). Treatment of cotton leafworm with plant leaves extracts containing tannins and phenolic compounds as the abundant extracted biocompounds, led to reduced weight of insects as compared with control insects fed on castor bean leaves (Hegazy *et al.,* 1992).

To attain an understanding of how phenolic acids affect weight gain, levels of two biochemical components i.e. total body proteins and carbohydrates were evaluated (Table, 1). The two components of larvae fed for 5 days on $80X10^{-3}M$ phenolic acids were significantly reduced as compared to control. The vice versa of total carbohydrates, the higher reduction of total proteins was observed for larvae fed salicylic acid than that fed tannic acid. Generally, total carbohydrates were more affected than total proteins.

The significant reduction of whole body proteins and carbohydrates necessitated the search of the cause of such depression. It was hypothesized that the used phenolics may inhibit dietary compounds (proteins and carbohydrates) or inhibits digestive enzymes. So it is of interest to determine, *in vitro,* a possible interaction with the digestive enzymes as proteins, and their substrates; casein and sucrose.

When phenolics were incubated with the protease or its substrate, there were significant inhibitions of the proteolytic activity (Table, 2). It was observed that tannic acid exerted its *in vitro* effects at relatively lower concentrations as compared to salicylic acid. The reaction mixture

Table 1. Total protein and carbohydrates for *S. littoralis* larvae exposed (5 days) to $80X10^{-3}M$ dietary phenolics.

Treatment	Total proteins (µg/larva)	Total carbohydrates (µg glucose/larva)
Control	2590 ± 10^{a}	847 ± 41.5^{a}
Tannic acid	2131 ± 12^{b}	314 ± 4.72^{b}
Salicylic acid	1824 ± 25^{c}	438 ± 30^{c}

Data represented as means± SD

Means in columns not followed by the same letter are significantly different at $P<0.05$.

Table 2. Effect of *in vitro* pre-incubation of phenolic acids with the enzyme protein or the substrate casein on the protease activity.

No. of µl of phenolic* solution in pre-incubation mixture	Pre-incubation mixture	Enzyme activity (O.D. units $X10^{-3}$/min/larva) mean±SD
0.00 (control)	-	95.6 ± 1.15^{a}
10 µl	Enzyme + Tannic acid	78.6 ± 2.25^{c}
	Casein + Tannic acid	66.9 ± 1.61^{e}
	Enzyme + Salicylic acid	75.63 ± 1.51^{d}
	Casein + Salicylic acid	81.56 ± 1.5^{b}
50 µl	Enzyme + Tannic acid	42.33 ± 2.0^{h}
	Casein + Tannic acid	40.0 ± 1.52^{h}
	Enzyme + Salicylic acid	56.66 ± 2.08^{g}
	Casein + Salicylic acid	60.33 ± 1.15^{f}

Means in columns not followed by the same letter are significantly different at $P<0.05$.

* The concentrations of tannic and salicylic acid solutions were 0.05 and 5%, respectively.

Table 3. Effect of *in vitro* pre-incubation of phenolic acids with the enzyme protein or the substrate sucrose on the invertase activity.

No. of µl of phenolic solution* in pre-incubation mixture	Pre-incubation mixture	Enzyme activity (as µg glucose/min/larva) mean±SD
0.00 (control)	-	66.6±2.08[a]
10 µl	Enzyme + Tannic acid	56.4±0.75[c]
	Sucrose + Tannic acid	61.9±1.7[b]
	Enzyme + Salicylic acid	60.4±0.52[b]
	Sucrose + Salicylic acid	60.9±1.01[b]
50 µl	Enzyme + Tannic acid	-
	Sucrose + Tannic acid	-
	Enzyme + Salicylic acid	35.6±1.21[e]
	Sucrose + Salicylic acid	39.6±1.52[d]

Means in columns not followed by the same letter are significantly different at $P<0.05$.
* The concentrations of tannic and salicylic acid solutions were 0.05 and 5%, respectively.

(1 ml) contained $2.87\mathbf{X10^{-3}}$ µM of tannic acid (10 µl), while I gm diet contained at least 20 µM. On the other hand, salicylic acid reaction mixture contained 3.6 µM (10 µl). Tannic acid might be more detoxified, *in vivo,* more than salicylic acid. The higher effect of tannic acid was on casein, while that of salicylic acid was on the enzyme protease. The inhibitory effects of both phenolics increased with the increase of the phenolic concentrations.

It was expected that invertase, as an enzyme composed of protein molecules, to be affected by phenolic acids, but it was not known the affinity to carbohydrates as sucrose. Table 3 demonstrates that pre-incubation of sucrose with tannic and salicylic acids led to inhibition of such substrate. However, the inhibition was lesser than that happened in invertase. The inhibition increased with the increase of salicylic acid concentration. It was not available to increase the concentration of tannic acid as done for salicylic acid, because the raising of its concentration significantly interfered with the reaction of glucose determination after hydrolysis of sucrose by the enzyme.

As shown in Tables (1&2), phenolic acids, *in vitro,* affected both proteins and carbohydrates, and their digestive enzymes, indicating their possible ability to get food less digestible. This emphasized by their *in vivo* reduction of total proteins and total carbohydrates, and their specific enzymes (Table, 4), where invertase activity showed more significant decrease than that of protease activity, specially in the case of tannic acid.

Phenolics may affect proteins as summarized by Felton *et al.* (1992) via 1) direct conjugation of specific amino acids (e.g. lysine) with phenolics, which result in the phenol-amine adduct becoming nutritionally unsuitable 2) binding of phenolic to

amino acids, which physically blocks the access of digestive enzymes to the protein substrate 3) protein precipitation 4) activated oxygen species (i.e. O^{-}_{2}, H_2O_2, OH),

Table 4. Digestive enzymes for *S. littoralis* larvae exposed (5 days) to $80X10^{-3}$M dietary phenolics.

Treatment	Protease activity (O.D. units $X10^{-3}$/min/larva)	Invertase activity (µg glucose/min/larva)
Control	94.28±2[a]	66.25±2.83[a]
Tannic acid	77.79±2.55[b]	33.83±3.40[b]
Salicylic acid	79.33±3.0[b]	51.3±1.52[c]

Data represented as means±SD

Means in columns not followed by the same letter are significantly different at $P<0.05$.

formed during oxidation of quinones and substituted phenols , which oxidize thiols and decarboxylate amino acids. Most reports have been discussed the affinity of phenolic acids to proteins (Reese and Beck, 1976; Ahmed, 1992; Summers and Felton, 1994).

In the present paper we throw light upon the antinutritive (less digestible) effect of phenolics on carbohydrates (sucrose). Indeed, no available study showed the mechanism of interaction of plant phenolics with carbohydrates that led them to be less digestible. However, oxidation of sugars by dehydrogenation in the presence of oxidants is well known (Nekrasov, 1978). The formation of O^{-}_{2} due to oxidation of phenolics, may lead to other reactive oxygen species and result in a direct oxidative challenge to carbohydrates.

The observed reduction in weight gain could be attributed, at least in part, to the effect on digestion after exposure to phenolic acids, but not excluding the prescence of other additional mechanisms. Reese and Beck (1976) found that *P*-benzoquinone inhibit ingestion in *A. ipsilon* larvae. Felton *et al.* (1992) reported that the reduced growth of *S. exigua* fed on plant phenolics is most likely attributable to the reduction in amino acid absorption and assimilation. This might be interpreted by the effect of phenolics on mid gut cells as reported by Ahmed (1992) due to a direct oxidative challenge to the digestive system of the actively feeding insect. The power of these compounds to regenerate oxidation effect seems to be of a significant value. Summers and Felton (1994) proposed that the toxicological effect of the O-dihydroxyphenolics, caffeic acid and chlorogenic acid, is due primarily to their ability to act as prooxidant. Finally it could be suggested that the oxidative stress of phenolic acids could affect digestive enzymes and their dietary substrates which ultimately could reduce larval growth.

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تأثير بعض الفينولات علي فسيولوجيا الهضم في يرقات دودة ورق القطن

طارق رئيس أمين[١]، صلاح أبو العينين[٢]، تقوي حامد اللقوة[١]

١. *معهد بحوث وقاية النباتات ، مركز البحوث الزراعية ، الدقي*
٢. *قسم الكيمياء ، كلية العلوم ، عين شمس*

حيث أن للفينولات القدرة علي إكساب المقاومة للنباتات ضد الإصابة بالآفات ، تم إختيار مركبين منها وهما حمض الساليسيلك وحمض التانيك علي العمر الرابع ليرقات دودة ورق القطن وذلك لمعرفة طريقة فعل هذه المركبات. وتم إضافة هذين الحمضين الي البيئة الغذائية لليرقات بتركيزات تتراوح بين ٢٠-١٦٠ ملليمول/كجم من وزن الغذاء. أوضحت النتائج أن المعاملة تؤثر علي نمو اليرقات مع إنخفاض المحتوي الكلي للبروتينات والكربوهيدرات. وبناء علي ذلك تم إفتراض تأثر عملية الهضم لما لهذه المركبات من القدرة علي إحداث الأكسدة داخل الجسم ولإثبات ذلك تم إجراء عدة تجارب داخل وخارج جسم الحشرة. وأظهرت النتائج أن هاتين المادتين ليس لديهما القدرة فقط علي تثبيط البروتينات (الكازين) ولكن أيضا علي تثبيط إنزيمات الهضم مثل البروتييز والأنفرتيز وبعض مواد التفاعل الأخري مثل الكربوهيدرات (السكروز). وأستنتج من ذلك أن نقص النمو نتيجة المعاملة بهذه المركبات يعزي علي الأقل جزئيا الي التأثير علي فسيولوجيا الهضم وذلك غير مستبعدا لوجود عوامل أخري مثل التأثير علي قدرة إبتلاع الطعام وعلي خلايا المعي المتوسط.

وبناء عليه يمكن القول أن قدرة هذه المواد علي إحداث الأكسدة داخل الجسم يؤثر علي إنزيمات الهضم ومكونات الطعام من بروتينات وكربوهيدرات الذي بدوره يؤثر في النهاية علي نمو اليرقات.