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

AMIN, T. R.¹, S. A. ABO-EL-ENEIN², AND TAKWA H. ELLAKWA³

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

(Manuscript received 31 August 2006)

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 160X10⁻³ 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 to 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 allelochemistry are non-nutrient compounds produced by one organism and affect another species (Whittaker, 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 (EI-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 $2 \times 10^{-3}$ M through $16 \times 10^{-3}$ 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 (Dubois *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 isosmotic saline (0.15 M KCl, pH 7). With the aid of a sharp razor, the dorsal side of the body was longitudinally opened, exposing the alimentary canal, cutting it first slightly anterior to the oesophagus 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 trypsin (protease) and sucrase (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 Swirski, 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 ΔH2O 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 \((r = -0.93 \text{ and } -0.883, \text{ respectively})\). 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.

![Diagram](image1)

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

![Diagram](image2)
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 80X10⁻³M 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 80X10⁻³M dietary phenolics.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total proteins (µg/larve)</th>
<th>Total carbohydrates (µg glucose/larve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>350±0.15*</td>
<td>87±0.15*</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>213±0.15*</td>
<td>134±0.15*</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>182±0.25*</td>
<td>49±0.25*</td>
</tr>
</tbody>
</table>

Data represented as mean±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.

<table>
<thead>
<tr>
<th>No. of µl of phenolic solution in pre-incubation mixture</th>
<th>Pre-Incubation mixture</th>
<th>Enzyme activity (D.O. units X10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.05 (control)</td>
<td>-</td>
<td>95±0.15*</td>
</tr>
<tr>
<td>10 µl</td>
<td>Enzyme + Tannic acid</td>
<td>78.6±4.25*</td>
</tr>
<tr>
<td></td>
<td>Cassein + Tannic acid</td>
<td>66.9±1.61*</td>
</tr>
<tr>
<td></td>
<td>Enzyme + Salicylic acid</td>
<td>75.63±1.51*</td>
</tr>
<tr>
<td></td>
<td>Cassein + Salicylic acid</td>
<td>81.56±1.5°</td>
</tr>
<tr>
<td>50 µl</td>
<td>Enzyme + Tannic acid</td>
<td>42.3±4.07*</td>
</tr>
<tr>
<td></td>
<td>Cassein + Tannic acid</td>
<td>40.6±1.52*</td>
</tr>
<tr>
<td></td>
<td>Enzyme + Salicylic acid</td>
<td>56.36±2.08*</td>
</tr>
<tr>
<td></td>
<td>Cassein + Salicylic acid</td>
<td>60.3±1.12°</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>No. of µl of phenolic solution* in pre-incubation mixture</th>
<th>Pre-incubation mixture</th>
<th>Enzyme activity (as µg glucose/min/lanx) mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 (control)</td>
<td></td>
<td>66.6±2.08*</td>
</tr>
<tr>
<td>10 µl</td>
<td>Enzyme + Tannic acid</td>
<td>56.4±1.79*</td>
</tr>
<tr>
<td></td>
<td>Sucrose + Tannic acid</td>
<td>61.9±1.29*</td>
</tr>
<tr>
<td></td>
<td>Enzyme + Salicylic acid</td>
<td>60.4±0.52*</td>
</tr>
<tr>
<td></td>
<td>Sucrose + Salicylic acid</td>
<td>60.9±1.01*</td>
</tr>
<tr>
<td>50 µl</td>
<td>Enzyme + Tannic acid</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sucrose + Tannic acid</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Enzyme + Salicylic acid</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sucrose + Salicylic acid</td>
<td>39.6±1.52*</td>
</tr>
</tbody>
</table>

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 0.0% and 9%, respectively.

(1 ml) contained $2.87 \times 10^{-3}$ µM of tannic acid (10 µl), while 1 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 block the access of digestive enzymes to the protein substrate 3) protein precipitation 4) activated oxygen species (i.e. O\(_2\), H\(_2\)O\(_2\), OH). Table 4. Digestive enzymes for S. littoralis larvae exposed (5 days) to 80X10\(^{-6}\)M dietary phenolics.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protease activity (O.D. units x10(^{-3})/min/larva)</th>
<th>Invertase activity (μg glucose/min/larva)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94.28±2(^a)</td>
<td>66.25±2.83(^a)</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>77.79±2.55(^b)</td>
<td>33.83±3.40(^b)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>79.33±3.0(^b)</td>
<td>51.3±1.55(^b)</td>
</tr>
</tbody>
</table>

Data represented as means±SD

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

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 presence of other additional mechanisms. Reese and Beck (1976) found that 3-phenylnaphthoquinone inhibit ingestion in A. ippon 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 oxidative 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.
REFERENCES


تأثير بعض الفيتولات على فسيولوجيا الهضم في بروقات دودة ورق القطط

طارق رئيس أمين، صلاح أبو العينين، نوري حامد اللوز

1. معهد بحوث وفلاحة النباتات، مركز البحوث الزراعية، الدقي
2. قسم الكيمياء، كلية العلوم، عين شمس

حيث أن الفيتولات القطرة على إكسب السلباتة للثباتات ضد الإصابة بالأفات، تم إختبار
مركبتين منها وهم: محسن الساليسليك وحمض الربنيك على العمر الرابع لبروقة دودة ورق القطط
والتي تم زرعها بطريقة عبئ هذه المركبات، وتعد هذه المركبتين من العناصر الفضائية لبروقة
بتركيزات تراوح بين 1-10 ملل مول/كم م وزن الغذاء. أظهرت النتائج أن معدلة أثرت
على نمو البروقات مع إنتاج المحتوى الكلي البروتينات والكربوهيدرات. وبناء على ذلك تم
إحراز ثورة عملية الهضم مما طلعتها المركبات من قدرة على إحداث الأكسدة داخل الجسم وإليهتم
ذلك تم إجراء عدة تجارب داخل وخارج الجسم المحرز، وأظهرت النتائج أن هناك تأثيرًا لنيسن
لديها القطرة فقط على تكثيف البروتينات (كولين). ولكن أيضاً على تأثير إنتاج البروتينات مثل
البروتيين والأفيونز. وتتطلب هذه المواد النشطة البديلة تأثير على المركبات المعرفي على الأطعمة التي تتأثر على فسيولوجيا
هضم وفقاً لذلك يجوز استخدامها كجزء من دورة إنقاذ المعدة وعليه خلايا
الدمي المتوسط.

وبناء على ذلك يمكن القول أن قدرة هذه المواد على إحداث الأكسدة داخل الجسم يؤثر على
إطارات الهضم ومكونات الطعام من البروتينات وكميات البروتينات التي يدورها يؤدي في النهاية على نمو
البروقات.